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9 Assessing metabolic activity at methane seeps: a testing ground for slow growing environmental systems

9.1 Introduction

Microbial communities mediate the planet's foundational biogeochemical cycles, mobilize nutrients, support higher trophic levels, and perform essential ecosystem services. High-throughput genetic sequencing and advanced computational tools have revolutionized our understanding of biodiversity and habitability over the last decade, providing a window into microbial systems. 16S rRNA gene surveys have uncovered rich stories of microbial diversity across a wide range of habitats, from coastal lagoons [1] to intercontinental dust [2] and seafloor hydrothermal systems [3]. Single-cell genomics, as well as computational binning of metagenomic databases, have filled in thousands of genomes and pointed to fermenting bacterial phyla [4] and the hydrocarbon metabolizing potential of Bathyarchaeota [5] and Verstraetearchaeota [6]. Taken together, the genomics revolution has reconfigured the tree of life [7, 8] and described compelling new habitats, from the human gut [9] to the deep subsurface [10].

Despite these exciting developments, an essential aspect of the role of microbes in ecosystem dynamics is missing from the genomics pipeline: metabolic activity. DNA based studies demonstrate genetic potential, but offer limited insight into cell viability or realized biochemical processes; indeed, molecular products derived from many genes are not present at any given time [11, 12], and relic genetic material can persist in soil for years, complicating the interpretation of broad genetic surveys [13, 14]. However, metabolic activity – the ability of organisms to conserve energy and transform their surroundings – is precisely what makes microorganisms so powerful: focusing on this parameter should be a central priority for environmental microbiologists seeking to understand our planet.

The challenge of measuring metabolic activity is particularly pronounced at marine methane seeps, where uncultured, slow growing, intricately interconnected microbial communities carry out globally relevant biogeochemical processes [15, 16]. The anaerobic oxidation of methane (AOM), catalyzed by primary producer consortia of anaerobic methanotrophic (ANME) archaea and sulfate-reducing bacteria (SRB), produces little energy [17, 18], yet these organisms support oases of secondary microbial mats, meiofauna, and clam beds [19, 20]. Geologic evidence of seep supported habitats extends back hundreds of millions of years [21], and methane associated pathways are believed to be among the most ancient of microbial metabolisms [22].

Although anoxic methane oxidizing enrichments have been nurtured [23], efforts to isolate ANME have been unsuccessful to date, as precise metabolic and environmental needs have been difficult to disentangle from consortia and community based relationships. The dearth of other seep hosted microbial isolates is likely primarily due to a lack of attempted approaches, though downflow hanging sponge reactors have proven successful in cultivating methane linked microbes from similar habitats [24].

As a result of these challenges, assessments of metabolic activity at methane seeps have embraced and advanced culture-independent tools. This outlook began as a necessity, but has increasingly been viewed as a strength. After all, integrated microbial communities are the default mode of life [25, 26], and the vast majority of microorganisms remain uncultured [8, 27]. The challenges associated with linking identity to function within this community centric framework have led to an array of creative solutions, which we describe below. In this context, methane seeps have served as an important testing ground for activity based tools and techniques that have since seen application at an array of terrestrial, marine, and subsurface sites.

Our assessment of activity oriented research at methane seeps encompasses multiple scales of investigation, from a broad view of community-wide changes to metabolism specific analyses seen through catabolic and anabolic lenses. The research community's focus on the distinguishing metabolisms of methane seeps – methane oxidation coupled to sulfate reduction – is reflected in the methods highlighted herein, though the opportunity for a wider scope of study is briefly discussed. We begin with system-wide parameters that inform metabolic activity through observation, such as bulk geochemical transformations and microbial colonization (Sect. 9.2). Catabolic activity studies focus largely on the sulfate-dependent AOM by tracking carbon- (methane, bicarbonate) and sulfur- (sulfate, sulfide) bearing molecules in lab based incubations (Sect. 9.3). Anabolism oriented work tracks biomolecule production associated with seep simulating conditions, frequently examining information-bearing molecules in order to link function with phylogenetic identity (Sect. 9.4). We address pressing empirical challenges (Sect. 9.5) before assessing metabolic activity based tools of the future (Sect. 9.6).

The culture-independent techniques that have been customized for investigations at methane seeps have exposed surprising new metabolisms and revealed the importance of seeps in biogeochemical processes. These tools have been adapted for a wide range of environmental microbiological investigations, helping a diverse community of researchers better understand how complex microbial systems impact ecosystems worldwide.

9.2 Observational approaches to quantifying seep hosted activity

In order to be most relevant from an observational, environment-wide perspective, activity measurements should be conducted *in situ* with minimal perturbation of the system. This is a challenging proposition in any real-world setting, but the difficulties of working in the deep sea make meaningful *in situ* work particularly daunting. Operationally, the high pressure, low temperature, time constrained, and robot mediated nature of experimental manipulation at seeps has limited the roster of reliable instrumentation. Hydrologic and microbiological complexity inherent to such habitats – e.g. variable fluid fluxes and flow paths [28], overlapping zones of methanogenesis and methanotrophy [29] – make it difficult to isolate variables. Nonetheless, modeling efforts and colonization studies offer insight into microbial activity at seeps.

9.2.1 Modeling rates of activity from geochemical profiles

One of the earliest methods of observation based rate quantification involved the derivation of bulk sediment hosted activity rates from geochemical profiles. In this approach, concentrations of relevant species (potentially including CH_4, SO_4^{2-}, H_2S, and HCO_3^-) from *in situ* [30] or recovered core [31] pore waters are measured, and mathematical models are used to determine steady state rates of chemical transformation. In the simplest version of a reaction transport model, rates of methane consumption (R) are determined from one-dimensional concentration (C) profiles and calculated temperature dependent diffusion coefficients ($D_{S,T}$), according to equation 9.1

$$R = D_{S,T} \left(\frac{\delta^2 C}{\delta z^2} \right) \tag{9.1}$$

where C is the dissolved methane concentration, z is depth, and $D_{S,T}$ is between $0.7–1\times 10^{-5}$ cm^2 s^{-1} for the most commonly applicable temperature range of 2–10 °C [32]. One challenge with such reductionist models is that they are underdetermined with regard to reactant transport: a high flow rate with rapid consumption could produce the same depth profile as low flow and slow uptake. For this reason, the incorporation of other physical parameters like advection rate and porosity, when measurable, are useful; fluid flow in particular signifies the dominant factor in determining the ultimate extent of AOM [33]. Several studies have developed such advection-diffusion-reaction models e.g. [34, 35], based on one-dimensional mass conservation [36, 37]. Treude et al., for example, use equation 9.2 when calculating rates of AOM (R) at Hydrate Ridge, OR [38], where ϕ is porosity, t is time, Θ is tortuosity, u is the advective flow rate, and other

variables are represented as in equation 9.1.

$$\phi \frac{\delta C}{\delta t} = \frac{\delta}{\delta z} \left(\phi \frac{D_{S,T}}{\Theta^2} \frac{\delta C}{\delta z} \right) - \frac{\delta C}{\delta z}(\Theta u) - \Theta R \tag{9.2}$$

Other factors, including biomass growth [33, 39] or kinetic [40] and thermodynamic limitations [41], can also be incorporated for higher fidelity treatment of well characterized sites (see Regnier et al., 2011, for a review on seep associated models [42]). Advection-diffusion-reaction models are useful in their quantification of net methane consumption, which is frequently the desired metric on an ecosystem-wide scale. However, reliable measurements of geochemical concentrations and advection rates (which can change substantially with the tidal cycle [43]) remain a challenge, and the elucidation of lineage-specific activity remains unaddressed.

9.2.2 Colonization Rates

An alternative approach to measuring *in situ* activity entails multitimepoint observations derived from samples recovered during distinct sampling campaigns. In one study, a 14-month incubation of sterile glass slides in Eel River Basin seep sediment demonstrated growth of bacteria whose δ^{13}C signatures indicated minimal incorporation of isotopically light, methane-derived carbon; ANME representatives were not observed [44]. A more detailed study characterized microbial colonization and diversity on sterile carbonate and wood substrates after 13 months at actively seeping and seemingly inactive sites [45]; similar experiments at hydrothermal vents have demonstrated Epsilonproteobacterial colonization of titanium rings after 20 days [46]. This time resolution circumvents high frequency temporal bias in seepage activity – which can vary over days [28] or weeks [47] – and begins to constrain the timescales over which communities can spread across newly amenable substrate. Colonization by *Thiotrichaceae* and *Helicobacteraceae* representatives at active seeps suggests a prevalence of sulfur-oxidizing niches, while ANME archaea point to anoxic, methane rich endolithic habitats and seeding of microbial constituents from below. The observation that newly colonized rocks and 'native' carbonates contained distinct microbial assemblages suggests that 13 months is insufficient time for a mature, steady-state community to develop; more extended time series studies could further illuminate this process of successional activity.

Repeated *in situ* sampling is useful for assessing activity and rate based features under natural conditions to measure parameters of interest, potentially including biomass growth or authigenic carbonate precipitation. As access to seep sites becomes more reliable – e.g. through cabled seafloor observatories – these modes of analysis are likely to become more ingrained in seep based science.
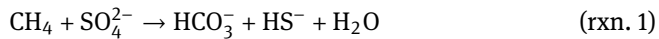
9.3 Catabolism based methods

Transporting sediment and carbonate rocks from seep impacted environments to the lab enables a much greater range of activity based experiments, allowing researchers to track specific aspects of catabolic or anabolic metabolism. These experiments are typically performed in micro- or mesocosm incubations (e.g. tens to hundreds of milliliters of substrate), sacrificing features of environmental realism (advective transport of reactants and wastes, temporal variation) but providing more consistent control of chemical concentrations and sampling.

The most common mode of activity measurement quantifies reactants and products of catabolism, the metabolic reactions that conserve energy in support of other cellular processes. These studies typically focus on methane and sulfate dynamics, so carbon, hydrogen, and sulfur are the best-established atomic tracking systems.

9.3.1 Tracking methane catabolism

Methane metabolism in anoxic sediments involves a multifaceted set of reactions. Most broadly, ANME archaea partner with SRB to enact sulfate dependent AOM [18] (Reaction 1), while methanogens (comprising seven orders of Euryarchaeota and possibly members of the newly characterized Bathyarchaeota [5] and Verstraetearchaeota [6]) produce methane from a number of precursors, most prominently hydrogen and carbon dioxide (Reaction 2) or acetate (Reaction 3).

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \qquad \text{(rxn. 1)}$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad \text{(rxn. 2)}$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \qquad \text{(rxn. 3)}$$

When quantifying rates of AOM, several reactants and products can be tracked. These procedures capture distinct aspects of a methane-processing pathway, and terminology is frequently used loosely, obscuring the true meaning of a study. We seek to clarify the situation by distinguishing between four commonly measured aspects of AOM as well as methanogenesis, which frequently occurs in colocated sediment horizons and whose activity can complicate interpretations (▶ Fig. 9.1).

9.3.1.1 Methane activation

Methane activation signifies the cleavage of at least one C−H bond but does not rule out the back-reaction to reform methane [48]. A back-reaction at any point in the reverse methanogenesis pathway would maintain the initial methane derived carbon atom, making isotopic labels attached to carbon ineffective in quantifying methane activation. Hydrogen isotopes, however, can provide insight, and techniques track-
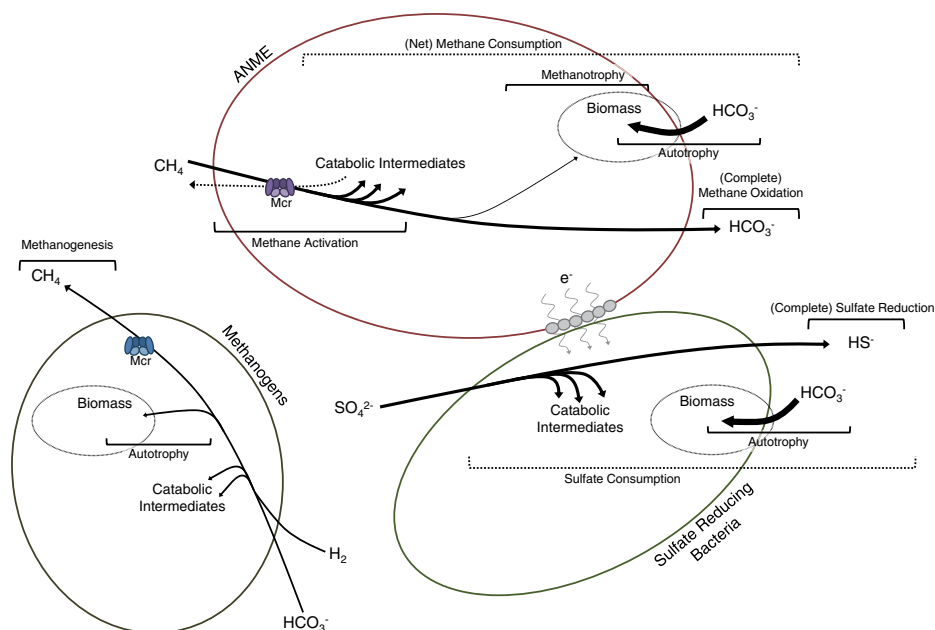
Fig. 9.1: A schematic diagram showing a network of catabolic and selected anabolic transformations amenable to activity oriented inquiry. Arrow thicknesses are intended to give a relative sense of metabolite fluxes under most AOM experimental conditions but are not to scale. See text for additional details

ing deuterium [49] and tritium [50] from methane into the aqueous phase have been developed.

The radiotracer ^{3}H–CH$_4$ was first synthesized in order to quantify aerobic methane oxidation rates in the Eel River Basin water column [50]. The developers viewed ^{3}H–CH$_4$ as an improvement over ^{14}CH$_4$ radiotracers because of its 2,000-fold higher specific activity, meaning that added concentrations could be kept as low as 3 nM and artifacts of experimental conditions could be avoided. Subsequent experiments have measured aerobic methane activation rates of North Sea water [51], Arctic fjords [52], and the eastern North Pacific [53]. Discrepancies between ^{14}C and ^{3}H based rates were attributed to different concentrations of added methane (< 2 nM ^{3}H–CH$_4$, 450 nM ^{14}CH$_4$) [52] or priming effects and method backgrounds [53]. ^{3}H–CH$_4$ studies have not fully engaged with the nuances involved with measuring hydrogen- versus carbon-linked atoms, typically equating ^{3}H presence with H^{14}CO$_3{}^-$ production [50–53], an approach that neglects biomass bound ^{3}H and ^{14}C, as well as back-reactions that would increase aqueous ^{3}H concentrations without full methane oxidation. To date, the ^{3}H–CH$_4$ method has only been used in aerobic methane activation studies.

An alternative method using monodeuterated methane (CH$_3$D) has been tested in both aerobic and anaerobic methane activating systems; following an incubation

period, D/H ratios of filtered water samples are measured on a stable isotope water analyzer [49]. When directly compared with the ^{14}C radiotracer method in AOM incubations, calculated rates of tracer appearance in the aqueous phase were consistently offset by a factor of two, demonstrating both the applicability of the method and the relevance of anabolic pools. To constrain the prevalence of the methane-forming back-reaction, the changing proportion of CH_3D and CH_4 was measured with nuclear magnetic resonance (NMR) spectroscopy, revealing that 4–16% of aqueous D was attributable to activated, back-reacted methane during AOM [49].

Using hydrogen as an isotopic probe of methane oxidation remains an immature field because the partitioning of methane derived hydrogen into catabolic and anabolic products is poorly understood. For this reason, a more thorough accounting of hydrogen atoms liberated through methane activation and oxidation would be useful; a combined study of D/H water ratios and anabolic incorporation of D (via nanoSIMS [54]) would begin to provide clarification.

9.3.1.2 Methanotrophy

Methanotrophy, in the strictest sense, refers to the incorporation of methane derived carbon into cellular constituents. However, because much of their biomass carbon is sourced from dissolved inorganic carbon (DIC) [55], ANME can best be described as mixotrophs that combine autotrophy (DIC-carbon) and methanotrophy (methane-carbon). A more complete assessment of methanotrophic measurement approaches is provided in Sect. 9.4.

9.3.1.3 Complete methane oxidation

Chemical oxidation strips electrons from an atom, and while the oxidation of methane's carbon from CH_4 to HCO_3^- entails a loss of eight electrons, any stage beyond the initial reaction of methane with the $CoM-S-S-CoB$ heterodisulfide constitutes methane oxidation. However, metabolites of intermediate oxidation states are rarely measured, and practitioners typically report DIC concentration as a proxy for methane oxidation (Reaction 1). We propose 'complete methane oxidation' as a more precise term associated with such measurements.

Tracking changes in DIC as a metric of methane oxidation rate – even when done while measuring methane concentration – is insufficient in the context of mixed microbial communities. DIC is the end product of many heterotrophic metabolisms, and given the heightened concentrations of higher hydrocarbons in seep hosted sediments [56, 57], the origin of fully oxidized carbon is difficult to determine unless isotopic labels are used judiciously. Oxidized carbon can also enter autotrophic pathways and be recycled into reduced compounds.

Tracking radiocarbon (^{14}C) from methane ($^{14}CH_4$) into DIC is perhaps the best-established method for quantifying rates of complete methane oxidation. The approach was first codified by Reeburgh [58] based on similar techniques measuring methane

production from ^{14}C-acetate or lactate [59]; procedural enhancements from Iversen and Blackburn [60] and Treude et al. [61] have since streamlined the process and minimized potential errors associated with incomplete analyte recovery.

After dissolved radioactive methane is injected, the sample is incubated at the desired conditions. Because the detection of ^{14}C atoms is so sensitive, experiments only require small volume amendments (typically a few tens of microliters) and brief incubation periods (hours to several days). To halt microbial activity, NaOH is added, and several parameters are measured. Total methane (CH_4) is determined via gas chromatography from a control, time-zero sample. Postincubation residual [14]CH_4 is measured by scintillation counting after headspace combustion at 850 °C and capture of the formed [14]CO_2 with phenylethylamine. Biologically formed [14]CO_2 is quantified by scintillation counting following acidification of the sample (driving any adsorbed or precipitated oxidized carbon into the gas phase) and capture of the resulting [14]CO_2 gas in phenylethylamine. When the volume of the sample (v) and duration of the experiment (t) are taken into account, the rate of methane oxidation is calculated as shown in equation 9.3.

$$\text{Methane Oxidation} = \frac{^{14}CO_2 \cdot CH_4}{(^{14}CH_4 + {}^{14}CO_2) \cdot v \cdot t} \tag{9.3}$$

Since its development, the ^{14}C method has arguably become the most pervasive approach used in rate quantifications; it has been employed in lab based incubations of marine sediments from the Gulf of Mexico [62], Saanich Inlet [63], Skan Bay [64], Cape Lookout Bight [65], Guaymas Basin [66], and the Chilean margin [61], as well as carbonate rocks from Hydrate Ridge [67]. A new approach allows researchers to introduce even less [14]CH_4 reactant [68] by exploiting the 10^3–10^9-fold more sensitive ^{14}C measurements of accelerator mass spectrometery [69].

[13]C can also be used to measure rates of complete methane oxidation, an approach that was most substantively developed for the examination of 'trace methane oxidation' by cultures of known methanogens [70, 71]. [13]CH_4 is added to the sample, and concentrations and isotopic ratios of headspace carbon dioxide and methane are measured by mass spectrometry (see Moran et al., 2007 for additional details [71]). Fractionation factors and back-reactions of labeled DIC to labeled methane should also be incorporated into rate equations depending on the amount of label and timescale of the experiment.

In the context of environmental communities, detection limits could render this approach challenging, particularly when ∼ µM addition constitutes a major perturbation, as in the water column above methane seeps [68]. With incubations from more methane replete environments such as anoxic seep sediments, [13]CH_4 has been used to probe the relative rates of AOM upon the addition of H_2 and methyl sulfides [72]. This approach revealed a decoupling of sulfate reduction and complete methane oxidation at low sulfate levels [73], and confirmed methane oxidation coupled to anthraquinone-2,6-disulfonate (AQDS) reduction [74]. Quantifying ^{13}C distributions among oxidized

and reduced pools via nuclear magnetic resonance spectroscopy has been the method of choice for detailed *in vitro* biochemical studies on methyl coenzyme M reductase systematics [48, 75].

9.3.1.4 Net methane consumption

Methane consumption involves a decrease in methane concentration but is end-product agnostic. Studies reporting this parameter frequently measure decreases in aqueous or headspace methane concentration and may or may not account for methyl coenzyme M reductase (Mcr) back-reaction or methanogenesis occurring in the same sample [29]. Thus, when isotopically unconstrained methane concentration is reported, "net methane consumption" is a more precise diagnosis.

Several efforts have quantified methane concentration within experimental incubations to determine net methane consumption rates. These include simultaneous tracking of methane loss (via a gas chromatography-flame ionization detector) and sulfide production to demonstrate 1:1 stoichiometry at a range of pressures [76], and mass spectrometry based concentration assessments at the inlet and endpoint of a 'simulated seep' flow-through reactor [77, 78]. The technique was also used, alongside stable isotope probing, omics studies, and supplementary geochemical measurements, to demonstrate a novel ANME lineage linking AOM to nitrate reduction [79]. Net methane consumption, as measured through changes in headspace or aqueous methane concentrations, can be a relatively straightforward way to capture system-wide microbially mediated processes, but additional details on interacting metabolic pathways or phylogenetic constraints on activity require more nuanced tools.

9.3.1.5 Methanogenesis

The anoxic sediments of methane seeps can host conditions that are energetically amenable to both methanogenesis and AOM [38, 80], particularly at sites of high organic load input [65] or serpentinization [81]. Both methanogens [82] and ANME [83] have also been demonstrated running their 'primary' reactions in reverse, complicating the interpretation of experiments with a unidirectional pathways focus. To better constrain simultaneous methane production and methane oxidation, catabolism oriented isotopic labels on oxidized carbon species can be incorporated into incubation conditions.

To determine the role of both aceticlastic and bicarbonate based methanogenesis in Gulf of Mexico seep sediments, Orcutt et al. added ^{14}C-bicarbonate and ^{14}C-acetate to incubation experiments and tracked $^{14}CH_4$ production with headspace combustion and scintillation counting. The results were integrated into parallel incubations quantifying complete methane oxidation rates, demonstrating that AOM was ten times as rapid as ^{14}C-bicarbonate reduction [29, 84]. Such studies highlight the complexity of methane metabolism and indicate how multiple pathways can interact to regulate seep geochemistry.

9.3.2 Tracking sulfur catabolism

Methane oxidation is frequently the primary interest of researchers investigating methane seeps, but given its intimate link with sulfate reduction, measuring sulfur related processes can help clarify interorganism relationships and reveal hidden metabolic linkages. Canonical dissimilatory sulfate reduction is a three-step process converting dissolved sulfate to adenosine 5′-phosphosulfate (APS), sulfite, and ultimately sulfide; the entire process is an eight-electron reduction, and its pairing with methane oxidation to HCO_3^- thereby results in a 1 : 1 stoichiometry.

Because of this concordance, sulfur catabolism measurements can be used to both track AOM and place methane into a broader context as a carbon source in anoxic zones. Most straightforwardly, sulfate concentrations can be quantified using ion chromatography [38, 62], high-performance liquid chromatography [85], or reaction with barium chloride [86], while sulfide can be assessed through the Cline assay [87]. These approaches have supported studies of the zonation of AOM driven sulfide mineralization [88] and the effect of temperature or pressure on sulfate-linked AOM rates [76].

Stable and radioisotopes have also been used to probe dissimilatory sulfate reduction, offering the benefit of more specific molecular tracking and higher sensitivity [89]. $Na_2^{35}SO_4$ is introduced to experimental incubations, and after the desired time period zinc acetate is added to stop microbial activity and fix $H_2^{35}S$ as $Zn^{35}S$. Inorganic sulfides are recovered by chromous acid digestion [90], and sulfide and aqueous sulfate activities are determined by scintillation counting. Sulfate reduction rates (*SRR*) are then calculated according to equation 9.4

$$SRR = \frac{[SO_4^{2-}] \cdot \alpha_{SO_4} \cdot (aH_2^{35}S)}{t \cdot v \cdot (a^{35}SO_4^{2-})} \tag{9.4}$$

where $[SO_4^{2-}]$ is the sulfate concentration, α_{SO_4} is the isotope fractionation factor for sulfate reduction, t and v are the incubation time and volume, and a values designate chemical activities of the relevant ^{35}S-containing pools.

Measures of sulfate reduction, when quantified alongside methane oxidation, help clarify the role of methane as a driver of overall heterotrophic activity and can expose intriguing aspects of the AOM-SR processes. Studies of sediments from geologically distinct seeps attribute varying levels of total sulfate reduction to methane oxidation, ranging from 7–11% in the Black Sea [34] to nearly 100% in high flux, organic poor sediments from the Bullseye seep [91]. Combining concentration based rate measurements with isotopic analysis has been particularly fruitful in developing interpretive frameworks for *in situ* methane and sulfate isotopic distributions. Such studies have shown that methane $\delta^{13}C$ values are linked to sulfate concentrations, $\delta^{34}S$ reflects methane concentrations [92], and sulfate $\delta^{18}O$ is suggestive of sulfide reoxidation [93, 94].

9.3.3 Tracking catabolism of 'nontraditional' electron acceptors

Historically, investigations of AOM metabolism have focused on sulfate as the associated electron acceptor [18, 95], a choice that reflects the dominant metabolic coupling in marine (high sulfate) methane seep settings. However, alternative electron acceptors have been implicated in other systems: nitrate in enrichment bioreactors inoculated with wastewater sludge [79, 96] and nitrite in terrestrial sediments [97].

AOM linked to metal reduction, meanwhile, was first demonstrated in seep sediment incubations [98]. Since then, strategic amendments with methane, sulfate, iron, manganese, as well as selective inhibitors like molybdate and bromoethanesulfonate, have pointed to cryptic cycling [99, 100] and suggested a role for metals in seep [94], marine [101, 102], and freshwater [103, 104] AOM. Studies pursuing metal reduction add quantification of metal species to the assessments of methane oxidation and sulfate reduction discussed above. Ferrous and total iron (following reduction with hydroxylamine hydrochloride) can be quantified with the ferrozine assay [105], while reduced manganese can be assessed spectrophotometrically after mixing with formaldoxime [106]. Nonetheless, the roles of biotic and abiotic processes in metal associated AOM remain difficult to interpret; reactive intermediates intercepted from putative metabolic reactions [107] or metal isotope distributions could provide useful insight in the coming years.

9.4 Anabolism based methods

Anabolism is the process through which molecules or elements are incorporated into biomass. This side of metabolic activity touches more tangentially on the ecosystem-wide, biogeochemical cycling implications of microbial communities; instead, it exposes components such as cell growth and proliferation, and, through the assessment of information-carrying molecules, provides a more granular look at phylogenetic identity and metabolic mechanisms. Here, we briefly review anabolism based studies of methane seep systems, whose uncultured core metabolism (AOM) has prompted the development and enhancement of several analytical tools. This assessment will cover a wide continuum of informational biomolecules, from cell counts to biomolecule-specific stable isotope probing and the hijacking of translational machinery to install chemically useful residues in incipient proteins.

9.4.1 Cell quantification

Counting the number of cells in a sample is among the most fundamental measures of the microbiological response to empirical conditions, demonstrating whether the microbial community is expanding or contracting, while also providing a basis for per-

cell rate calculations. In the context of seep sediments, the efficient, unbiased isolation of cells is an initial obstacle, since biomass can adsorb to charged clay particles or be shielded from *in situ* lysis reagents [108]. The use of a Percoll density gradient, in concert with thermal treatment and sonication, effectively concentrates microbial biomass from seep sediments [109, 110] and carbonate rocks [67] (see Dawson et al. for a more detailed protocol [111]). Alternatively, chemical treatment followed by sonication and Nycodenz and/or polytungstate density centrifugation resulted in high cell extraction efficiencies in Arctic Ocean subseafloor sediment [112] and Nankai Trough subduction zone sediments [113]. These processes do not appear to disrupt spatially associated consortia [15], but the retention of active cells following the procedures has not yet been demonstrated.

Isolated biomass can be used for direct cell counts, lineage-specific characterization through fluorescence *in situ* hybridization (FISH), and quantitative PCR (qPCR). Cell counts with general DNA dyes (e.g. acridine orange, DAPI) have demonstrated the proliferation of ANME-SRB consortia in Hydrate Ridge seep sediment after two years of lab based incubations. Supplementing the analysis with FISH probes to confirm phylogenetic identity, a consortia doubling time of approximately seven months was calculated [114]. qPCR was used as a proxy for cell counts to demonstrate lineage-specific growth rate enhancements among ANME [78].

Loss of DNA during extraction could limit the interpretive power of qPCR results. While water column studies have noted a concordance between qPCR and other cell quantification methods [115], a study from nonseep associated anoxic marine sediments, which compared results from general DNA stain, FISH, CARD-FISH (CAtalyzed Reporter Deposition-FISH), and qPCR approaches, came to a different conclusion. Cell abundances determined by general stains and the sum of domain-level CARD-FISH numbers were similar, but qPCR derived gene copy numbers demonstrated a highly variable underestimate of overall abundances (i.e. cell counts from general DNA staining) [116]. These types of paired analyses suggest that physical substrates such as marine sediment could play a complicating role in the recovery of DNA and associated assessments of microbial activity and proliferation.

9.4.2 Stable isotope probing

Stable isotopes of hydrogen, carbon, oxygen, and sulfur have proven effective in characterizing catabolism in microbial communities from methane seeps (see Sect. 9.3.1–9.3.2 above). In the context of anabolic processes – many of which involve informational molecules that help link identity with potential or realized function – protocols based on carbon, nitrogen, and, to a lesser extent, hydrogen incorporation are well established. Here, we briefly demonstrate the types of analyses enabled by stable isotope probing (SIP) along the continuum of informational richness of anabolic analyte, from whole cells to lipids, DNA, RNA, and proteins.

The design of a SIP study depends on the specific questions being addressed as well as several general considerations. First, the atomic proportion of the biomolecule of interest should be evaluated; mean cellular biomass, for example, has an approximate stoichiometry of $CH_{1.66}O_{0.46}N_{0.14}$ [117], so tracking carbon atoms provides a higher potential signal than nitrogen atoms. Second, the source material for anabolic pathways determines the proportion of labeled atoms that are ultimately incorporated into biomolecules of interest. For example, an estimated 50% of ANME lipid carbon is derived from methane while the remainder comes from bicarbonate [55], making both precursors equally promising molecular probes of carbon incorporation. Third, experimentalists must evaluate the proclivity of potential atomic labels to exchange with the aqueous phase during metabolism. In particular, it is difficult to directly trace hydrogen atoms from reactant to biomolecular product, as many hydrogen bonds are relatively labile. Fourth, the times of biomolecule turnover will influence the concentration of the label and the duration of the experiment: DNA generally requires cell division to incorporate a label, while RNA, proteins, and lipids are continually regenerated [118]. Similarly, the duration of the experiment should be tuned to avoid nonspecific or otherwise undifferentiable cross-feeding, a process whereby an isotopic label moves among multiple trophic levels. Finally, technical considerations related to instrumental readout may limit experimental possibilities. The geometrical constraints of nanoSIMS detectors, for example, make simultaneous hydrogen and sulfur detection extremely challenging, and experiments should be designed with such limitations in mind.

9.4.2.1 Intact cells

In 2001, investigators began tracking the methane dependent incorporation of ^{13}C and/or ^{15}N into individual cells with the newly available technique of secondary ion mass spectrometry (SIMS). To pinpoint the identity of these cells, FISH probes were developed to target 16S rRNA genes with varying degrees of phylogenetic specificity [119]. The combination of these tools – FISH-SIMS – facilitated the discovery of AOM-mediating consortia from methane seep sediments, initially through descriptions of natural isotopic abundances [15, 120], and later through the addition of isotopically enriched substrates [121]. Subsequently, FISH enhancements that bolstered signal (CARD-FISH[122, 123]), enriched for certain lineages (magneto-FISH [124]), or targeted functional genes (mRNA-FISH [125]), as well as more sensitive SIMS instruments (nanoSIMS [126]) have broadened the realm of empirical possibility.

NanoSIMS counts ions of several predesignated mass/charge ratios at a resolution of ~50 nm [127] across planes of material that are ablated away. By compiling these points and generating a 3D map of dozens or hundreds of closely associated cells, patterns of elemental uptake can be measured and the cross feeding relationships between different lineages can be inferred. FISH-nanoSIMS studies using stable isotope substrates have implicated ANME in nitrogen fixation [128], revealed consortia-

wide differences in nitrate uptake based on distinct SRB lineages [109], proposed novel metabolic associations between consortia constituents [129], and demonstrated metabolic decoupling between ANME and SRB [74]. By extending the position of one of the instrument's detectors and circumventing the 22 : 1 mass range restriction, simultaneous analysis of H, C, and N isotopes was recently performed [54]. In this mode, biomolecule exchangeability with the aqueous phase becomes an asset, as deuterium uptake reflects baseline activity levels that can permit more targeted questions of anabolic activity [54]. NanoSIMS has also helped clarify dynamics of metabolic activity in other seafloor studies, revealing carbon fixation by worm symbionts in shallow sediments [130] and demonstrating viable autotrophic cells in Pleistocene-aged sediments off the coast of Japan [131].

9.4.2.2 Lipids

Lipid-SIP tracks ^{13}C into phospholipid fatty acids and is among the more sensitive SIP techniques [132]. Because lipids are the primary components of cell membranes, this approach is sensitive to rapid changes in microbial populations [133]. While the phylogenetic resolution of lipids is lower than other SIP relevant biomolecules, ANME-1, -2, and -3 can be distinguished based on the presence of glycerol dialkyl glycerol tetraethers, crocetane, and particular pentamethylicosenes respectively [134, 135]. Sulfate-reducing partners are marked by varying ratios of C_{15} and C_{16} fatty acids [134].

Isotopic analysis of these phylogenetically constrained lipids allows researchers to determine the relative proportions of heterotrophic and autotrophic anabolism when samples are incubated with deuterated water (whose signal is incorporated into all metabolic activity) and ^{13}C-DIC (which is only incorporated into lipids in the case of autotrophy) [136]. When applied to ANME-1/HotSeep enrichments from Guaymas Basin sediment, this approach indicated that between 8–25% of both bacterial and archaeal lipid carbon comes from methane [137]. An earlier study found that proportion to be approximately 50% in ANME-2 dominated communities from Hydrate Ridge; partnering SRB were almost entirely autotrophic [55]. Subsequent studies have tracked deuterium from water into different ANME-1 lipids to clarify biosynthetic pathways and appreciate the nuances of distinct enrichment patterns among different lipids within the same lineage [138]. Continued study of molecule-specific biosynthetic processes, as informed by stable isotope additions, will help establish which molecules could serve as useful bellwethers of anabolic activity in environmental or microcosm settings.

9.4.2.3 DNA

DNA-SIP experiments incubate samples with ^{13}C-labeled substrate for a period of time, extract genetic material, separate labeled and unlabeled DNA by ultracentrifugation in a CsCl solution, and characterize the enriched fraction [139]. The method is well positioned to link phylogenetic identity to a marker of growth in the form of

genome replication, which is required in order for labeled substrate to be incorporated into the genetic material [140]. This prerequisite is both a liability (it can be slow and unevenly distributed) and a strength (it ensures that reproduction has occurred, if biomass expansion is a key focus). In the context of methane seep microbial communities, the several-month doubling times of ANME-SRB consortia would require extended experiments in order to recover a representative distribution of labeled DNA. Potentially of greater concern is the fact that all metabolic activity that occurs between cell divisions – the sum of a cell's biochemical imprint on its surroundings – goes unseen by DNA-SIP.

Few researchers have pursued DNA-SIP in the context of marine methane seeps, likely because they have been dissuaded by the long doubling times and the efficacy of FISH-SIMS in identifying organisms responsible for methane oxidation. However, in many locations, methane represents just a fraction of the electron donors that support sulfate reduction [141], so casting a wider net for alkanes that serve as reductants is an important undertaking. For example, a paired DNA- and RNA-SIP study of SRB from Amon mud volcano and Guaymas Basin sediment identified four clades that oxidized and incorporated ^{13}C from butane and dodecane [142]. In a study of aerobic methanotrophy in surface layer sediment at the Coal Oil Point seep off California, members of the *Methylophaga* and *Methylophilaceae* lineages – neither of which had been associated with methane oxidation – assimilated labeled carbon into their genomes after just three days of incubation with oxygen and ^{13}CH$_4$ [143]. Other hydrocarbon metabolizing studies have targeted heavy DNA fractions from mixed communities incubated with benzene [144] and toluene [145] in an effort to specify which members could encode degradative pathways [146].

Despite its limitations, DNA-SIP study of methane seep communities could clarify the timescales over which various constituents replicate, offering data to complement qPCR or FISH based quantification efforts. When used in concert with other, more metabolism oriented techniques (see Sect. 9.4.2.4–9.4.2.5 below), it could reveal fundamental lineage or metabolism dependent relationships between activity and propagation.

9.4.2.4 RNA

Because transcription is a hallmark of viable cells, RNA-SIP is a useful way to measure activity and, by sequencing and annotating isotopically labeled transcripts, the metabolically directed nature of that activity can be discerned [147, 148]. It is also substantially more sensitive than DNA-SIP: an early comparison found that ^{13}C accumulated in RNA nearly ten times as quickly as in DNA [149], while next generation sequencing platforms can detect transcripts with just 1.5 atom percent ^{13}C [150], or fully labeled RNA at mixing ratios as low as 0.001% [151]. This discrepancy was illustrated by the detection of ammonia oxidizing archaea from soil inoculum through labeled RNA; due to slow doubling times, no signal was found in the DNA [152]. Over time,

cross feeding results in diffusion of a label across trophic levels of a community. If sampling points are chosen strategically, primary feeders can be identified first, followed by selective and generalist heterotrophs [153].

RNA-SIP played an important role in the identification of SRB actively engaged in nonmethane hydrocarbon degradation [142]: heavy transcripts were reverse transcribed and terminal restriction fragments were linked with *Desulfosarcina* lineages that dominated mud volcano sediment amended with ethane, propane, and butane [154]. Fortunato and Huber used biomass from hydrothermal vent fluid to assemble a metatranscriptome enriched in bicarbonate derived ^{13}C [155]. The approach revealed dynamic community responses to incubation conditions: Epsilonproteobacteria shifted from using oxygen as an electron acceptor to using nitrate at moderate temperatures, and *Methanococcus*-derived methane metabolism transcripts dominated at higher temperatures. The proven ability to generate a community-level metatranscriptome from seafloor habitats [155] and deep sediments [148] opens the door to similar investigations at methane seeps.

9.4.2.5 Proteins

Proteins constitute the biochemical machinery of the cell, making the elucidation of actively produced proteins via SIP-proteomics an insightful window into the metabolic needs of microbes. In an environmental context, detection limits are estimated to be at approximately 10% label incorporation [156], and distinct incorporation values derived from multiple time points provide useful data on assimilation pathways and community dynamics [157]. Labeling can be conferred through substrates or common nutrients (such as ^{13}C-glucose or ^{15}NH$_3$) or through the addition of labeled amino acids; the former approach typically generates more meaningful results, as biosynthetic pathways can be partially reconstructed, while the latter offers more predictable mass shifts and highly accurate quantitation [158]. Unlike the SIP methods described above, proteomics requires metagenomic knowledge – ideally derived from the same sample or environmental system – against which to search mass spectra results.

Initial protein-SIP efforts examined interspecies interactions in toluene degrading cocultures [159], while more recent studies have moved into progressively more complex communities and environmental systems. Using sediment from Hydrate Ridge and Gulf of Mexico seep environments, Krüger et al. showed methane dependent uptake of labeled ammonium into proteins after only three weeks of incubation [160]. Bulk ^{15}N-enriched protein analysis as a measure of ANME biosynthesis was shown to be more sensitive than incorporation of ^{13}C from methane into lipids; the dominance of the Mcr protein in the ANME proteome may be a factor [161]. A SIP-metaproteomics study of Hydrate Ridge sediment detected thousands of proteins, including the full 'reverse methanogenesis' pathway attributed to ANME lineages. Multiple time points and ^{15}N enrichment values revealed that the addition of methane prompted ANME and SRB to synthesize proteins and led to decreases in transcription machinery as-

sociated with other metabolisms linked to oxygen or nitrate reduction [162]. Dozens of Mcr orthologs produced during the course of the experiment also enabled the detection of abundant posttranslational modifications on Mcr, exposing an additional dimension of functional diversity with an as-yet-undetermined bearing on metabolic activity.

9.4.3 BONCAT

Bioorthogonal noncanonical amino acid tagging (BONCAT) is a recently developed approach that allows protein synthesis in uncultured cells to be visualized [163–166], co-opting anabolic processes in order to identify active organisms. BONCAT is based on the *in vivo* incorporation of a synthetic amino acid that exploits the substrate promiscuity of specific tRNA-amino acyl synthetases. Only two known artificial amino acids are able to hijack the natural translational machinery without recognized biases against specific taxonomies or physiologies or the need for genetic modification of the host cell: *L*-homopropargylglycine (HPG) and *L*-azidohomoalanine (AHA), which both replace *L*-methionine (Met) during protein synthesis [167]. In contrast to AHA, HPG is not prone to chemical transformation under the alkaline, highly sulfidic conditions typically encountered in marine sediments [165]. After incorporation into new proteins, the amino acid can be fluorescently detected via azide-alkyne click chemistry. This allows biosynthetically active cells to be detected, either while still alive or in the chemically fixed state [163–165]. When used in conjunction with rRNA-targeted FISH, BONCAT allows cell identity and protein synthesis activity to be linked (▶ Fig. 9.2). BONCAT has been demonstrated to correlate well with other, independent proxies of cell growth, specifically the incorporation of ^{35}S-methionine or ^{15}NH$_4$$^+$ often used in microautoradiography [166] or SIMS [165] experiments.

The power of BONCAT for seep associated ecophysiology experiments was recently demonstrated in a study that tracked the translational activity of syntrophic consortia catalyzing AOM (▶ Fig. 9.2) [163]. By combining BONCAT, fluorescence-activated cell sorting (FACS), whole genome amplification, and 16S rRNA gene sequencing, hundreds of individual, biosynthetically active archaeal-bacterial partnerships were identified. The study revealed that representatives of all major clades of archaeal methanotrophs occurring in a single methane seep sample were active under controlled incubation conditions. Unexpectedly, ~14% of AOM consortia were anabolically active even in the absence of methane, either suggesting that energy sources other than methane oxidation could fuel the metabolism of this partnership or that energy storage compounds accumulated during times of plenty were mobilized. BONCAT-FACS also led to the discovery of a previously unrecognized interaction of ANME with members of the Verrucomicrobia, a poorly understood phylum that is widely distributed in marine sediment [168]. The nature of interactions between these two partners is as yet unknown.
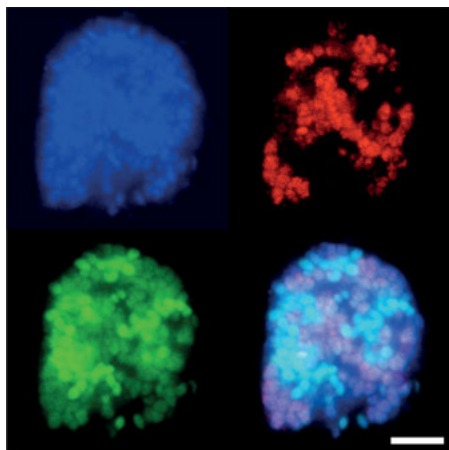
Fig. 9.2: Detection of protein-synthesizing cells in an ANME-SRB consortium. Sediment from the Hydrate Ridge methane seep site was incubated for 114 days in the presence of 50 µM HPG before being analyzed by BONCAT-FISH. *Blue* – DNA-stain DAPI. *Red* – an archaea-specific FISH probe. *Green* – translationally active cells as detected by BONCAT. *Lower right* shows an overlay of all channels. Scale bar equals 10 µm

BONCAT promises to be a valuable component of the activity based measurements toolbox. The approach is particularly attractive when studying substrates that are not available as isotope-labeled derivatives. In contrast to isotope-labeling studies, which require specialized instrumentation, BONCAT relies on standard epifluorescence microscopy. The approach is simple to establish, comparatively high throughput, and uses inexpensive reagents that are readily available from a number of vendors [164].

9.5 Key outstanding issues and challenges

Over the last two decades, researchers have made substantial progress in uncovering the details of methane seep ecosystems through the lens of microbial metabolic activity. New metabolisms, subseafloor habitats, and interspecies interactions have been described, while analytical tools developed in service of seep related investigations have proved useful in other fields. Nonetheless, several obstacles currently obscure our understanding of key aspects of how seeps operate and what they mean for global systems. In particular, while work to date has focused largely on lab based microcosm incubations and the integrated microbial communities therein, future researchers face challenges in environmental realism and varying temporal and spatial scales.

9.5.1 Linking the lab with the real world

Experiments conducted in the lab offer clear advantages of accessibility and controllability, and allow scientists to access a wide range of empirical tools. However, such work necessarily involves a degree of separation from the natural environment un-

der investigation, and the severity and implications of that disconnect remain largely unaddressed. The role of pressure and temporal and spatial variability on metabolic activity of seep ecosystems warrants additional attention.

9.5.1.1 Pressure

Given the range of depths at which marine methane seeps occur [169], pressure is an important variable that is frequently neglected in lab based experimental systems. When pressure has been considered, its impact has remained variable and sample dependent. Using Hydrate Ridge sediments from ~800 m depth, methane partial pressures of 1.1 MPa led to a five-fold increase in sulfate reduction rates compared to atmospheric pressure [76]; a different study conducted at 9.0 MPa found an 80% AOM rate enhancement [49]. Experiments with Guaymas Basin sediment reported a general concordance between pressure and methane dependent sulfate reduction rate, but the relationship broke down at higher temperatures [170].

In experiments with seep sediment from the Japan Trench, however, methane driven sulfate reduction rates showed no relationship to changing pressure [171]. Investigations into the cause of any pressure related effects have similarly mixed results. Some researchers propose that rate increases are caused by greater methane solubility and bioavailability [172], while other experiments have demonstrated a six- to ten-fold AOM rate increase at 10 MPa when methane concentrations in the experimental and control (atmospheric pressure) incubations were held constant [141]. Given the range of published data, *in situ* pressure should be maintained throughout the sampling and experimental process for the most representative results. Activity associated tools focusing on different elements and molecules of the AOM system, or examining transcriptomic and proteomic responses to pressure, will also help to clarify the nuances of this environmentally relevant variable on seep communities.
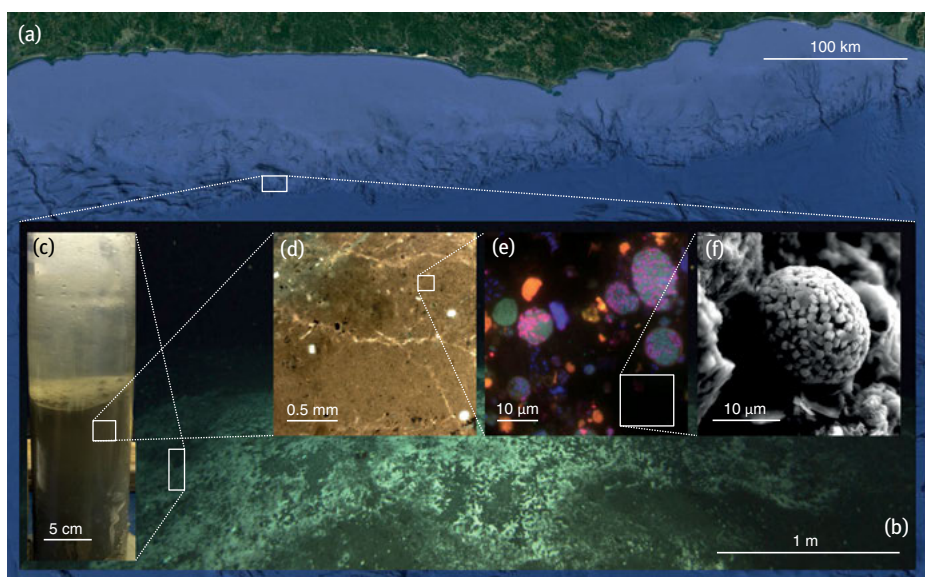
9.5.1.2 Integrating across spatial scales

One of the greatest challenges facing microbial ecologists is the integration of biochemical transformations at the nano- and microscale over larger areas to understand the impacts of kilometer-scale ecosystems. At the microbial level, synergistic relationships between neighboring cells can result in metabolite transfer or genome streamlining [173]; understanding these spatial relationships can help make sense of lineage-specific omics signals. There is also evidence that metabolic complementarity confers fitness advantages among co-occurring organisms, particularly in energy limited conditions [174], making colocalization an important trait for studies of biogeochemical cycles [175].

The spatial arrangement of, and metabolite transfer between, ANME and SRB within consortia has been a central focus of seep associated microbiology, and many of the nanoSIMS oriented studies described above (see Sect. 9.4.2.1) have been predicated on the preservation of these structures. While sample preparation and processing do

not appear to irrevocably disrupt aggregates, any spatial associations that may be lost are not well constrained. Furthermore, the focused pursuit of the ANME-SRB coupling mechanism over the last several years has relegated other interspecies interactions to the sidelines. Given the density of redox boundaries in methane seep sediments and the diversity of seep communities, this system likely contains a number of unseen interactions that play important roles in ecosystem dynamics.

Moving from a scale of dozens of microns to hundreds of meters – the necessary vantage distance from which to view a typical seep (▶ Fig. 9.3) – requires a number of poorly substantiated assumptions. Most notably, *in situ* methane concentrations (at various subseafloor depths, over meter scales horizontally, and as a function of time) are lacking. Understanding these variations, as well as methane- and sulfate-bearing subsurface fluid flow, is essential in determining how relevant microbial activity at seeps is for ecosystem services on a global scale. The current uncertainties have led to wide ranging estimates of the significance of global AOM in the carbon cycle and climate regulation [16, 176].

Fig. 9.3: Integrating data at methane seeps across several orders of magnitude of spatial scale is a major challenge. Seagoing research expeditions target a limited segment of a geographical region (a), and submersible or robotic investigations happen on the landscape scale (b). Centimeter-scale push cores (c) are retrieved, enabling investigations of porosity and grain packing via thin sections (d), phylogenetic identity of microbes via microscopy (e), or mineralogy via scanning electron microscopy (f). (Images courtesy of a: Google Earth; b: Victoria Orphan / Lisa Levin / Anthony Rathburn / WHOI; c, d: Jeffrey Marlow; e: Shawn McGlynn; f: Aude Picard)

9.5.1.3 Temporal variations

Another opportunity for improving lab based approximations of real-world settings is through consideration of temporal variation at methane seeps. Advective flow varies widely across different timescales, from hours to millennia. Benthic flux meters have captured shifts in fluid flow over the course of several weeks, from several meters per year upward to several centimeters per year downward, into the seafloor [28, 47]. Variations result from a complex combination of tidal forcing, compaction driven shifts in conduit geometry, hydrate dissociation, tectonic forces, local stratigraphy, sedimentation rate, and carbonate crust formation [28, 177].

Appreciating how localized concentrations of methane throughout a mound may change over time is important for two reasons. First, it bounds the extent of methane dependent microbial activity, providing a more realistic view of broader consumption: existing global estimates of AOM activity do not consider time dependent changes in activity [16, 176]. Second, examinations of temporal variation could demonstrate how community composition and metabolic potential respond to environmental changes. In other words, if it takes decades for a methanotrophic community to reestablish itself after a period of seep quiescence, then momentary methane concentrations could overestimate actual levels of methane consumption.

Not all microbial activity at seeps is directly dependent upon methane, and as subsurface flow changes, different lineages may take advantage of shifting niches. A transplantation study of carbonate rocks from active seeps to seemingly quiescent sites showed a persistence of most taxa over 13 months, though relative abundances, particularly of putative sulfide oxidizers, did change [45]. The timescales over which microbial constituents colonize newly formed seeps – and how long it takes them to construct authigenic carbonate mounds – is unknown; sampling communities at seep complexes with a range of mound ages (e.g. Hydrate Ridge [178]), would help clarify colonization and succession processes.

9.6 Metabolic activity and tools of the future

A number of emerging technologies are poised to facilitate major breakthroughs in activity based environmental microbiology. Some of these new tools are particularly well suited for ANME-SRB consortia, while others will be more generally applicable to uncultured systems. Below, we highlight several compelling opportunities on the horizon alongside specific questions related to seep hosted microbial activity that could thus become accessible.

9.6.1 Preserving microscale spatial arrangements

Analyzing microbial communities while maintaining their spatial configurations will clarify how different organisms interact. Reconstructing interspecies interactions from homogenized results of metaomics studies would allow researchers to propose interactions based on gene, transcript, or protein complementarity, but such inferences are difficult to substantiate, especially because a cell's immediate diffusive environment severely restricts its relevant neighbors at any given time. In order to preserve spatial arrangements, one promising option could be to solidify a sample of sediment or carbonate rock with molten agar, and embed thin section slices in resin. This approach has been used on preconcentrated and density gradient separated consortia to provide detailed images of aggregate cross sections [179].

In concert with methods that retain microscale arrangements, imaging mass spectrometry (IMS) can generate two- and three-dimensional maps of protein [180] or lipid [181] markers. Matrix assisted laser desorption/ionization (MALDI) is a 'soft ionization' technique that mobilizes large molecules for analysis in a mass spectrometer without fragmenting them; this method, upstream of a time-of-flight mass analyzer, is the most common IMS configuration [182]. These methods have been used primarily in animal tissue analyses for biomedical purposes [183]; microbial applications have largely been for diagnostic purposes based on pure culture spectra [184]. The limiting factor that precludes mixed microbial community analysis has been spatial resolution of MALDI laser beams. Recently, however, resolution has improved to 1 μm [185], which is within the realm of usability for microbial consortia and biofilms. Determining the number of resolvable molecules from a given 'pixel' and their quantitation remain substantial obstacles [186].

9.6.2 Single-cell growth rate

Growth rate in seep systems – as determined by increasing biomass – is most commonly determined through cell counts and/or nanoSIMS ^{15}N incorporation. These are both approximate methods that integrate over weeks, months, or years of incubation time; direct links between immediate, microscale environmental conditions such as methane concentration, pH, redox potential, or salinity are difficult to access.

In order to determine single-cell or single-aggregate growth rates, micro- and nanoscale cantilevers can be used. These devices are narrow mechanical sensors; a fluid channel extends out and back along the perimeter in an elongated U-shape. By vibrating the cantilever, it is possible to correlate the resonant frequency to the mass of the material flowing through the channel [187]. Using a series array of cantilevers, recent designs have been able to track a single cell for a few minutes, and microbial cell

growth rates of just 0.02 pg per hour (~2% of a cell's biomass [188]) can be detected. Since the flow is unidirectional, the throughput is orders of magnitude higher than previous iterations of the technology, allowing for a more representative sampling of a population [189]. To date, only eukaryotes and cultured bacteria have been tested, but presumably, mixed microbial systems could be examined as well. For example, maintaining appropriate conditions (activities of dissolved substances, temperature, etc.) through the flow path and depositing cells in an array of microchambers for single-cell sequencing would allow researchers to link identity and genetic makeup with growth rate under environmentally relevant parameters.

9.6.3 Microcalorimetry

Heat production is widely believed to be the result of all metabolic reactions and a wasteful by-product of biological transformations [190]. Researchers have calculated 'growth efficiency' by combining experimental determinations of enthalpy and growth yields with calculations of standard enthalpy values [191]. Advancements in microfluidics and materials science have enabled smaller, more precise microcalorimeters. One recently developed device can measure heats of reaction from 3 nL volumes with 4.2 nW resolution, and was used to quantify heat generated by *in vitro* urease reactions [192]. Experiments with subseafloor crustal fluids comparing experimental and reference ampules in an isothermal calorimeter determined the temperature range of activity from heterotrophic sulfate reduction [193]. Associated Gibbs energy calculations yielded ~ −13 kJ/mol sulfate, a seemingly viable value near previously proposed minimal limits [194].

While the theoretical basis and physiological meaning of biological heat production are not fully developed (for example, endergonic metabolism has been reported in *M. barkeri* [195]), the practice of measuring heat output through calorimetry is an intriguing capability [196, 197]. In particular, the approach is metabolism agnostic, as heat production from all metabolic reactions is integrated into a calorimetric measurement. Community-scale shifts in metabolic activity and substrate use can be observed over time, as demonstrated in a study of heterotrophy in tidal flat sediments [198]. The use of ^{13}C-labeled substrate linked electron acceptor depletion to changing heat outputs, while RNA-SIP revealed the microbial constituents involved. With heat production as a nearly universal product of metabolism, cryptic cycling in metal associated AOM [100] could be untangled. When coupled with activity measurements of specific metabolisms and cross referenced with thermodynamic calculations, the degree to which such metabolisms (e.g. methane oxidation, or nitrogen fixation) contribute to the overall complement of biological activity could be determined.

9.6.4 Raman spectroscopy

Microspectroscopy is an appealing technique because of its broad range of potential analytes, speed, noninvasive nature, single-cell resolution, and compatibility with other stains [199]. Raman spectroscopy excites a sample with photons of a given wavelength and detects those that undergo Stokes scattering, resulting in a lower energy, higher wave number emission [200]. The magnitude of the decreased energy is attributable in part to the masses of the atoms in the illuminated molecule. Because higher atomic masses lead to higher energies and lower wave numbers, stable isotope incorporation of $> 10\%$ can be discerned. In a study of groundwater biofilm, Raman spectroscopy was combined with FISH and ^{13}C amendments to reveal naphthalene-degrading *Pseudomonas* cells with substantial cell-to-cell variation [199]. Incorporation of D from D_2O was used as a general marker of activity to identify key players in a mouse microbiome sample and to separate them for downstream molecular analyses [201].

SIP-Raman is an important addition to the arsenal of activity based tools, but label-free spectroscopic methods could be especially helpful for *in situ* rate measurements. To the degree that specific molecules can be linked to certain absorption bands, the effect of integrated metabolic activity on localized concentrations can be queried. Infrared signatures of molecules like methane, ethane, and carbon dioxide would help researchers visualize and quantify how critical metabolites and greenhouse gases flow through seep systems. Strategies to accommodate such sensors at pressure and to 'see through' complex aqueous solutions will need to be developed, but spectroscopic techniques could allow researchers to study intact ecosystems in exciting new ways.

9.6.5 Replication rates from metagenomic data

Computational treatment of metagenomic data can parse genome location-specific relative copy numbers to determine replication rates in a mixed microbial community. As a genome is copied during cell division, DNA polymerase moves from the origin of replication toward the terminus. Thus, genetic material recovered from rapidly growing populations will display a higher proportion of the origin-adjacent sequence. This approach was pioneered using gut microbiome samples [202]; when applied to environmental communities, investigators demonstrated that Candidate Phyla Radiation organisms [203] from a subsurface aquifer exhibited rare, and as yet unexplained, episodes of high growth rate [204]. Inferences of lineage-specific replication rates could open up a vast trove of metagenomic datasets to activity oriented inquiry, though interpretation of such results in a community context will require additional work.

9.6.6 Single-cell omics

The convergence of technical advances in cell sorting, microfluidics, and sequencing has created a new field of single-cell omics analyses. There are many advantages to pursuing molecular data from individual microbes in a mixed community: core genomes and coexisting subpopulations can be clarified [205], and novel diversity and new metabolic features can be uncovered [7]. In the context of activity based analyses, single-cell genomics, transcriptomics, and proteomics can all provide useful information. When paired with BONCAT-FACS [163] or SIP-Raman [201], individual genomes could be linked with biosynthetic activity, moving beyond FISH-nanoSIMS to provide a full genomic context for differentially active cells. High-throughput single-cell transcriptomics has thus far been restricted to eukaryotic samples [206], and early single-cell proteomic efforts are antibody based, limiting coverage to a few dozen proteins [207, 208].

In addition to technical difficulties associated with sequencing such small quantities of material, isolating cells in an intentional way remains challenging. Microwell dilution [209] or microfluidic droplet based methods [210] have minimal ability to discriminate between cells, while FACS can sort based on limited fluorescence associated information [211]. Integrating methods of isolating cells with traits of interest (e.g. possessing a certain gene, exhibiting high growth rates, or in close spatial association with other cells of interest) will be an important priority for single-cell analyses.

9.7 Conclusions

In the decades since the discovery of seafloor methane seeps, the wide array of activity based tools has exposed a new world of metabolic possibility and revealed seeps as key capacitors in the biogeochemical processing of methane. The novel syntrophic coupling of ANME and SRB has been thoroughly explored, demonstrating a compelling case of energy sharing and electron transfer. Methanotrophic activity, in both sediments and carbonate rocks, is a substantial sink for methane that prevents vast quantities of a strong greenhouse molecule from entering the water column and, potentially, the atmosphere.

However, substantial uncertainties remain, especially pertaining to the global impacts of seep associated methane consumption and the role of non-AOM metabolisms in community structure and broader ecosystem functioning. Planet-wide calculations of methane consumption have depended largely on scaling up lab based rates from point sources; understanding the nuances of subsurface flow and localized concentrations will drastically redraw the methane map. A wider set of microbial players is also well within reach [5, 6, 212]. Producers and consumers of higher hydrocar-

bons (molecules which are found in high concentrations at methane seeps) have been largely ignored, and the ways in which AOM based primary production influences other trophic levels will no doubt reveal myriad other unforeseen interactions and metabolic networks.

Methane seeps are remarkable habitats – one of the few examples of chemosynthetic oases on the seafloor – with a global reach. The tools described here will help researchers assess metabolic activity in all its forms and further clarify the role of microbial communities in driving marine methane seeps and shaping our planet.

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