

Exploring Versatility of Energy Metabolism and Dynamics of Anabolism and Growth in Anaerobic Methanotrophic Consortia

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ABSTRACT

Two main questions are asked in this thesis, how environmental microorganisms respond and persist in an energy-limiting condition, and how we can investigate and disentangle the dynamics of these microbes' activity and growth at a high spatiotemporal resolution. To the end, this thesis focuses on a symbiosis of methane oxidizing archaea (ANME) and sulfate reducing bacteria (SRB), who mediate the anaerobic oxidation of methane (AOM), an important process in the global methane cycle. For the former question, Chapter 2 first found that carbon monoxide (CO) was able to serve as an alternative electron donor for ANME-SRB, and notably CO can even reverse the direction of AOM in ANME archaea to produce methane by the reduction of CO₂. Chapter 3 then explored and verified from the other side the potential role of pyrite (nano)particles in supporting AOM via a predicted reaction at the pyrite-water interface to generate sulfate and iron oxides as electron sinks. For the latter question, Chapter 4 took advantage of stable isotopic probing combined with the high sensitivity and spatial resolution of nanometer-scale secondary ion mass spectrometry (nanoSIMS) approach, and proposed a pipeline of multi-isotope imaging to record and *in situ* read out the single cell activity in the past. Chapter 5 as an in progress work attempts to disentangle the native division process in the yet uncultured ANME archaea by means of serial block face electron microscopy (SBEM) and deep learning imaging analysis. Taken together, this work provides more evidence of the versatile energy metabolism for ANME-SRB symbiosis and at the same time offers solutions to capturing the dynamics of activity and growth in natural microorganisms for the field of environmental microbiology.

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Chapter 1

INTRODUCTION

Environmental microbiology is rooted in the interactions of microorganisms with each other in the intra- and inter-species matter and with their living environment. These interactions profoundly impact microbial physiology, metabolism, and ultimately growth. Challenges arise in this field mainly from the complexity in the context of both the environment and microbes themselves. To this end, questions are intriguing about how natural microbes physiologically adapt to the changing environment, particularly for energy sources, and how their native metabolic activity and growth behave and further are being characterized *in situ* in great spatial and temporal resolution. For these questions, this thesis will cover.

A well-studied yet uncultured microbial consortia of anaerobic oxidation of methane (AOM) was focused, which typically consist of syntrophic species, anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria (SRB) living in the deep-sea methane seep sediments (Knittel & Boetius, 2009; Orphan et al., 2001). The net AOM reaction with sulfate produces scant energy on the edge of thermodynamic feasibility for their survival in the deep ocean (Boetius et al., 2000). Environment is dynamic, particularly in terms of the energy nutrient levels, i.e., methane and sulfate, for ANME and SRB (Orphan et al., 2004; Tryon et al., 2002; Tryon et al., 1999).

Marine ANME archaea are energetically versatile in methane oxidation by using multiple terminal electron acceptors besides sulfate, e.g., metal oxides (manganese (birnessite) and iron (ferrihydrite)) (Beal et al., 2009), humic acids and quinone-like compound 9,10-anthraquinone-2,6-disulfonate (AQDS) (Scheller et al., 2016). These findings inspired us to rethink scenarios where there is a lack of methane or sulfate and how the partnership of ANME/SRB survives by adapting their physiology to utilize alternative electron donor(s) or hunt electron sinks via interacting with the outer environment.

Building on these, in Chapter 2 we examined the possibility of carbon monoxide (CO) serving as an alternative electron donor for ANME/SRB consortia, which was reasonably based on the nature of low reduction potential of CO/CO₂ pair (Grahame & DeMoll, 1995) and the presence of necessary CO metabolizing machinery in

ANME/SRB genomes (this thesis). In parallel with the exploration of other electron sources, in Chapter 3 we investigated possible electron sinks for ANME/SRB who were living down to deep sediments, a region away from the top-down diffusion of sulfate. An ignoring mechanism of reaction between a well-known mineral, pyrite, with water molecules was particularly studied. What's more, beyond studies of alternative energy sources for AOM system in the environment yet under the same topic of nutrient limitation for microbes, a model sulfate reducing bacterium *Ddesulfovibrio vulgaris* was focused in Appendix A. We quantitatively explored the impact of external bioavailable nitrogen input (ammonium) and nitrogen fixation activity on the biomass productivity, and in particular the energy metabolism in the transition phase between these two nitrogen modes.

An important yet understudied and challenging topic in environmental microbiology is to track microbial activity and even growth, especially for uncultured microorganisms and consortia at a spatial-temporal resolution *in situ*. Unlike isolated pure cultures, cells grow fast and are trackable, which allows us to easily visualize the growth by using time-lapse microscopy. When it comes to methane seep sediments, the difficulty is amplified exponentially. Several factors have to be considered. First, ANME/SRB consortia haven't been isolated yet till now due to their slow growth rate with a typical generation time of several months (Orphan et al., 2009). Second, ANME/SRB, microbes of interest, are living in methane seep sediments, which is a complex system mixed with multiple microbial species and tons of inorganic and organic compounds and materials (Orphan et al., 2004). This makes it even harder to visualize them directly with microscopes. Last but not least, temporal resolution is another dimension of information of interest, which will be explored and discussed in this thesis.

The non-toxic nature of stable isotopes combined with the high sensitivity and spatial resolution of nanometer-scale secondary ion mass spectrometry (nanoSIMS) has great potential to quantitatively study microbial processes in bulk natural samples (McGlynn et al., 2015). In Chapter 4, we developed a proof-of-concept experiment which was rooted in nanoSIMS protocol and further combined with a self-curing pulse-chase pipeline with the purpose of recording and *in situ* read out of the biosynthetic activity in the past. Then we moved deeper in Chapter 5, where we explored the division process in the yet uncultured anaerobic methane oxidizing archaea by means of serial block face electron microscopy and deep learning imaging analysis.

These studies in this thesis primarily focused on the ANME/SRB consortia system and discussed two long-standing questions, how they persist in energy-scant environments and how they behave and grow spatiotemporally. Notably in the latter question, this thesis provided novel and inspiring protocols in the work of yet uncultured microorganisms for the field in environmental microbiology.

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*Chapter 2***BEYOND METHANOTROPHY: ANME-SRB CONSORTIA USE
CO AS AN ALTERNATIVE ELECTRON SOURCE**

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2.1 Abstract

Efficient energy metabolism is essential for microorganisms living on the thermodynamic edge. Anaerobic methane-oxidizing archaea (ANME-2) and sulfate-reducing bacteria (SRB) consortia represent a globally relevant syntrophic association capable of growing with minimal amounts of free energy and persisting under methane-limited conditions. Their potential for physiological plasticity, including use of alternate electron donors is poorly understood. Carbon monoxide (CO) is predicted to be a thermodynamically favorable electron donor due to its low reduction potential and presence in the environment. Here we show that environmental ANME-SRB consortia can oxidize CO in the absence of methane, using combination of stable isotope geochemical tracers, metatranscriptomics, and single cell activity measurements (FISH–nanoSIMS). Oxidation of CO was coupled with sulfate-reduction, and, in its absence, through CO₂ reduction by ANME-2. This resulted in the production of methane at one ninth the rate of methanotrophy under conditions supporting anaerobic oxidation of methane. Single cell analysis of anabolic activity indicates CO respiration likely supports cell maintenance rather than active growth, consistent with the observed downregulation of energy generating complexes in ANME (e.g., *mtr*, *rnf*, etc.). The versatile capability of CO oxidation by anaerobic methanotrophic consortia broadens the dimension of carbon cycling in methane seeps and highlights potential mechanisms of resilience under changing geochemical regimes.

2.2 Introduction

Energy availability matters in the deep sea. The anaerobic oxidation of methane (AOM) coupled to sulfate reduction is an important microbial process for controlling methane flux from ocean sediments worldwide (Knittel & Boetius, 2009; Reeburgh, 2007). This process is mediated by anaerobic methanotrophic archaea (ANME), primarily found coupled with the reduction of sulfate living with sulfate-reducing bacteria (SRB) (DeLong, 2007; Knittel et al., 2005; Orphan et al., 2002). Methane-derived energy and carbon from AOM chemolithoautotrophy thus forms a foundation for the broader community at methane seeps (Fisher, 1990). Marine ANME archaea are energetically versatile in methane oxidation by using multiple terminal electron acceptors besides sulfate, e.g., metal oxides (manganese (birnessite) and iron (ferrihydrite)) (Beal et al., 2009), humic acids and quinone-like compound 9,10-anthraquinone-2,6-disulfonate (AQDS) (Scheller et al., 2016). However, the oxidation of alternative electron donors, besides methane, by ANME archaea is rarely investigated.

Members of the ANME-2 within the order *Methanosarcinales* are evolutionarily related to methylotrophic methanogens such as *Methanosarcina* (Chadwick et al., 2022). Previous studies showed that *Methanosarcina* species were able to grow on carbon monoxide (CO) as an energy source (Krzycki et al., 1982; Rother & Metcalf, 2004), opening the possibility that related ANME-2 could also utilize CO. Previous reports pointed out that CO might be prevalent in marine sediments, resulting from the thermal decomposition of organic compounds such as humic acids and phenolic compounds or its release as a byproduct from microbial pathways (Alpert & Banerjee, 1971; Bidwell & Fraser, 1972; Kochetkova et al., 2011; Mara et al., 2023; Maugh, 1972; Radler et al., 1974; Sokolova et al., 2009; Wilson et al., 1970). CO is a thermodynamically favorable electron donor with a low reduction potential of -558 mV (pH 7.0, CO_2/CO) (Grahame & DeMoll, 1995). CO oxidation is broadly distributed both environmentally and phylogenetically, with examples known from *Moorella thermoacetica* (Daniel et al., 1990; Kerby & Zeikus, 1983), purple sulfur bacteria *Rhodopseudomonas rubrum* (Uffen, 1976, 1983) and *Carboxydotherrmus hydrogenoformans* (Svetlichny et al., 1991), some aerobes like *Oligotropha carboxidovorans* (Meyer & Schlegel, 1983), some uncultured microbes such as the soil microbial community contributing the consumption of the atmospheric CO (Bay et al., 2021), some archaea in the phylum *Candidatus Hydrothermarchaeota* in sub-seafloor crustal area (Carr et al., 2019), and also some sulfate reducing bacteria (Parshina et al., 2005). Further, genomic surveys showed a widespread distribu-

tion of anaerobic CO dehydrogenases (CODH) across ANME species including the mono-functional CODH and bi-functional CODH involved in the Wood–Ljungdahl pathway, which all support the potential for CO metabolism for ANME.

This study investigated whether members of environmental ANME-2 and their syntrophic SRB partners can metabolize CO through the combination of microcosm experiments, geochemical and isotopic analysis, metatranscriptomics, and single-cell resolved fluorescence in situ hybridization and nanoscale secondary ion mass spectrometry (FISH–nanoSIMS).

2.3 Results

Both ANME and SRB encode carbon monoxide dehydrogenases

The existence of CO dehydrogenases in ANME-SRB consortia was first investigated through analysis of high-quality genomes representing the recognized marine ANME groups (ANME-1, ANME-2a, ANME-2b, ANME-2c, and ANME-3; 49 binned genomes) (Chadwick et al., 2022) and their syntrophic SRB lineages (Seep-SRB1a, Seep-SRB1g, Seep-SRB2, and HotSeep-1; 65 binned genomes) (Murali et al., 2023). A phylogenetic tree was generated based on this curated dataset of CO dehydrogenase/acetyl-CoA synthase complex subunit (*cdhA*) and monofunctional anaerobic CO dehydrogenase (*cooS*) (Fig. 1). Both categories of CO dehydrogenases (*cdhA* and *cooS*) were found broadly present in diverse ANME and SRB lineages, except homologs of *cooS* which were not detected in the reconstructed genomes of ANME-1 (Fig. 2.1, see the full tree in Fig. 2.9). In addition, several lineages were found to host two copies of *cooS*, including ANME-2a CONS7142G09b1 and Seep-SRB1g str. CR10073A, both of which were verified to occur on contigs with other ANME-2a or Seep-SRB1g genes respectively (Fig. 2.1). The broad distribution of CO dehydrogenases encoded by diverse ANME and SRB lineages served as the motivation for more in depth physiological study of the potential for CO metabolism by anaerobic methanotrophic consortia.

ANME-SRB have the catabolic capability to respire CO and sulfate

To test whether ANME-SRB consortia can respire CO, we performed a series of anaerobic microcosm experiments using methane seep sediments collected from the Costa Rica margin (see Methods). The sediments were dominated by members of *Methanomarinus* (ANME-2b; 53.63%) along with ANME-2a-2b (9.12%) and *Methanogaster* (ANME-2c; 2.75%) groups, and their partners Seep-SRB1 (6.21%) and Seep-SRB4 (0.23%) based on 16S rRNA amplicon sequencing (Fig. 2.10).

We tested the ability to reduce CO coupled with sulfate using ^{13}CO at three different concentrations, 0.1-, 0.4-, and 1.0-bar partial pressure in biological triplicates. A positive control (methane + sulfate; $n = 3$) and a killed control ($n = 1$; 0.1-bar ^{13}CO + sulfate) were also run in parallel (Table 2.3). Labeled ^{13}CO was used to quantify the rate of CO oxidation concomitant with the production of sulfide (HS^-) over the course of 24 days. These experiments demonstrated active CO oxidation alongside the reduction of sulfate, however CO oxidation rates showed a decrease with increasing CO partial pressures (Fig. 2.2). At 0.1 bar, CO was oxidized at a rate of $34.97 \pm 0.68 \mu\text{M d}^{-1} \text{ cm}_{\text{sed}}^{-3}$, equating to approximately half the rate of methane oxidation ($64.63 \pm 0.68 \mu\text{M d}^{-1} \text{ cm}_{\text{sed}}^{-3}$) measured in our positive controls (Table 2.4). This rate decreased from $23.21 \pm 4.67 \mu\text{M d}^{-1} \text{ cm}_{\text{sed}}^{-3}$ to $12.00 \pm 0.07 \mu\text{M d}^{-1} \text{ cm}_{\text{sed}}^{-3}$ in parallel microcosm incubations when CO concentrations increased from 0.4 bar to 1.0 bar in the headspace (Table 2.4), possibly attributed to toxicity of CO at high concentrations as has been reported in other organisms (Rother & Metcalf, 2004; Schöne et al., 2022).

CO oxidation involves another electron sink besides sulfate

An examination of the electron transfer and mass balance for the CO and sulfate redox reaction indicates there were significantly higher rates of CO oxidation in the incubation experiments than could be explained by sulfate reduction alone. The measured rate of CO_2 production ($34.97 \mu\text{M d}^{-1} \text{ cm}_{\text{sed}}^{-3}$) was 7 times greater than the predicted rate ($5.32 \mu\text{M d}^{-1} \text{ cm}_{\text{sed}}^{-3}$) calculated from sulfide production based on Eq. 2.1 (Table 2.4). This discrepancy suggests that under conditions with CO and sulfate, sulfate accounts for only a portion of the electrons from CO oxidation, implying there must be additional electron acceptor(s) in the sediment microcosm beyond sulfate.

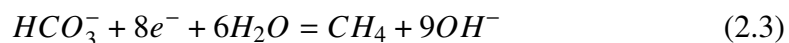
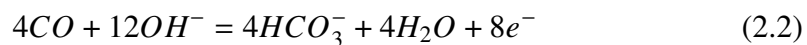


Based on this, we identified two likely alternative electron acceptors, CO and CO_2 (present as bicarbonate (HCO_3^-) in the medium), were sufficient in high enough concentrations to account for this discrepancy. First, CO itself could be reduced by nitrogenases present in ANME and/or SRB (Dekas et al., 2009; Metcalfe et al., 2021). Previous work demonstrated that both vanadium- and molybdenum-nitrogenases are able to reduce CO to diverse hydrocarbons including ethylene (C_2H_4), ethane (C_2H_6) and propane (C_3H_8) (Hu et al., 2011; Lee et al., 2010). However, GC-MS analysis of our microcosm experiments did not detect any of

these alkanes (data not shown) and thus we presume CO reduction by nitrogenase is minimal to nonexistent.

Another possibility is CO₂ (HCO₃⁻) as an electron acceptor. Therein, a number of products are predicted: i) formate; ii) acetate; or iii) methane as reported for *M. acetivorans* grown on CO (Rother & Metcalf, 2004). Formate or acetate was not detected by either NMR or IC (detection limits: formate on NMR, *ca.* 3 μM; acetate on IC, *ca.* 5 μM; data not shown). We then focused on methane production. Theoretically this reaction is thermodynamically favorable, as the reduction potential of CO₂/CH₄ (-245 mV at pH 7.0) (Scheller et al., 2016), is higher than that of CO₂/CO (-558 mV, pH 7.0). Microcosm incubations amended with 0.1 bar CO and 5 mM sulfate were used to confirm that CO continued to be consumed over time, along with the production of sulfide from sulfate (Fig. 2.3a, b). In these bottles, we observed methane production however the concentration did not accumulate above *ca.* 0.2 μmol in the headspace (Fig. 2.3c). This methane concentration, lower than predicted, may be the result of subsequent oxidation by sulfate-coupled AOM (i.e., cryptic methane cycling). Indeed, we observed that methane was able to be oxidized while CO was also present by spiking in ¹²CO during a ¹³CH₄ + sulfate time course experiment (Fig. 2.4).

To further investigate methane production from CO oxidation in the absence of sulfate-coupled AOM, we followed up with a series of sulfate-free microcosm experiments supplied with 0.4 bar partial pressure CO (Table 2.3). These conditions should diminish the activity of the syntrophic SRB and halt any subsequent oxidation of the produced CH₄ by ANME. By supplying HCO₃⁻ as the sole terminal electron acceptor, a continuously increasing amount of methane was produced coupled to CO consumption at a rate of 0.36 μmol CO d⁻¹ cm_{sed}⁻³ (mean, n = 2; Fig. 2.5). Parallel microcosm experiments without addition of HCO₃⁻ to the medium were also run. These results showed CO oxidation was also coupled with methane production under these conditions and at comparable rates of 0.35 μmol CO d⁻¹ cm_{sed}⁻³ (mean, n = 3). This suggests that self-generated CO₂ may also serve the terminal electron acceptor. Most importantly, the ratio of CO oxidation rate to methane production rate calculated from the five replicates was 3.85 ± 1.93, close to the predicted stoichiometry of 4:1 in Eq. 2.4.





To assess whether methane was sourced from CO₂, amendments of ¹³CO (≥ 99 atom % ¹³C) and ¹²C-HCO₃⁻ (100%) were used (incubation set #4; Table 2.3). In these experiments the presence of ¹³C in the bicarbonate pool would be through CO oxidation (Eq. 2.4; Fig. 2.5c), and any methane production from CO₂ reduction should reveal a mixed ¹²C/¹³C ratio if both processes are occurring. Results from these isotope labeling experiments confirmed this hypothesis, producing a portion of ¹³C-labeled methane with a value of 40.64 ± 0.68% (n = 3) (Fig. 2.11).

ANME and SRB consortia show anabolic activity by FISH–nanoSIMS when supplied with CO as the electron donor

Having demonstrated that CO oxidation occurs in seep sediments coupled with the reduction of either sulfate or CO₂, we then asked whether this bulk anaerobic CO respiration in turn supported anabolic activity by ANME-2 and their SRB partners in the absence of methane addition. Single cell anabolic activity was measured using stable isotope probing with ¹⁵N-ammonium as a marker for biosynthetic activity combined with FISH–nanoSIMS (Krüger et al., 2008; McGlynn et al., 2015; Orphan et al., 2009). In these 4-month CO incubations, sulfate and bicarbonate served as electron accepters along with ¹⁵N-ammonium addition.

A total 4,979 FISH-identified cells within 42 consortia of ANME-2b (ANME-2b-729 oligonucleotide probe) and their SRB partner (likely Seep-SRB1g (Metcalf et al., 2021); hybridized with a Desulfosarcina/Desulfococcus group probe (DSS658)) were analyzed from Set #1 of incubation experiments (Table 2.3). Cellular ¹⁵N enrichment was measured in both ANME-2b and syntrophic SRB cells (Fig. 2.6a,b, Table 2.1), suggesting that both were anabolically active during CO oxidation coupled with sulfate reduction in the absence of external methane addition. Notably, high concentrations of CO in the headspace resulted in lower levels of cellular ¹⁵N enrichment, consistent with some degree of toxicity (Fig. 2.6b, Table 2.1). Anabolic activity decreased to a greater extent with increasing concentrations of CO for both ANME (1.0 bar CO) and SRB (0.4 bar and 1.0 bar), with enrichment levels below the baseline of ¹⁵N fractional abundance (here determined to be 0.53; Table 2.1). This observation is consistent with reports from CO metabolizing methanogens where higher levels of CO inhibited methanogenic activity (Daniels et al., 1977; Rother & Metcalf, 2004). When CO concentrations were provided at

0.4 bar, however, approximately 22% of the ANME-2b cells analyzed (186 out of 831 cells) remained anabolically active at a considerable level, while the fraction of active SRB cells dropped dramatically (6 out of 488 cells). This may point to a higher CO tolerance by ANME-2b relative to their SRB partner.

To further test whether ANME-2b was responsible for the measured CO oxidation and methane production and exclude the potential contribution of other microbial groups (i.e., methanogens), an additional set of FISH–nanoSIMS experiments were performed using sulfate-free conditions over a 3-month period (Figs. 2.6c and 2.6d, Table 2.1). Cellular ^{15}N enrichment for 2,464 ROIs (cells) belonging to ANME-2b and their SRB partners were resolved. ANME-2b cells showed an average ^{15}N fractional abundance of 0.67 ± 0.26 (470 of 1,684), while co-occurring SRB cells were anabolically inactive, with the exception of a few cells (6 out of 780); (Fig. 2.6d). The observation of ANME-2b anabolic activity in the absence of activity by their SRB partner supported their direct involvement in methane production during respiration of CO as an energy source.

We assessed the relative differences between CO oxidation and anaerobic methanotrophy supporting ANME growth using the cell-specific growth rates from FISH-nanoSIMS and bulk sediment respiration rates (Table 2.2). With CO addition (0.4 bar), methane production by ANME-2b occurred with an average rate that was approximately 1/9 of the sulfate-coupled AOM rate determined in parallel incubations supplied with methane and sulfate (Table 2.2). However, the apparent cellular energy gain from rate of growth was minimal, with ANME-2b growth on CO (0.4 bar) and CO_2 nearly 20 times less than the canonical AOM coupled sulfate reduction (Table 2.2). This suggests the observed ANME-2b CO oxidation may be used to support maintenance activity, rather than growth.

Spatial patterns of cellular activity grown on CO

The spatial patterns of activities from ANME-2b and syntrophic SRB were examined (Fig. 2.12). Under CO/sulfate/bicarbonate conditions, ANME-2b cells on average were more active (^{15}N atom % enrichment) than their corresponding SRB partners within each consortium, which differs from the standard methane/sulfate condition where the average activity of archaeal and bacterial populations were correlated, and close to 1:1 as demonstrated previously (McGlynn et al., 2015; Scheller et al., 2016) (Fig. 2.12a). This is reasonable that ANME specifically utilized bicarbonate as an additional electron acceptor other than sulfate when respiring CO as an electron

source. Besides, ANME-2b and syntrophic SRB living on CO showed distance-independent trends in cellular activity (Figs. 2.12b, c). This is contrast to what is predicted in the case of syntrophic exchange or sharing of a diffusible intermediate (Alperin & Hoehler, 2009; Milucka et al., 2012; Orcutt & Meile, 2008), that is, a weak syntrophic interaction in the CO-living consortia.

Metatranscriptomic analysis of ANME-2b/SRB response to CO

Metatranscriptomics was performed to investigate the underlying metabolic response of CO metabolism compared with methane (1.5 bar methane, 0.1 and 1.0 bar CO) using either sulfate or bicarbonate as the electron acceptors (n = 3 biological triplicates for each treatment, 9 incubations total). Nearly 90% of the reads from these experiments were affiliated with the dominant AOM consortia members, ANME-2b and syntrophic SeepSRB-1g, consistent with the 16S rRNA amplicon sequencing results (Fig. 2.10). In the remaining pool of transcripts, neither did we resolve any other active taxonomy at a reasonable level, nor did we see an active transcription of *mcr* genes from methanogens. The dominance of transcripts affiliated with AOM consortia supports our interpretation that ANME-2b and their syntrophic SRB partner were responsible for CO oxidation. Based on these findings, we focused our subsequent transcriptomic analyses on ANME-2b and Seep-SRB1g. To better illustrate the transcriptomic activity, both differential gene expression (hereafter as DE) (DESeq2) and percentile ranking (Sleuth TPMs (transcripts per million reads)) were analyzed.

For ANME-2b, the expression of most genes did not change significantly between CO and methane treatments in terms of overall transcripts per million reads (TPM) distribution (Fig. 2.13). Approximately 2.79% of ANME genes were significantly higher expression and 13.21% were lower in the microcosms with 0.1 bar CO compared with the control AOM conditions supplied with CH₄ and sulfate (Fig. 2.14). Of the genes found to be significantly upregulated in the CO condition compared to the CH₄ condition, most were associated with stress response, including defense mechanisms, toxin/antitoxin systems, and stress-related ABC exporters (ABC-type antimicrobial peptide transport system) (Supplementary Data S1). This indicates that CO is a less favorable substrate for ANME-2b respiration compared with CH₄, and may be toxic to some degree.

Interestingly, the expression of the ANME-2b carbon monoxide dehydrogenase, *cooS*, occurred at similar levels in the methane/sulfate vs. CO/sulfate or

CO/bicarbonate conditions, with mean values of $52.3 \pm 5.1\%$ (CH_4), $48.2 \pm 3.4\%$ (0.1 bar CO), and $55.1 \pm 3.4\%$ (1.0 bar CO), (Supplementary Data S1). In contrast to *cooS*, significantly lower expression levels were found for the full *cdh* operon, which is an integral component of the Wood-Ljungdahl pathway ($66.3 \pm 13.6\%$ (CH_4) vs. $49.9 \pm 11.6\%$ (0.1 bar CO), $p < 0.001$ (two-tailed t-test)); (Supplementary Data S1). This suggests a different response of the two types of CO dehydrogenases when supplied with an external source of CO.

In support of the potential for cryptic methane cycling suggested from our geochemical analyses and isotope labeling experiments, we observed a high level of expression for the majority of genes associated with methanotrophy/methanogenesis and carbon fixation pathways by ANME-2b within the CO/sulfate or bicarbonate condition, that was at a comparable level with the methane/sulfate treatment supporting AOM (Fig. 2.7, Supplementary Data S1). This included the methyl-coenzyme M reductase (*mcr*) operon ($>99.8\%$ among all conditions), 5,10-methylenetetrahydromethanopterin reductase (*mer*) (mean: 99.1% (CH_4), 97.4% (0.1 bar CO), 96.8% (1.0 bar CO)), and formylmethanofuran dehydrogenase (*fwd*) operon (mean: 96.6% (CH_4), 94.0% (0.1 bar CO), 93.8% (1.0 bar CO)) (Fig. 2.7, Supplementary Data S1). In contrast, N^5 -methyl-H4SPT:HS-CoM methyltransferase (*mtr*) operon, a key energy converting step in the methanogenesis pathway, had a significantly lower expression level in CO/sulfate/bicarbonate conditions compared to the parallel methane/sulfate treatment (0.1 bar CO vs. CH_4 ; percentile: $84.7 \pm 3.3\%$ vs. $98.2 \pm 0.6\%$, mean $\pm$ s.d.; differential analysis: *ca.* 25-fold decrease on average) (Figs. 2.7 and 2.15, Supplementary Data S1).

Further analysis of ANME-2b expression patterns revealed that other key genes involved in the pathways of energy conservation and syntrophic electron transfer (Chadwick et al., 2022) also had a lower expression level with CO compared to methane, both in terms of ranking and DE analysis (Figs. 2.7 and 2.15). For example, the NADH-ubiquinone/plastoquinone oxidoreductase (*fpo*) operon, involved in F_{420} conversion, was down-regulated under CO/sulfate/bicarbonate treatments (ranking: 84.9% (0.1 bar CO) and 83.9% (1.0 bar CO) vs. 96.7% (CH_4)), representing a 9-fold decrease in differential expression (DE) (0.1 bar CO vs. CH_4 ; Figs. 2.7 and 2.15, Supplementary Data S1). In addition, expression of genes associated with the Na^+ -translocating ferredoxin:NAD $^+$ oxidoreductase (*rnf*) were also significantly lower in CO/sulfate/bicarbonate treatments (ranking: mean, 71.4% (CH_4), 43.4% (0.1 bar CO), 49.4% (1.0 bar CO); DE: *ca.* 4-fold decrease (0.1 bar CO vs. CH_4);

Figs. 2.7 and 2.15, Supplementary Data S1). The decrease in transcription of *mtr*, *fpo*, and *rnf* indicates that while these energy conserving pathways remain during CO oxidation in ANME-2b, this respiratory process is clearly a deviation from their primary methanotrophic metabolism.

The transcriptional response of the syntrophic SeepSRB-1g partner revealed an opposite response to CO in comparison with ANME-2b, where 21.00% of expressed genes were significantly elevated in treatments with 0.1 bar CO with either sulfate or bicarbonate as the electron acceptor compared with the methane/sulfate treatment (Fig. 2.13), and only 1.52% of genes were significantly down-regulated (Fig. 6). This expression pattern changed substantially in the high CO condition (1.0 bar CO), where the proportion of genes down-regulated increased to 14.42%, and only 2.32% of genes were highly expressed (Fig. 2.14).

Like ANME-2b, SeepSRB-1g actively expressed CO dehydrogenases among CH₄ and CO treatments, where both the *cooS* and the *cdh* operons had comparable rankings in expression between methane/sulfate and CO/sulfate/bicarbonate (0.1 bar CO) condition (Fig. 2.7, Supplementary Data S2). At higher CO concentrations however, *cdh* operon gene expression was significantly down-regulated (1.0 bar CO). Most genes involved in SRB respiratory pathways were highly expressed in all conditions (percentile ranking >80%) including the quinone-interacting membrane-bound oxidoreductase complex (*qmo*), dissimilatory-type sulfite reductase (*dsr*), sulfate adenylyltransferase (*sat*) and adenylyl-sulfate reductase (*apr*); (Fig. 2.7, Supplementary Data S2). However, the expression level for genes belonging to the multi-subunit Na⁺/H⁺ antiporter (*mnh*) operon changed dramatically between methane/sulfate (mean ranking: 9.7%, CH₄) and CO/sulfate/bicarbonate conditions (mean ranking 86.5%, 0.1 bar CO), representing an average 188-fold increase in expression (0.1 bar CO vs. CH₄) (Figs. 2.7 and 2.15, Supplementary Data S2). Genes previously identified as involved in syntrophic electron transfer and carbon fixation showed equivalent expression levels in treatments with 0.1 bar CO vs. CH₄/sulfate.

2.4 Discussion

CO oxidation by ANME-2b/syntrophic SRB

Here, we used multiple lines of evidence to demonstrate that ANME and syntrophic SRB are both capable of and responsible for CO oxidation in seep sediment incubations. Central to this conclusion was verifying that CO oxidation was carried out

by anaerobic methanotrophic consortia rather than by co-occurring methanogens or other carboxydophilic microbes, given the complex and diverse microbial community present within our sediment microcosms. Several lines of evidence support this finding.

First, in incubations with CO as the sole energy source and external methane was absent in the headspace, CO oxidation was observed alongside the reduction of both sulfate and CO₂ (bicarbonate) as electron acceptors, resulting in the production of sulfide and methane (Figs 2.2 and 2.3, Table 2.4). FISH–nanoSIMS analysis confirmed the anabolic activity of ANME-SRB under these conditions. Additionally, GC-MS analysis verified that methane production was linked to CO₂ reduction (Fig 2.6a and 2.6b, Table 2.1). However, the detection of activity by ANME-SRB on its own did not unequivocally identify them as the primary CO oxidizers, as it remained possible that another microorganism was oxidizing CO and generating methane which in turn fueled AOM by ANME-SRB in the presence of sulfate.

To further investigate the direct role of ANME-2 and SRB in CO oxidation, we performed a parallel experiment where the same seep sediments were incubated in a sulfate-free medium, to minimize sulfate-coupled AOM. In this condition, CO₂ served as the sole electron acceptor and coupled with the oxidation of CO, producing methane. Notably, ANME-2b cells were anabolically active in these treatments, evidenced by stable isotope probing and FISH–nanoSIMS analysis of cellular ¹⁵N enrichment (Figs 2.6c and 2.6d, Table 2.1). Previous studies have reported that ANME are not able to oxidize methane with CO₂ as the electron acceptor (Yu et al., 2021). Here our results confirmed the capability of ANME-2b to oxidize CO coupled with methane production. Together, these findings indicate that ANME-SRB remain active even in the absence of an external methane source.

Furthermore, 16S rRNA amplicon sequencing after four months of incubation did not recover known methanogens in our microcosm experiments (Fig. 2.10). While certain methanogens like *Methanosarcina* sp. are known to produce acetate and formate as major products along with methane during growth on CO (Rother & Metcalf, 2004), neither metabolite was detected in our experiments. Moreover, metatranscriptomic analysis revealed no transcription of CO dehydrogenases or *mcr* genes associated with methanogenic archaea, which was overwhelmingly dominated by ANME-2b and Seep-SRB1g transcripts. Collectively, these findings reduce the likelihood that non-ANME-SRB microbes had any meaningful contribution to the CO oxidation occurring in our experiments. Taken together, this evidence

strongly supports that anaerobic methanotrophic consortia, specifically ANME-2b/SeepSRB-1g, are capable of respiring CO as an energy source and producing methane from the reduction of CO₂.

Physiologically, both catabolic and anabolic activity exhibited a decreasing trend with increasing CO concentration, although cell-specific activity remained high at low CO levels (0.1 bar; Figs 2.2 and 2.3 and 2.6, Table 2.1, Table 2.4). This trend is consistent with previous reports of microbial inhibition in response to CO exposure (Ragsdale, 2004; Rother & Metcalf, 2004) and is also supported by the higher expression levels of stress-related genes under CO conditions (Table 2.4). A CO partial pressure of 0.1 bar in the headspace corresponds to 112.83 μ M dissolved in seawater at 4°C (Douglas, 1967; Schmidt, 1979), which is two to three orders of magnitude higher than CO concentrations reported from a cold methane seep (hundreds of nM) based on data from the International Ocean Discovery Program (IODP) Expedition 385 in the Guaymas Basin (Mara et al., 2023). Thus it is likely that CO metabolism by anaerobic methanotrophs may be more pronounced in the natural environments where CO supply is lower.

Growth or maintenance?

Methane production by ANME-2b was observed at a rate of approximately one-ninth of the AOM rate in parallel incubations with 0.4 bar CO (Table 2.2). This observation raised the question of whether ANME-2b archaea are obligate methanotrophs or if they can sustain anabolism through CO oxidation coupled to methane generation. We calculated the potential for anabolic biosynthesis and energy generation from redox reactions between the terminal electron donor and acceptor over time using a combination of our geochemistry and nanoSIMS data (Table 2.2). Notably, ANME-2b methane production from CO (0.4 bar) and CO₂ resulted in minimal biomass yield per unit of potential energy gained, around 20 times lower than that of canonical sulfate-coupled AOM. This implies that CO metabolism primarily supports maintenance of ANME-2b activity, rather than enabling significant growth. Several factors may explain the low efficiency of energy utilization for biosynthesis.

Methanogenesis may not a favorable energy conservation pathway for ANME grown on CO. Studies of CO metabolism by *Methanosarcina acetivorans* have shown that acetate and formate production via the acetyl-CoA pathway (involving substrate-level phosphorylation) is the predominant CO-dependent catabolic pathway, rather

than methanogenesis (Rother & Metcalf, 2004; Schöne et al., 2022). In these studies, more than 75% of the CO was converted to acetate, while less than 10% was released as methane during carboxydrotrophic growth. However, in our microcosm experiments with ANME-2b, neither acetate nor formate was detected during CO oxidation. Furthermore, phosphotransacetylase (*pta*) and acetate kinase (*ackA*), two critical genes involved in substrate-level phosphorylation, are absent from ANME genomes (Chadwick et al., 2022). Therefore, energy conservation via the acetyl-CoA pathway is unlikely to be occurring through the same mechanism as reported in methanogens.

Another possibility is the presence of an uncharacterized cytoplasmic enzyme associated with the methanogenesis pathway that catalyzes the methyl-H₄SPT reaction without contributing to energy conservation. During CO oxidation, we observed a substantial downregulation of genes from the methanogenesis pathway (Fig. 2.15). In particular, the N⁵-methyl-H₄SPT:HS-CoM methyltransferase (*mtr*) operon, which catalyzes the energy-conserving (sodium-pumping) methyl transfer reaction, exhibited significantly lower expression under CO conditions (Fig. 2.15, Supplementary Data S1). This suggests a diminished role for the Mtr complex compared with other steps in the methanogenic pathway, potentially explaining the limited energy conservation available for biosynthesis.

The observed ANME-2b expression pattern is consistent with reports of a substantial decrease in *mtr* transcripts for *M. acetivorans* when grown on CO compared to methanol as the energy substrate (Lessner et al., 2006). Concurrent with the decrease in *mtr*, the expression levels of the *mts* system in *M. acetivorans* (*mtsD*, *mtsF*, and *mtsH*) was significantly upregulated under carboxydrotrophic conditions (Lessner et al., 2006). Based on these findings, Ferry et al. proposed a potential bypass for Mtr via direct cytoplasmic methyl transfer at the expense of minimal energy conservation (Oelgeschläger & Rother, 2009). *Mts* genes encode a predicted cytoplasmic methyl-tetrahydromethanopterin:CoM (CH₃-THMPT:HS-CoM) methyltransferase, which was predicted to serve as an alternative pathway when *mtr* expression is downregulated under CO conditions. Biochemical enzymatic assays using exogenous protein expression demonstrated CH₃-THMPT:HS-CoM methyltransferase activity (Vepachedu & Ferry, 2012). However, follow up studies using in vivo mutation and physiological experiments did not support this hypothesis, here concluding that Mtr is central to methanogenic metabolism and cannot be bypassed easily (Schöne et al., 2023).

Supporting this interpretation, homologs to *mts* were not detected in the ANME-2b genome, indicating that the proposed bypass mechanism in (Lessner et al., 2006; Oelgeschläger & Rother, 2009) is unlikely to compensate the down-regulation of *mtr* in ANME-2b. Consequently, the question why ANME-2b *mtr* expression is low with CO and whether this step can be bypassed remains unresolved. Overall, the absence or inhibition of key energy conservation machinery in ANME-2b under CO conditions likely accounts for the low efficiency of energy conservation for anabolism. Instead, the moderate level of methane production from CO₂ reduction coupled with CO oxidation may serve as a mechanism for CO detoxification and maintenance of metabolic activity under conditions unfavorable for AOM. Similar low assimilation efficiencies have been reported in other microorganisms, such as sulfate-reducing bacteria during respiration of extracellular metal oxides (Bond & Lovley, 2002; Coleman et al., 1993; Lovley et al., 1993), and methanogenic hydrocarbon-oxidizing archaea metabolizing long-chain alkanes to methane (Cheng et al., 2013; Fowler et al., 2012; Zhou et al., 2022).

The question of whether methanotrophic ANME lineages possess net methanogenic capabilities has been a topic of debate for the past decade (Bertram et al., 2013; Beulig et al., 2019; House et al., 2009; Lloyd et al., 2011). To date, there is no direct evidence supporting energy conserving net methanogenesis in ANME lineages under in situ conditions, despite possibility holds that some ANME-1 lineages harbor hydrogenases to potentially support hydrogenotrophic methane production (Chadwick et al., 2022). However, due to the inability of using H₂, acetate or methyl compounds for ANME-2 lineages as energy source (Chadwick et al., 2022), our experiments provide evidence for CO-dependent methane production coupled with CO₂ reduction by ANME-2b archaea. The ANME-2b and other ANME-2 lineages may use this process to sustain basic metabolic activity (e.g., maintenance energy), although it is still uncertain whether this translates to sufficient anabolic activity to support cell division. While high CO levels (e.g., partial pressures of 0.1 bar or 0.4 bar), appeared to trigger stress responses in ANME consortia based on metatranscriptomic data, it remains possible that at lower CO concentrations likely existing in nature, growth rates and metabolism may be more favorable.

CO metabolism in anaerobic methanotrophic consortia

CO oxidation and energy metabolism in ANME

While CO oxidation was demonstrated by ANME-2b, its activity was relatively low compared with conditions supporting methane oxidation. Transcriptomic analysis revealed an overall downregulation of gene expression, with most low expression genes associated with the electron transfer chain (Figs. 2.7 and 2.15). These findings align with cell-targeted FISH-nanoSIMS data, which showed lower anabolic activity under CO conditions compared with methane incubations (Table 2.1). The core ANME-2b catabolic process here is the oxidation of CO, the terminal electron donor, by the cytoplasmic CO dehydrogenase.

Unexpectedly, we did not observe greater expression of CO dehydrogenases transcripts (i.e., *cooS*) in ANME under CO conditions compared to incubations with methane (Figs. 2.7 and 2.15). A similar trend has been reported in *M. acetivorans*, where one *cooS* copy was downregulated in CO-grown versus acetate-metabolizing cells (Lessner et al., 2006). However, more direct enzymatic assays have shown that cultures of CO-metabolizing *M. acetivorans* exhibit approximately three-fold higher CO dehydrogenase activity compared to methanol-grown cultures (Rother & Metcalf, 2004). This suggests the possibility that ANME-2b may regulate *cooS* activity at the posttranscriptional or enzymatic level rather than through changes in gene expression when respiring CO.

When sulfate served as the terminal electron sink, electrons derived from CO were presumably transferred extracellularly to the syntrophic SRB partner to complete the reaction. The electron transfer pathway for CO metabolism appears to share the same pathway as methane oxidation (AOM) for shuttling electrons across the cytoplasmic membrane and into the extracellular space (see Fig. 2.8b, green arrows in the upper panel). Membrane-bound enzymes Rnf and/or Fpo, are hypothesized to reduce ferredoxin (Fd^{2-}) and F_{420}H_2 which subsequently deliver electrons to the membrane-associated electron carrier methanophenazine (Mp). Mp then transfers electrons to membrane-bound multi-heme cytochromes c, which facilitate electron transfer to the syntrophic partner through Direct Interspecies Electron Transfer (DIET) (McGlynn et al., 2015). Notably, both the Rnf and Fpo operations were significantly downregulated under the sulfate-CO condition compared to the sulfate-methane treatment (Fig. 2.15, Supplementary Data S1), suggesting a metabolic preference for methane. However, despite the comparatively low expression levels, these pathways appeared to remain active.

Percentile profiling of whole transcripts for each condition showed that the *fpo* operon had a comparable expression level ($80.14 \pm 7.42\%$ (0.1bar-CO) vs. $96.83 \pm 2.05\%$ (CH₄)), while the *rnf* operon exhibited a lower percentile ranking in the CO treatment ($35.78 \pm 12.16\%$ (0.1bar-CO) vs. $74.50 \pm 8.75\%$ (CH₄)) (Fig. 2.7, Supplementary Data S1). Although both membrane enzyme complexes are predicted to be involved in electron transfer, the expression data suggests a potentially greater role for Fpo during CO oxidation.

Expression patterns were also analyzed in the context of methane production, with CO₂ represented the terminal electron sink. In this scenario, electrons carried by Fd²⁻ and F₄₂₀H₂ as electron donors are transferred from formyl-methanofuran dehydrogenase (Fwd) to F₄₂₀-dependent N⁵,N¹⁰-methylene-H₄MPT dehydrogenase (Mtd) and methylene-H₄MPT reductase (Mer) during CO₂ reduction to methane. Importantly, the *mcr* operon exhibited one of the highest percentile expression rankings (*ca.* 99.8%, 0.1bar-CO) (Fig. 2.7, Supplementary Data S1), remaining essentially unchanged compared to the methane condition. The high transcription activity of Mcr is consistent with its role in methane generation by ANME-2b and, possibly, in the cryptic consumption of newly synthesized methane under conditions where both CO and sulfate were provided (Fig. 2.3).

CO oxidation and hypothesized energy metabolism in the syntrophic Seep-SRB1g

Under incubation conditions with sulfate and CO, both CO and newly produced methane may serve as electron donors for ANME-2, potentially enabling DIET between the syntrophic partners. In this scenario, respiratory electron transfer would follow the same pathways as under standard sulfate-coupled AOM.

Electrons are transferred to their syntrophic SRB partner via multi-heme cytochrome c proteins, which couples these electrons to sulfate reduction. Electrons delivered from extracellular proteins in the SRB partner are believed to be routed through the periplasm and used to reduce quinone pools in the cytoplasmic membrane. These electrons are ultimately used for energy generation through the dissimilatory sulfate reduction pathway.

Expression data from the SRB partner supports this scenario. Genes encoding extracellular multi-heme cytochrome c proteins were significantly upregulated in the syntrophic Seep-SRB1g under CO conditions. This includes OmcX (fold change: 2.72 (1.0 bar-CO vs. CH₄), $P_{adj} = 5.78E-08$) and TmlA (fold change: 3.15 (1.0bar-

CO vs. CH₄), $P_{adj} = 0.027$) (Fig. 2.15, Supplementary Data S2). Both genes are predicted to be involved in extracellular electron transfer (Murali et al., 2023). Here, TmlA is hypothesized to form a distinct porin:multiheme cytochrome c conduit, differing from the OetI-type conduit that has been shown to be highly expressed in previous studies of ANME-SRB consortia (Krukenberg et al., 2018; Yu et al., 2021). This conduit is uniquely present in the Seep-SRB1g genomes (Murali et al., 2023), and its upregulation under CO + sulfate conditions suggests a distinct physiological role from the OetI-type conduit, and characteristic of the ANME-2b/Seep-SRB1g partnership.

The syntrophic SRB cannot directly use methane as their electron donor, requiring a metabolic partnership with ANME to obtain electrons extracellularly sourced from methane-oxidation. However, when CO replaces methane as the electron donor in the system, two major changes in the ANME-SRB partnership are predicted: (i) CO oxidation by CO dehydrogenase in the cytoplasm serves as the primary electron source for both ANME and SRB. Notably, this predicts that respiratory electron transfer in SRB would be stimulated intracellularly rather than extracellularly. The shift in the direction of electron flow likely necessitates metabolic adjustment to optimize electron transport pathways. (ii) The ability to respire CO and sulfate enables the syntrophic SRB to sustain catabolic activity independent of its ANME partner.

CO oxidation is facilitated by CO dehydrogenases, which reduce ferredoxin. Electrons from reduced ferredoxin and NAD⁺ can also be used in generating sodium motive force through membrane-bound Rnf and Mnh complexes. Mnh functions as a Na⁺/H⁺ antiporter, transporting Na⁺ or H⁺ in response to Rnf activity. Notably, Mnh was significantly upregulated under the CO/sulfate condition, supporting its role in this process. For instance, MnhB exhibited a fold change of 19.63 ($P_{adj} = 0.00000078$) under 0.1bar CO versus CH₄ condition, while MnhD showed the highest increase with a fold change of 593 ($P_{adj} = 1.15E-11$) (Fig. 2.15, Supplementary Data S2). Percentile ranking profiling revealed little mnh expression in the methane condition (approximately 0%), but high activity under CO conditions (80-95% of all genes in Seep-SRB1g genome) (Supplementary Data S2). This observation strongly suggests that syntrophic Seep-SRB1g are more dependent on ferredoxin for energy conservation in the presence of CO than methane.

The sodium motive force generated by this process energizes the membrane and is predicted to drive ATP synthesis via ATP synthases as sodium ions are pumped

through the membrane. The ATP generated is subsequently used to activate sulfate by Sat, which reduces sulfate to adenosine 5'-phosphosulfate (APS). Electrons from reduced quinones are transferred to AprAB via Qmo complexes, catalyzing the reduction of APS to sulfite (SO_3^{2-}). Additional electron transfer occurs through DsrMKJOP complexes, from quinones to DsrC and ultimately to DsrAB, where sulfide (HS^-) is generated. The mechanism for DsrC cycling plays an important role in providing an endogenous electron source, as mentioned above.

Flx-Hdr complex serves as a key component in this process, oxidizing two molecules of NADH to reduce one molecule each of ferredoxin and DsrC (Murali et al., 2023). This coupling effectively recycles ferredoxin from Rnf activity, while generating reduced DsrC for dissimilatory sulfate reduction. The preference for ferredoxin, as reflected in the high expression of Mnh genes, along with the endogenous cycling of DsrC, offers a potential mechanism for the syntrophic Seep-SRB1g to live independently on CO with sulfate.

2.5 Conclusion

In this study, we leveraged microcosm experiments combined with geochemical monitoring, single cell activity analysis by FISH–nanoSIMS, and metatranscriptomic profiling to demonstrate the oxidation of carbon monoxide by anaerobic methanotrophic consortia consisting of ANME-2b archaea and Seep-SRB1g sulfate-reducing bacteria beyond methanotrophy.

Physiologically, the low reduction potential of the CO/CO_2 redox pair may favor atypical metabolic pathways under certain conditions. The CO oxidation derived from CO dehydrogenases in ANME-2b starts the electron flow intracellularly and further proceeds via various electron carriers like ferredoxin and F_{420} in the energy metabolism. Concomitant to the cycling of those carriers partially in turn boosts the reduction of CO_2 and the production of methane at end through the methanogenesis pathway in ANME-2b (Fig. 2.8). The cycling maintains the cellular redox balance for ANME archaea, which potentially favors their persistence in deep-sea sediments in the absence of external methane.

Ecologically, the widespread distribution of CO dehydrogenases in both ANME and SRB genomes suggests that both partners in the syntrophic association have the potential to oxidize CO. This metabolic flexibility enables ANME-2b to generate methane while simultaneously lowering CO concentrations to levels that allow the anaerobic oxidation of methane (AOM) to proceed. The offset was demonstrated

between predicted free energy yield and ability of the ANME archaea to convert this into anabolic activity under CO condition comparing to that of CH₄. This eventually controls the growth rate and biomass yield and maintain the size of the population, which may ultimately benefit the methanotrophic community who live in the energy-limited environments. The discovery of CO oxidation by anaerobic methanotrophic consortia in methane seep sediments provide new insights into microbial contributions and interactions influencing carbon cycling in marine ecosystems. The capacity of these consortia to utilize both CO and methane underscores their ecological significance in biogeochemical processes that regulate carbon fluxes in the deep ocean.

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Author contributions

V.J.O. conceived and supervised the project; Y.G. and V.J.O designed the methodology; Y.G. performed the experiments; D.R.U. and Y.G. performed the metatranscriptomic data treatment and analysis; R.M., V.J.O and Y.G. performed the metabolic analysis of ANME-2b and syntrophic SRB; Y.G. generated the original draft of the manuscript, and all authors provided advice and contributed to the final drafting of

the manuscript.

Competing interests

The authors declare no competing interests.

2.6 Materials and methods

Sediment collection and processing

Methane seep sediments covered with white microbial mats were collected by push core (PC6) in the dive AD4912 on 27 May 2017 by DSV Alvin, launched from R/V Atlantis on research cruise AT37-13 in Jaco Scar, off Costa Rica, at 1,811-m water depth (lat 9.1163, long 284.8372). The sediment core was sliced into 3-cm-thick horizons shipboard which were stored individually in separate Whirl-Pak bags and kept anaerobically at 4 °C in sealed Mylar bags sparged with Ar until they were shipped to the laboratory. Sediments (serial number #10079) from Costa Rica were particularly maintained and used in this study.

Artificial seawater composition

Artificial seawater (ASW) (final concentration) was used in the study modified from reference (Yu et al., 2021). The composition of the medium was: NaCl 457 mM, Na₂SO₄ 10.0 mM, MgCl₂ 47 mM, HEPES (pH 7.5) 25 mM, NH₄Cl 2.0 mM, KCl 7.0 mM, NaHCO₃ 5.0 mM, CaCl₂ 1.0 mM, K₂HPO₄ 1.0 mM, SeO₃²⁻ 0.01 μM, WO₄²⁻ 0.007 μM, 0.1% trace element solution, containing per liter: nitrilotriacetic acid 150 mg, MnCl₂ · 4H₂O 610 mg, CoCl₂ · 6H₂O 420 mg, ZnCl₂ 90 mg, CuCl₂ · 2H₂O 7 mg, AlCl₃ 6 mg, H₃BO₃ 10 mg, Na₂MoO₄ · 2H₂O 20 mg, SrCl₂ · 6H₂O 10 mg, NaBr 10 mg, KI 70 mg, FeCl₃ · 6H₂O 500 mg, NiCl₂ · 6H₂O 25 mg.

Microcosm setup

All manipulations of the sediment incubations were done anaerobically. Sediments (serial number #10079) from Costa Rica were first divided into two 100-ml serial vials and maintained with sulfate/methane prior to establishment of the formal microcosm experiments. Following the protocol of experiments, sediments were evenly allocated into 30-ml serum vials with 2-ml settled sediments per vial, and then overlaid with 8-ml artificial seawater to a final volume of 10-ml slurry. The killed control vial was first fixed with 4% PFA overnight and then overheated at 90 °C for 4 hrs. The seawater above the sediment was used with the artificial seawater (see below) that contained 10 × less Ca²⁺, no sulfate, no sulfide, and 25 mM HEPES buffer at pH 7.5. ¹⁵NH₄⁺ (final concentration 1mM, 99% ¹⁵N, Cambridge Isotope Laboratories, Tewksbury, MA, USA) was added separately as the heavy isotopic tracer probing for nanoSIMS. Electron donors and acceptors were added separately. The electron acceptor, Na₂SO₄, was added based on the protocol

with a final concentration of 5.0 mM. Methane (1.0 bar, with 5% of ^{13}C -methane, Sigma-Aldrich) or ^{13}CO (0.1bar, 0.4bar, and 1.0 bar, Sigma-Aldrich) was added as electron donors based on the protocol with N_2 as the balance gas to a final total overpressure of 1.0 bar in the headspace. All the manipulated incubations were monitored in dark at 4 °C, meanwhile 0.5-ml supernatants were taken every 6 days in the anaerobic chamber full of N_2/H_2 . 20 μl of the filtered supernatant was added to 380 μl $\text{Zn}(\text{OAc})_2$ (500 mM) to preserve the sulfide for future detection. Around 100 μl went to sealed DIC vials which were flushed with He and were supplied with 200 μl of H_3PO_4 (42.5%). This was for the measurement of newly formed DIC and was found to be the most accurate to determine the methane consumption in the microcosm experiments (Scheller et al., 2016). The rest of the harvested supernatant was flush frozen in liquid nitrogen and stored at -80 °C for metabolite measurement. The incubations above were refreshed with new medium every one month when the nutrients were used up. 1.0 ml of 2% paraformaldehyde in 3 $\times$ PBS was then added to the remaining 0.5 ml sediment slurry (final concentration 1.3% PFA), resuspended and fixed overnight at 4 °C. Incubation was ended after 4 months. Slurry samples (1 ml from each vial) were taken and fixed in 2% PFA overnight at 4 °C (final concentration 1% PFA). The fixed sediment was subsequently washed 3 times with 3 $\times$ PBS, followed by a single wash in EtOH/PBS (1:1) and then re-suspended in EtOH/PBS (1:1) to a final volume of 0.5 ml that was used for microscopy and nanoSIMS. The rest of slurry was flush frozen in liquid nitrogen and kept in -80 °C for DNA extraction.

Manipulation of incubations for monitoring methane production and CO consumption was performed in an independent microcosm set up. Sediments from the same push core were inoculated. Cultures were developed in 10-ml serum vials, where the headspace was filled with N_2 , ^{13}CO , and Ar. The addition of Ar was used as the internal standard for GC measurement. The medium used in the incubation was same as above. DIC samples were taken before GC measurement at sampling time points.

Geochemistry determination

Analysis of sulfide was carried out in technical triplicate via the methylene blue method (Cline, 1969). Quantification was carried out in a plate reader (TECAN SunriseTM) by monitoring the absorbance at 670 nm. Quantification of newly formed DIC based on $^{13}\text{CH}_4$ was done by a GC-IR-MS GasBench II (Thermo Scientific). Gasbench data analysis was using the approach described in (Scheller et al., 2016).

Gas samples were quantified by a GC-TCD equipped with a HP-MOLSIV column (SN US3351826H, Agilent Technologies).

To test whether it occurred that nitrogenases reduced CO in the system, GC-MS analysis was performed using an Agilent/HP 5890 GC–5972 MSD system equipped with a capillary HP-Plot/Q column (Agilent Technologies). Multiple hydrocarbons were monitored including methane (CH_4), ethylene (C_2H_4), ethane (C_2H_6) and propane (C_3H_8) (Hu et al., 2011; Lee et al., 2010).

NMR spectra were acquired on a Bruker NEO 400 NMR spectrometer. D_2O was added to aqueous samples to make them 10% D_2O . To check for the presence of formate, ^1H spectra were acquired using the standard Bruker parameter set WATER, employing a 1D NOESY pulse sequence with a 10 millisecond mixing time. In order to confirm the identity of the formate and estimate its initial concentration, a sample containing formate was spiked with authentic formate to raise the sample concentration by 100 micromolar, and the WATER experiment was repeated.

Extracellular metabolites determination

Extracellular metabolites were quantified via parallel Dionex Integrion HPIC (ThermoFisher, Waltham, MA, USA) ion chromatography systems with anion and cation columns running in parallel housed at the Caltech Environmental Analysis Center. The ion chromatography system was run as described previously (Green-Saxena et al., 2014; Speth et al., 2022) with the following modifications.

A Dionex AS-DV autosampler loaded samples diluted 1:50 in 18 M Ω water to serial anion and cation columns and detectors, which are maintained at 30 °C. Anions were resolved by a 2 $\times$ 250 mm Dionex IonPac AS19-4 μm analytical column protected by a 2 $\times$ 50 mm Dionex IonPac AG19-4 μm guard column (ThermoFisher, Waltham, MA, USA). A potassium hydroxide eluent generator cartridge generated a hydroxide gradient that was pumped at 0.25 ml min $^{-1}$. The gradient was constant at 10 mM for 5 min, increased linearly to 48.5 mM at 27 min, then increased linearly to 50 mM at 40 min. A Dionex AERS 500 suppressor provided suppressed conductivity detection running on recycle mode with an applied current of 31 mA. Cations were resolved by a 2 $\times$ 250 mm Dionex IonPac CS16-4 μm analytical column protected by a 2 $\times$ 50 mm Dionex IonPac CG16-4 μm guard column. A methanesulfonic acid eluent generator cartridge generated a methanesulfonic acid gradient that was pumped at 0.16 ml min $^{-1}$. The gradient was constant at 10 mM for 5 min, nonlinearly increased to 20 mM at 20 min (Chromleon curve 7, concave up), and nonlinearly increased

to 40 mM at 40 min (Chromeleon curve 1, concave down). A Dionex CERS 2 mm suppressor provided suppressed conductivity detection with an applied voltage of 4.2 V. Chromatographic peaks were integrated by Chromeleon 7.2.9 using the Cobra algorithm, and were correlated to concentration by running known standards and generating 4-point calibration curves.

Sample preparation for aggregate embedding, sectioning, FISH, and nanoSIMS

Sample preparation was carried out following protocol as described previously (McGlynn et al., 2015). Briefly, PFA-fixed consortia were detached from the sediment particles via sonication on ice with microtip probe (Branson). Aggregates were Percoll separated and concentrated by centrifugation, immobilized in molten noble agar (3%). The biomass in agar blocks were then dehydrated via a series of ethanol concentrations of 50%, 75%, 90%, 100%, 100%, and 100%. The dehydrated blocks were embedded in glycol methacrylate (Heraeus Kulzer-Technovit 8100). Sections of *ca.* 1 μ m thickness were cut and stretched on a water droplet on a polylysine coated slide with teflon wells (Tekdon Inc) and analyzed by FISH. Images of the FISH-stained consortia were collected, and the location of these consortia were mapped for subsequent nanoSIMS analysis as described below.

FISH and probes used in this study

The FISH hybridization followed the protocol as described previously (Scheller et al., 2016). The samples were hybridized in hybridization buffer containing 35% formamide and incubated at 46 °C for 2 hrs, followed by a wash step at 48 °C for 15 min. The FISH probes were used in this study (5 ng/ μ l for each): ARCH915 (Alexa488), dual labeled: 5' to 3' = GTGCTCCCCGCCAATTCCT (Amann et al., 1990); ANME-2b-729 (Cy3), dual labeled: 5' to 3' = CGTTCTCGTAGGGCGCCT (McGlynn et al., 2015); DSS658 (Alexa647), dual labeled: 5' to 3' = TCCACTTC-CCTCTCCCAT (Lösekann et al., 2007; Manz et al., 1998).

nanoSIMS measurements

Sections with aggregates and cells were first coated with 20-nm-thick Au (High Resolution Sputter Coater 208HRD, Cressington). NanoSIMS measurements were performed on a CAMECA nanoSIMS 50L in the Center for Microanalysis at the California Institute of Technology. Gold-coated samples were presputtered with a 130-pA primary Cs⁺ ion beam with aperture diaphragm D1 = 1 until the ¹⁴N¹²C⁻ ion counts stabilized. Data were collected using a 5-pA or 10-pA beam with

D1 = 2 and entrance slit (ES) = 2. Masses were collected corresponding to the $^{12}\text{C}^-$, $^{13}\text{C}^-$, $^{14}\text{N}^{12}\text{C}^-$, and $^{15}\text{N}^{12}\text{C}^-$ ions, using a tuning protocol as described previously (Chadwick et al., 2019; McGlynn et al., 2015). Acquisitions were collected from diverse-size square raster areas, with 512×512 pixels, and 3 to 4 planes were collected per area. Dwell time settings resulted in acquisition times from 30 min to 2 hrs per plane depending on the size of the raster.

nanoSIMS data analysis

NanoSIMS data processing and analysis were done in Matlab (R2024b) and R (R4.4.2 version). NanoSIMS.im data files were initially processed using the Look @ NanoSIMS Matlab GUI (Polerecky et al., 2012) to align planes and export raw data. To align isotopic enrichment data of nanoSIMS with the taxonomic information in the space resolution, the processed nanoSIMS raw $^{14}\text{N}^{12}\text{C}^-$, and $^{15}\text{N}^{12}\text{C}^-$ images were used for the region of interest (ROI) drawing by hand combining with FISH images as reference. This was performed in the software Fresco (Adobe) on tablet. Regions of acquisitions that contained ANME-SRB cells were outlined based on the manual cell ROIs, and the elemental information was assigned to each ROI, which generated the single-cell level isotopic enrichment.

DNA extraction and iTag community analysis

DNA was extracted from the sediments stored after 4-month incubation using DNeasy PowerSoil Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Illumina iTag 16S rRNA gene sequencing protocol was followed as described in the study (Yu et al., 2021). Briefly, PCR amplification, barcoding and sequencing of the 16S rRNA hypervariable region (V4-V5) was performed using PCR primers 515f and 906r and amplification conditions as previously described (Yu et al., 2018). Sequencing data was processed using QIIME v1.8.0 (74), clustered at 99% sequencing identity using UCLUST v7.0.1001 (Edgar, 2010), and the taxonomic identity of the most abundant sequence in each cluster was assigned using a custom SILVA database modified from SILVA Ref NR 99 Database Release 115 (Mason et al., 2015; Quast et al., 2013). iTag community analysis was performed using dada2 and R.

RNA extraction and metatranscriptomic analysis

To obtain fresh materials for RNA analysis, incubations using sediments from the same push core were developed in parallel following the same protocol as mentioned

above. Conditions here used for RNA analysis included electron donors methane, 0.1bar-CO and 1.0bar-CO. Each condition contained biological triplicates. Samples were withdrawn and used for RNA extraction immediately.

RNA was extracted using RNA PowerSoil Total RNA Isolation Kit (Qiagen, Valencia, CA, USA). TURBO DNA-free Kit (ThermoFisher) was used to remove genomic DNA and purified using RNeasy Plus MicroKit (Qiagen). rRNA was removed using Pan-Pro (RNA-Seq) riboPOOL 24 reaction Kit (siTOOLs Biotech). Then, RNA was prepared for sequencing using Superscript IV VILO Master Mix (ThermoFisher/Invitrogen). The library was prepared by Next UltraTM II Directional RNA Library Prep Kit (NEB), and was sequenced on a NextSeq2000 (Illumina) platform in the Millard and Muriel Jacobs Genetics and Genomics Laboratory at the California Institute of Technology, generating 2×150 bp paired-end reads with an average insert length of 300 bp. A depth of 40 M reads per sample was sequenced.

Transcript abundance was quantified using kallisto (**bray_near-optimal_2016**) against a single reference database consisting of one ANME-2b MAG (Ga0402030_bin1) and one SRB1g MAG (Ga0402030_bin2) generated previously by a single-aggregate sequencing study (Chadwick et al., 2022; Murali et al., 2023). These MAGs were the best representative as they originate from an adjacent horizon of the same sediment core and are the best-quality pair of ANME-SRB MAGs (99.4% complete, 1.3% redundant and 98.7% complete, 2.2% redundant, respectively). Counts were read into DESeq2 (Love et al., 2014) in R for normalization and analysis for differential abundance results presented. Percentile rankings of each gene were also converted from the kallisto TPM data on a per-sample basis. Focal genes were annotated with a combination of KEGG (Kanehisa, 2019; Kanehisa & Goto, 2000; Kanehisa et al., 2023) and Pfam databases (Paysan-Lafosse et al., 2024) and manual BLAST (Altschul et al., 1990) to known sequences.

CO dehydrogenase homology searches and phylogeny analysis

A local database of genome sequences from ANME and syntrophic SRB was assembled from two previous studies (Chadwick et al., 2022; Murali et al., 2023) from our lab, representing all the genomes of ANME-SRB consortia available currently. CO dehydrogenase/acetyl-CoA synthase complex subunit alpha (*cdhA*, MA_RS05310) and anaerobic carbon-monoxide dehydrogenase catalytic subunit (*cooS*, MA_RS06785) from *Methanosarcina acetivorans* C2A were used as template to search for homologs in ANME genomes; anaerobic carbon-monoxide de-

hydrogenase catalytic subunit (*cooS*, DVU_RS09895) from *Desulfovibrio vulgaris* Hildenborough was used to blast against all syntrophic SRB genomes for homologs. Protein sequences were aligned using MUSCLE (Edgar, 2004) and then a phylogenetic tree was built using IQ-Tree2 using -m MFP with 1,000 bootstraps (Minh et al., 2020). The tree was displayed by iTOL (Letunic & Bork, 2021).

Data availability

The raw 16S rRNA amplicon sequencing and metatranscriptomic sequencing reads can be found on the National Center for Biotechnology Information database under BioProject ID PRJNA1227537.

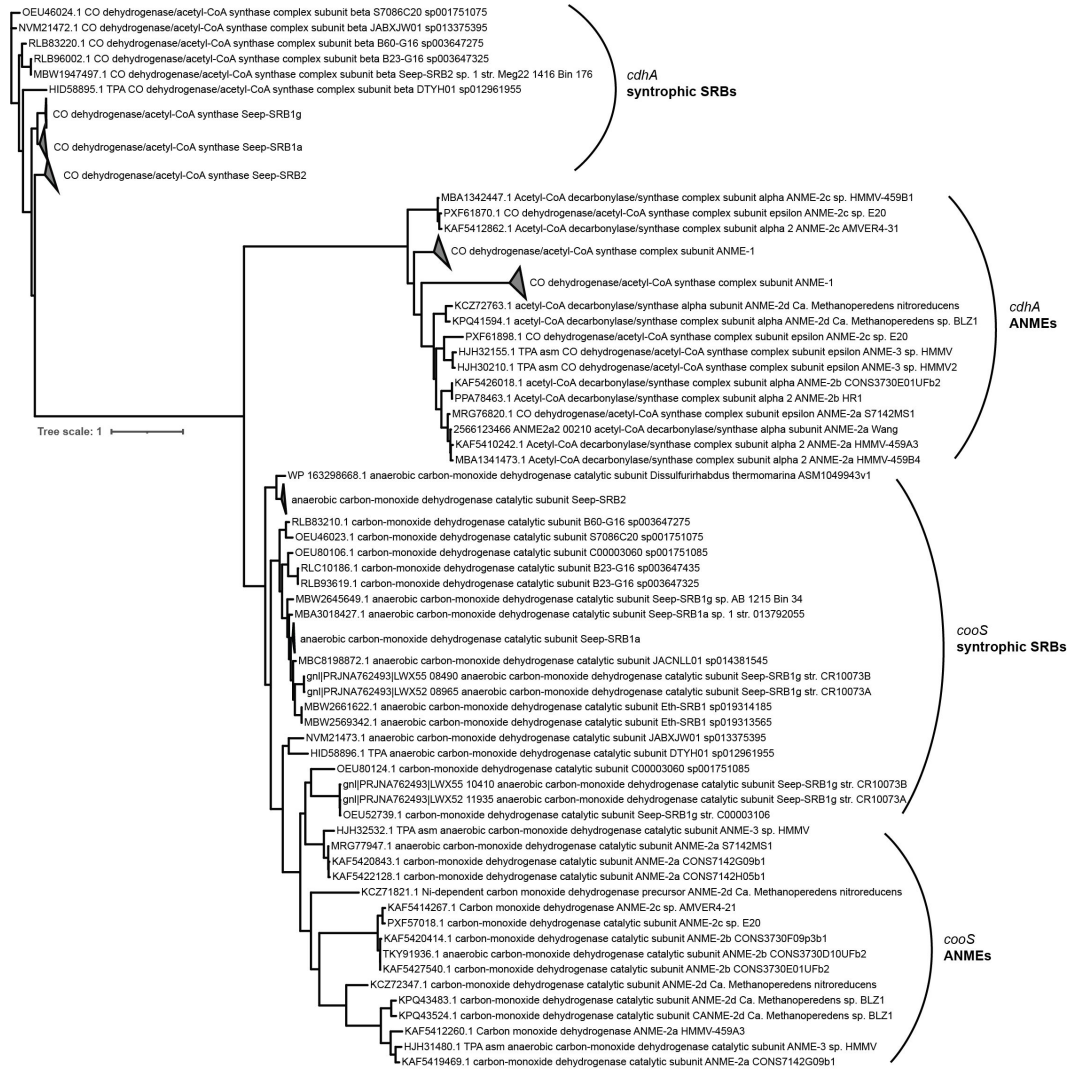


Figure 2.1: The phylogeny of CO dehydrogenase/acetyl-CoA synthase (CODH/ACS) complex alpha subunit (*cdhA*) and monofunctional anaerobic CO dehydrogenase (*cooS*) in the lineages of ANME and syntrophic SRB. The CODH homologs were retrieved from genomes in previous studies (Chadwick et al., 2022; Murali et al., 2023). All homolog alignments were examined manually after identification with BLAST. We operationally distinguished *cdhA* from *cooS* homologs by the following principle: a hit was labelled *cdhA* if it is part of a CODH/ACS complex cluster (operon), and otherwise *cooS*.

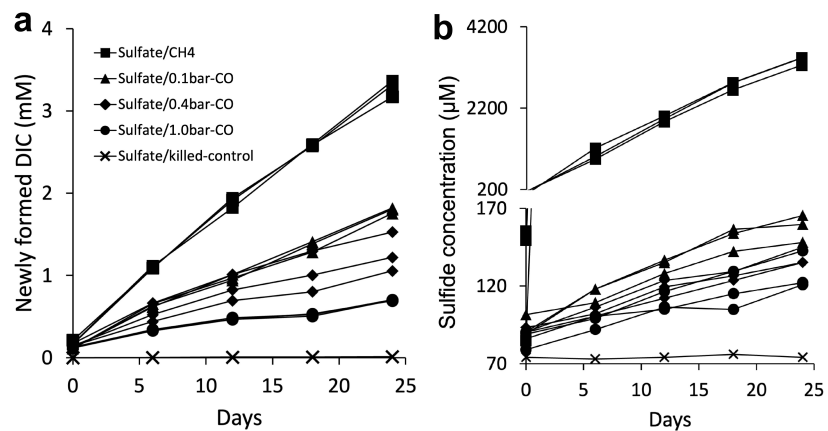


Figure 2.2: Time course of catabolic activities of anaerobic methanotrophic consortia in methane seep microcosm experiments. **a**, The oxidation of $^{13}\text{CH}_4$ or ^{13}CO to ^{13}C -dissolved inorganic carbon (DIC); **b**, the reduction of sulfate reflected by the concentrations of sulfide (HS^-).

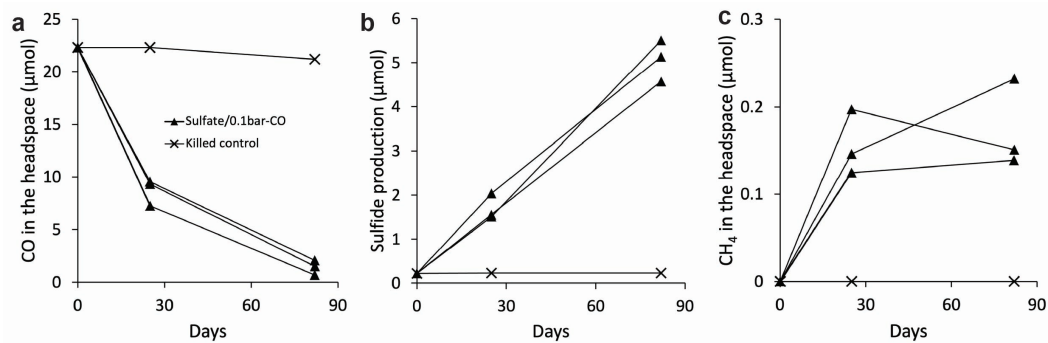


Figure 2.3: CO consumption coupled to sulfide and methane production monitored over time from microcosm incubations. Biological triplicates were developed as the experimental condition paired with one incubation as the killed control.

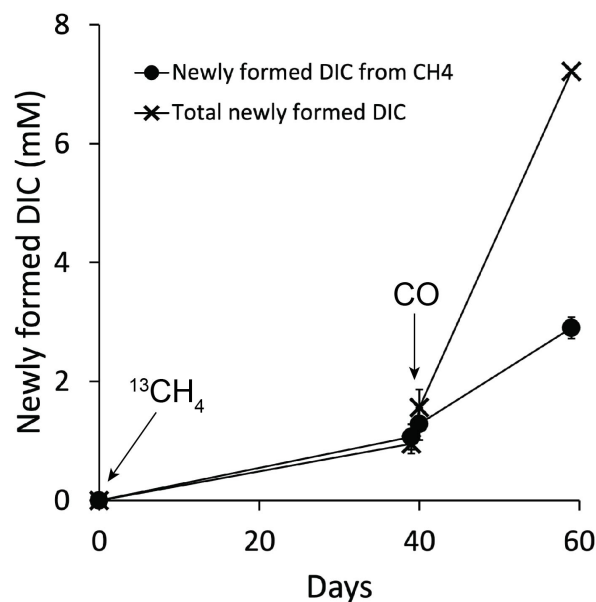


Figure 2.4: DIC production was monitored in the incubations when both methane and CO were present. $^{13}\text{CH}_4$ and sulfate were added to the cultures from the beginning. ^{12}C - and ^{13}C -DIC comprised of the total newly formed DIC, where ^{13}C -DIC came from $^{13}\text{CH}_4$ oxidation. Unlabeled CO was amended at day 40 of incubations. At this point, a mix of $^{13}\text{CH}_4$ and CO was present in the headspace and acted as the potential electron donors. Practically, the subtraction of ^{13}C -DIC from the total newly formed DIC would represent the activity of the CO oxidation. This experiment was performed in biological triplicates (mean $\pm$ s.d., $n = 3$). Where not visible, the error bar is smaller than the symbol.

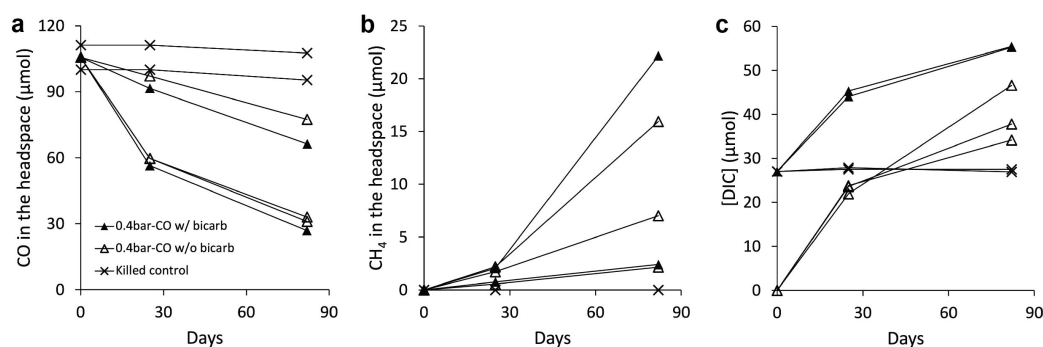


Figure 2.5: CO consumption coupled to methane production over time in microcosms without sulfate and with CO_2 as the sole added terminal electron acceptor. Biological duplicates were performed under the condition with bicarbonate as the sole electron acceptor (closed triangle). Biological triplicates were developed under the condition without any external electron acceptor (open triangle). **a**, CO concentration development in these conditions over time; **b**, methane generation over time. Unit was normalized to the total molar amount as μmol .

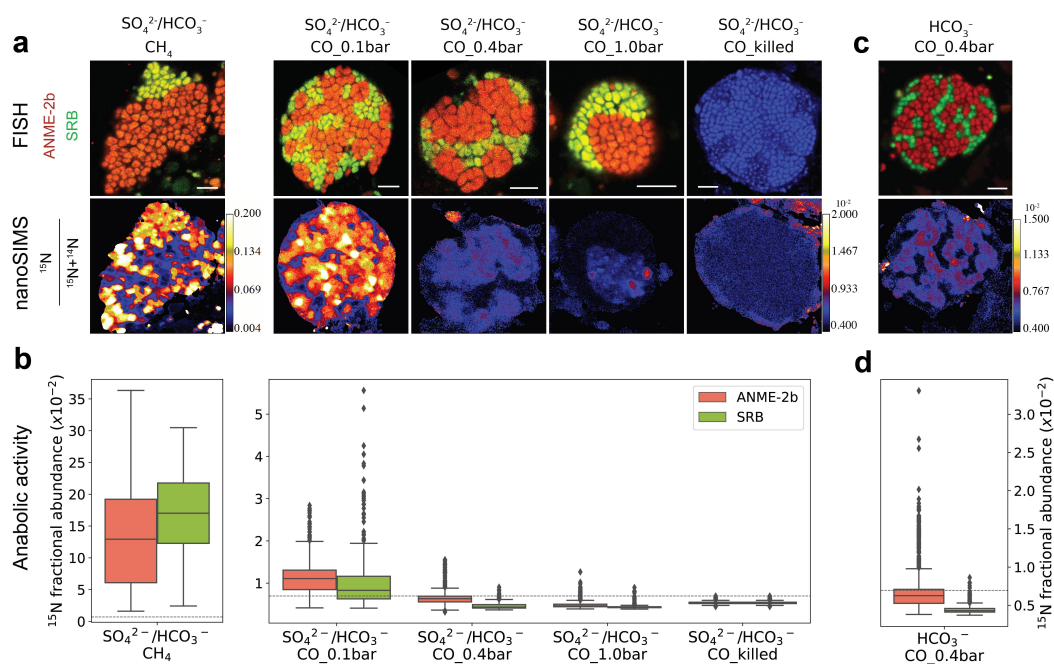


Figure 2.6: Anabolic activity summarized from paired FISH–nanoSIMS experiments for ANME-2b lineage and its syntrophic SRB. Representative FISH–nanoSIMS images from seep sediment microcosms. In panel **a** and **c**, the top row shows FISH pictures for ANME-2b (red) and their syntrophic SRB partners (green); the bottom shows a representative nanoSIMS image for each condition. The ¹⁵N fractional abundance in all the cell ROIs analyzed in this study is presented in panels **b** and **d**, where the dashed line was the thresholding value for active cells. Statistical details were summarized in Table 1. (**a**, **b**) Samples from sulfate/HCO₃⁻-containing bottles were taken down after 122-day incubations, and (**c**, **d**) sample from HCO₃⁻-containing bottles (sulfate free) was taken down after 90-day incubations. Scale bar: 5 μ m.

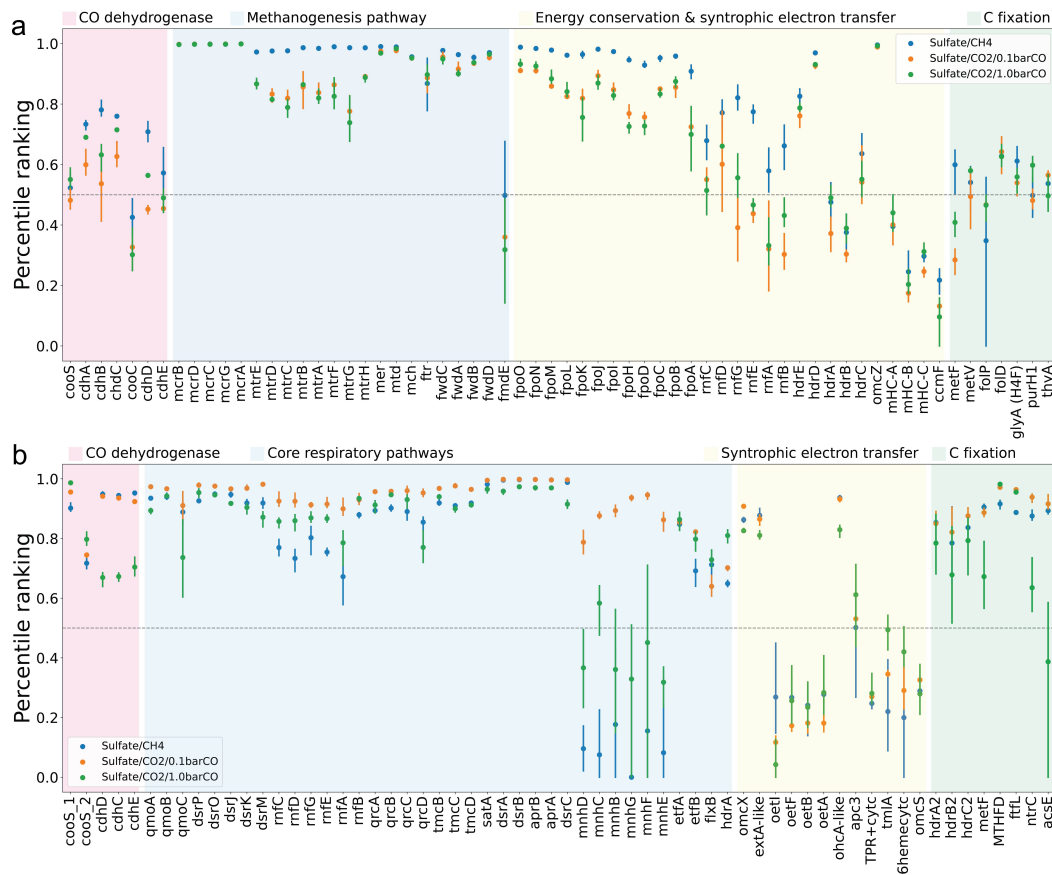


Figure 2.7: Rank of transcriptomic expression of selected genes involved in the energy metabolism in ANME-2b and syntrophic SeepSRB-1g. **a**, Selected genes in ANME-2b: CO dehydrogenase, methanogenesis pathway, energy conservation and syntrophic electron transfer, and carbon fixation. **b**, Selected genes in SeepSRB-1g: CO dehydrogenases, core respiratory pathways, syntrophic electron transfer, and carbon fixation. The detailed profiling of those genes is summarized in Supplementary Data S1 and S2.

Figure 2.8: A proposal for carbon monoxide oxidation and energy metabolism in ANME-SRB consortia. Brief (a) and detailed (b) schematic showing energy metabolism in ANME-2b and SRB consortia. In b, proposed catalyzing steps connected with black arrows are the conventional energy metabolic pathways of AOM; green arrows indicate the electron flow across ANME-SRB consortia with CO and sulfate as the electron donor and acceptor. CODH, carbon monoxide dehydrogenase; CoM-S-S-CoB, heterodisulfide; $F_{420}/F_{420}H_2$, oxidized/reduced co-factor F_{420} ; Fd/Fd $^{2-}$, oxidized/reduced ferredoxin; Mcr, methyl-S-CoM reductase; Mtr, N 5 -methyl-H4SPT:CoM methyltransferase; Mer, N 5 ,N 10 -methylene-H4SPT reductase; Mtd, N 5 ,N 10 -methylene-H4SPT dehydrogenase; Mch, N 5 ,N 10 -methenyl-H4SPT cyclohydrolase; Ftr, formyl-MF:H4SPT formyltransferase; Fmd, formyl-methanofuran dehydrogenase; Hdr, heterodisulfide reductase; HS-CoB, coenzyme B; HS-CoM, coenzyme M; Mp/MpH $_2$, oxidized/reduced methanophenazine; Fpo, $F_{420}H_2$:methanophenazine oxidoreductase; Rnf, rhodobacter nitrogen fixation, energy converting Fd $^{2-}$: acceptor oxidoreductase; Mnh, multisubunit Na $^+$ /H $^+$ antiporters; Tmc, transmembrane channel-like protein; Qrc, quinone-reductase complex; Dsr, dissimilatory sulfite reductase; Qmo, quinol oxidizing complex; Mrp, multiple resistance and pH adaptation antiporters; Flx, flavin oxidoreductase; Apr, adenosine 5'-phosphosulfate reductase; APS, adenylyl-sulfate/adenosine-5'-phosphosulfate; Sat, sulfate adenylyltransferase; Q/QH $_2$, oxidized/reduced quinol.

Figure 2.9: The full phylogeny of CO dehydrogenase/acetyl-CoA synthase (CODH/ACS) complex alpha subunit (*cdhA*) and monofunctional anaerobic CO dehydrogenase (*cooS*) in the lineages of ANME and syntrophic SRB.

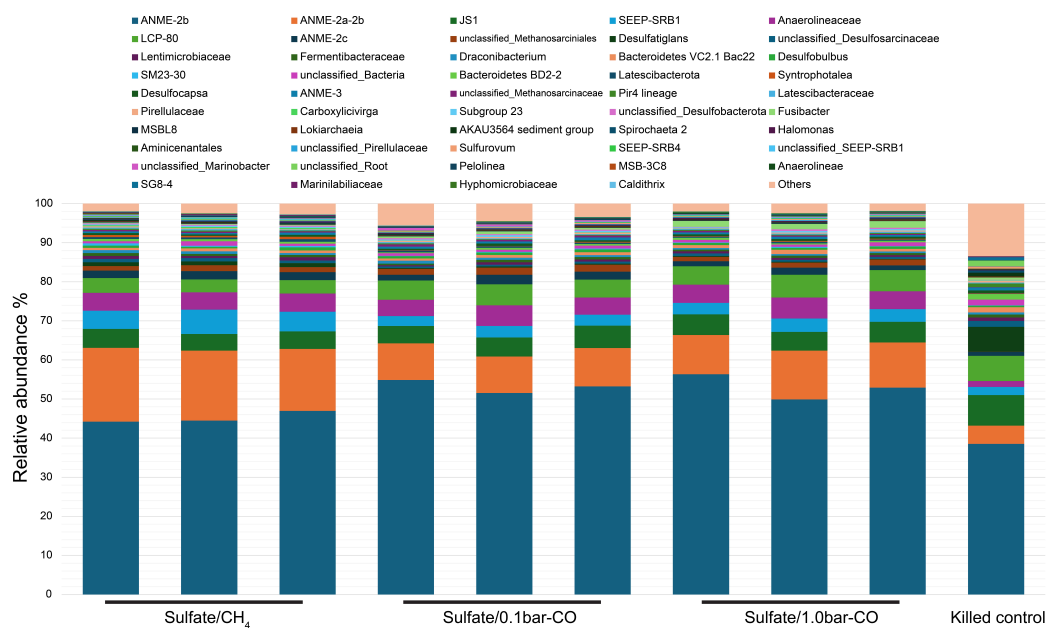


Figure 2.10: Community analysis of methane seep sediments using samples after 4-month incubation with CO using partial 16S rRNA gene amplicon sequencing. Taxonomic classification is based on the SILVA small subunit rRNA database v138. Taxonomies with more than 1% were shown.

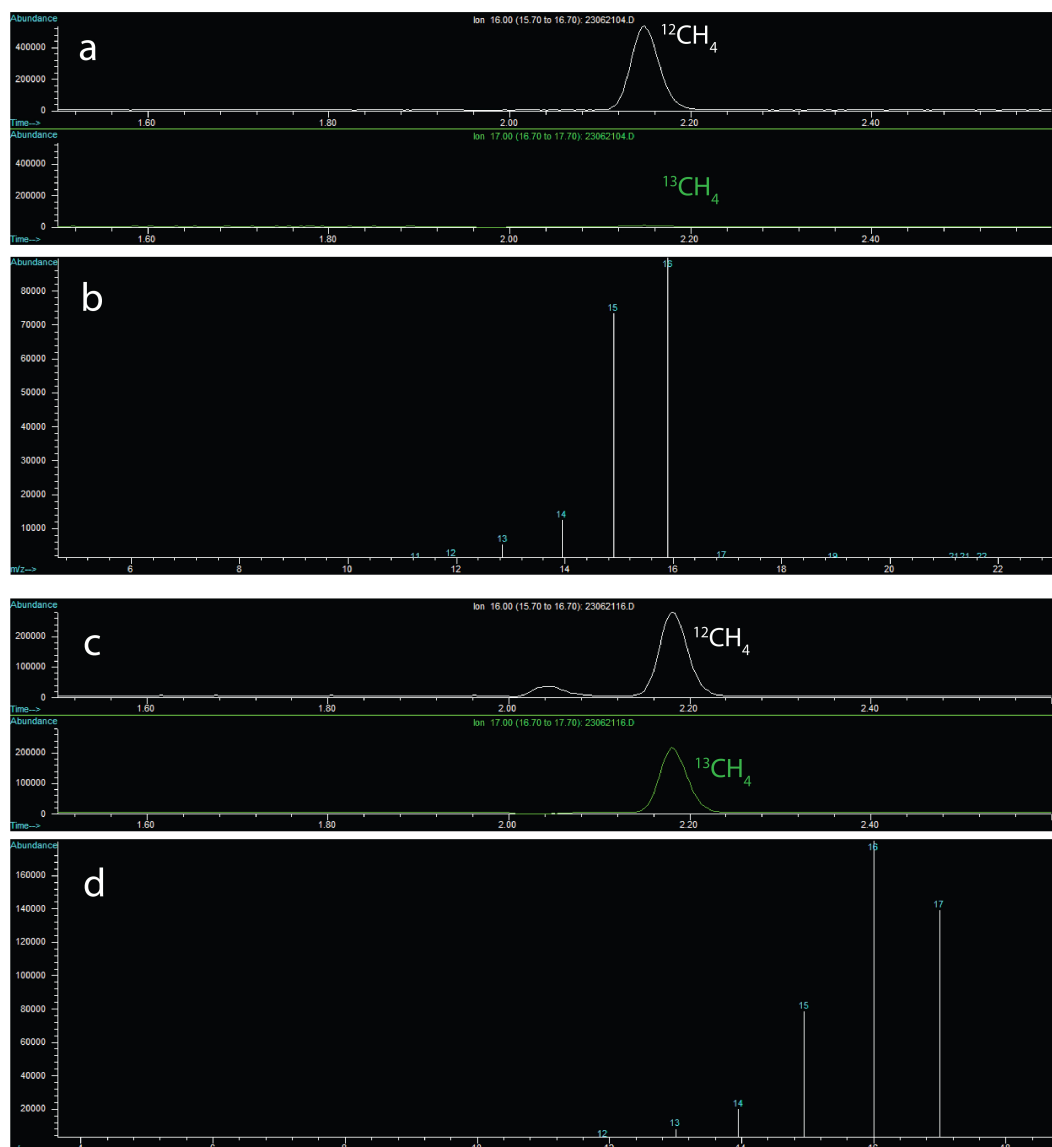


Figure 2.11: GC-MS measurement of methane production from CO oxidation. **a**, Gas chromatography of ^{12}C -methane. **b**, The mass-to-charge (m/z) ratios at which ^{12}C -methane was traced. **c**, A representative gas chromatography of ^{12}C - and ^{13}C -methane from CO/bicarbonate manipulated experiments. **d**, The mass-to-charge (m/z) ratios at which ^{12}C - and ^{13}C -methane was traced. In panel **d**, m/z of 17 represents $^{13}\text{CH}_4$, 16 for a combination of $^{12}\text{CH}_4 + ^{13}\text{CH}_3$, 15 for a combination of $^{12}\text{CH}_3 + ^{13}\text{CH}_2$, etc.

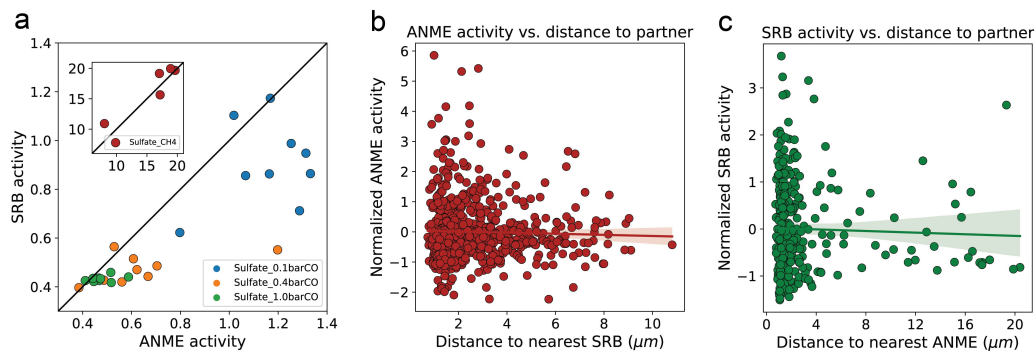


Figure 2.12: Activity relationships between ANME-2b and SeepSRB-1g in AOM consortia. **a**, Population-level average of SRB activity versus ANME activity for individual AOM consortia. The 1:1 line is shown in black. **b**, **c**, Normalized activity of single ANME or SRB cells, respectively, plotted against distance to the nearest syntrophic partner. Shaded areas illustrate the 95% confidence intervals in slopes and intercepts of the linear regressions.

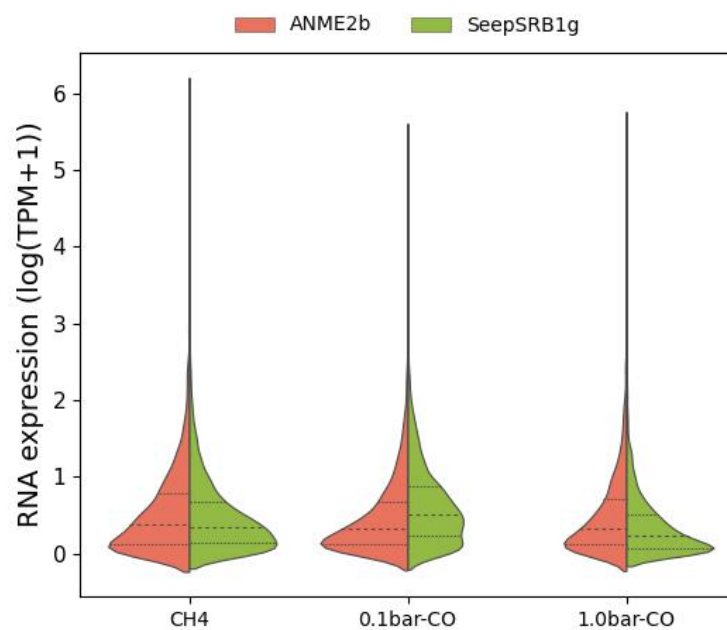


Figure 2.13: The distribution of gene expression of ANME-2b and syntrophic SeepSRB-1g in different treatments. The comparison was based on normalized transcript per million reads (TPM). Dashed lines indicate the quartile of the distribution.

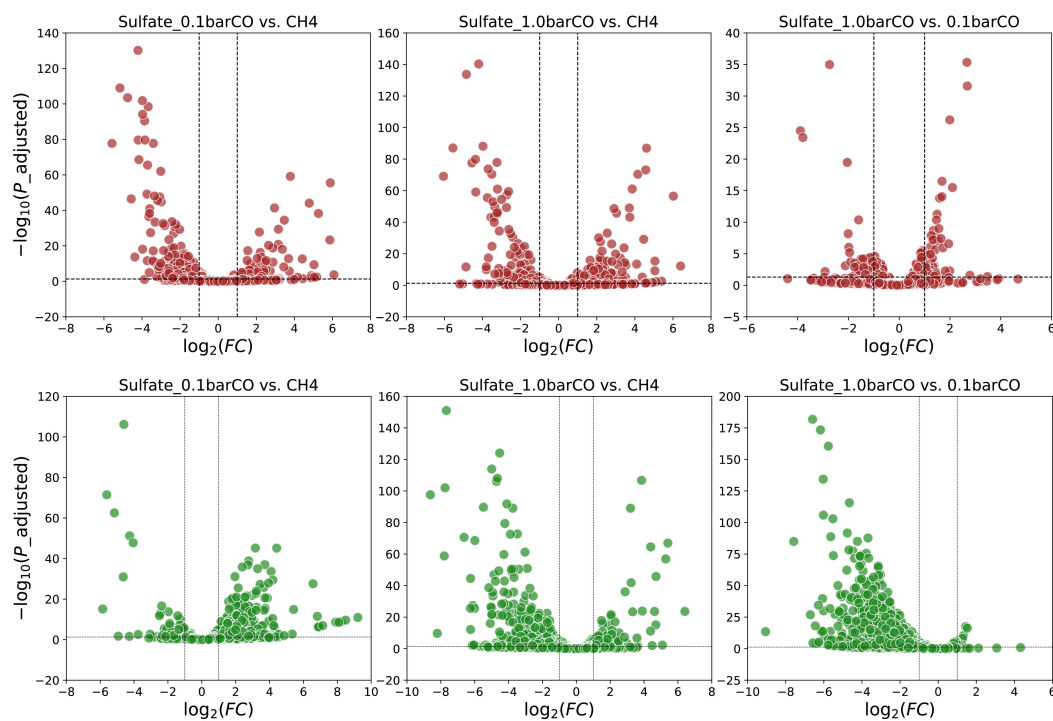


Figure 2.14: Volcano plots of gene expression for ANME-2b (upper panel, red) and SeepSRB-1g (lower panel, green). Conditions include 0.1bar-CO vs. CH₄, 1.0bar-CO vs. CH₄, and 1.0bar-CO vs. 0.1-barCO. The cutoffs were $P_{\text{adjusted}} = 0.05$ and $\log_2(FC) = 1$.

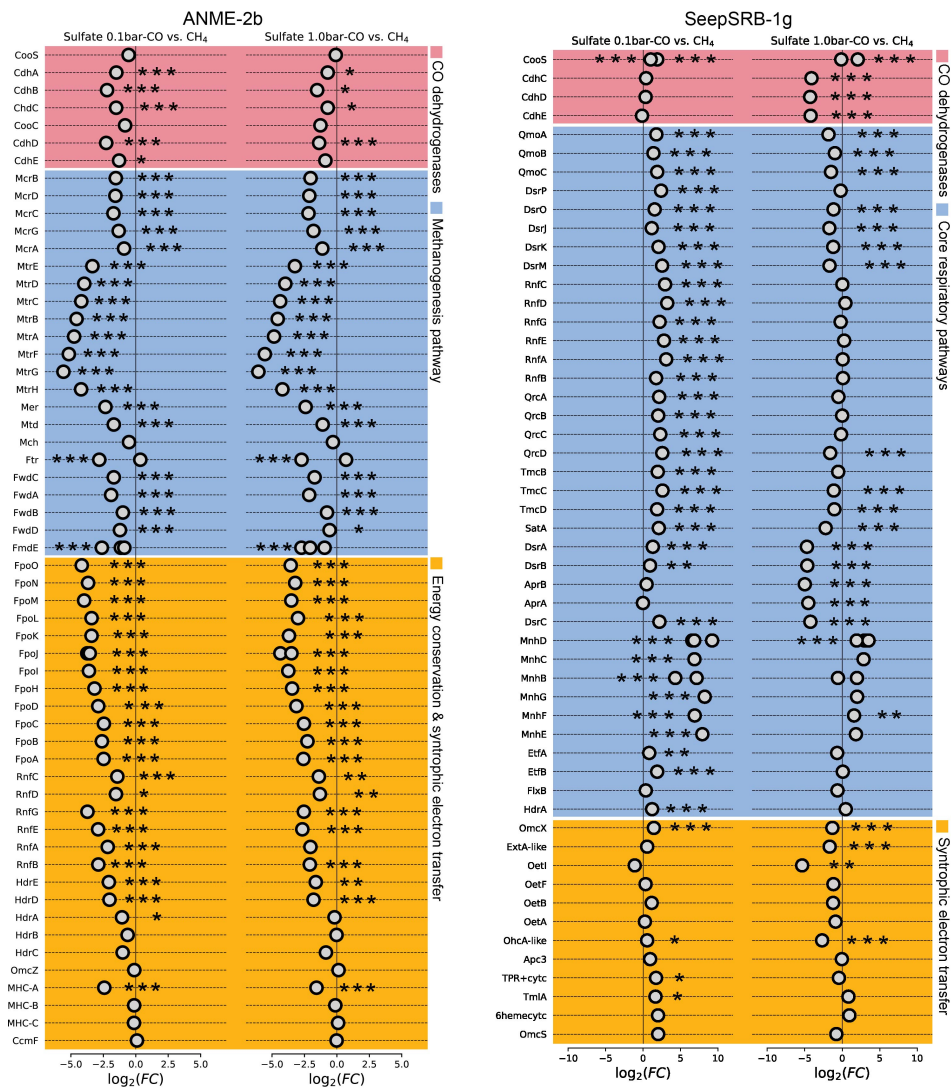


Figure 2.15: Transcriptomic expression of selected genes involved in the energy metabolism in ANME-2b and syntrophic SeepSRB-1g. (Left) Selected genes in ANME-2b: CO dehydrogenases (pink), methanogenesis pathway (blue), and energy conservation and syntrophic electron transfer (yellow). (Right) Selected genes in SeepSRB-1g: CO dehydrogenases (pink), core respiratory pathways (blue), and syntrophic electron transfer (yellow). The gene expression is centered log-ratio transformed. Differential expression of genes between CO condition and CH₄ condition is reported as log₂-fold-change (log₂FC). The pairwise statistical test is based on DESeq2 with the Wald test and adjusted by applying FDR. * $P_{adjusted} < 0.05$; ** $P_{adjusted} < 0.01$; *** $P_{adjusted} < 0.001$. The detailed profiling of those genes is summarized in Supplementary Data S1 and S2.

Table 2.1: ^{15}N enrichment of ANME-SRB consortia at single cell level from FISH-nanoSIMS analysis

	e ⁻ acceptor	sulfate CH ₄	sulfate HCO ₃ ⁻ CO_0.1 bar	sulfate HCO ₃ ⁻ CO_0.4 bar	sulfate HCO ₃ ⁻ CO_1.0 bar	sulfate HCO ₃ ⁻ CO_killed	HCO ₃ ⁻ CO_0.4 bar
ANME-2b	^{15}N fractional abundance ^a	13.65 ± 8.33	1.16 ± 0.40	0.63 ± 0.17	0.49 ± 0.09		0.67 ± 0.26
	% of active cells ^b	(100%)	(94.27%)	(22.38%)	(3.85%)		(27.91%)
	no. of cells analyzed ^c	(368)	(576)	(831)	(805)		(1,684)
SRB	^{15}N fractional abundance	16.72 ± 6.29	1.05 ± 0.71	0.46 ± 0.08	0.44 ± 0.05	0.53 ± 0.03 (776)	0.44 ± 0.05
	% of active cells	(100%)	(64.82%)	(1.23%)	(1.23%)		(0.77%)
	no. of cells analyzed	(194)	(452)	(488)	(489)		(780)
	no. of aggregates	5	10	10	10	7	20

^a ^{15}N fractional abundance, $^{15}\text{N}/(^{15}\text{N} + ^{14}\text{N}) \times 100\%$;

^b The percentage of active cell ROIs in each condition (the ones with ^{15}N fractional abundance higher than any ROI in the killed condition);

^c The number of single cells analyzed in each treatment.

Table 2.2: The ratio of single cell ^{15}N assimilation to bulk potential energy generation by redox reaction

Condition	CO/bicarbonate ^a	CH ₄ /sulfate
CH ₄ production or oxidation rate nmol CH ₄ d ⁻¹ cm ⁻³ _{sed}	0.061 ± 0.054	0.52 ± 0.02
ANME-2b single cell N-15 atom assimilation per energy $^{15}\text{F J}^{-1} \text{ cm}^{-3}$ ^b	0.010 ± 0.0038 ^c	0.21 ± 0.13 ^d
no. of cells analyzed	1,684	368

^a $^{15}\text{F} = ^{15}\text{N}/(^{15}\text{N} + ^{14}\text{N})$;

^b Data from Figure 3 with 0.4bar-CO;

^c The bulk energy yield was based on the theoretical redox reaction from Eq. 2.4.;

^d The bulk energy yield was based on the reaction:



Table 2.3: Summary of microcosm experiments in this study.

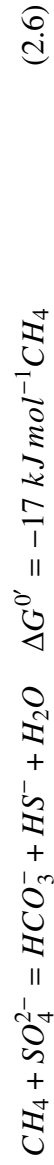
Microcosm	Electron donor	Electron acceptor	Geochemical measurement	nanoSIMS (Y/N)	Result illustration
Set #1	CH ₄	sulfate+bicarbonate	sulfide, DIC	Y	Figs. 2, 6;
	0.1bar-CO	sulfate+bicarbonate	sulfide, DIC	Y	
	0.4bar-CO	sulfate+bicarbonate	sulfide, DIC	Y	
	1.0bar-CO	sulfate+bicarbonate	sulfide, DIC	Y	
Set #2	0.1bar-CO	sulfate+bicarbonate	sulfide, DIC, CO, CH ₄	N	Fig. 3
Set #3	CH ₄ , CO	sulfate+bicarbonate	DIC	N	Fig. 4
Set #4	0.4bar-CO	bicarbonate	DIC, CO, CH ₄	Y	Figs. 5, 6

Table 2.4: The catabolic activity of ANME-SRB consortia with CO, CH₄ and sulfate.

	($\mu\text{M d}^{-1} \text{ cm}^{-3}_{\text{sed}}$)	0.1bar-CO	0.1bar-CO	0.1bar-CO	CH ₄
Sulfide production		1.33 $\pm$ 0.32	1.01 $\pm$ 0.13	0.88 $\pm$ 0.23	67.63 $\pm$ 2.76
Newly formed DIC		34.97 $\pm$ 0.68	23.21 $\pm$ 4.67	12.00 $\pm$ 0.07	64.63 $\pm$ 2.64
Ratio of [DIC production] to [Sulfide production]		27.48 $\pm$ 7.79	23.02 $\pm$ 3.86	14.19 $\pm$ 3.60	0.96 $\pm$ 0.07
Predicted DIC formation from sulfide production		5.33 $\pm$ 1.30 ^a	4.04 $\pm$ 0.53 ^a	3.53 $\pm$ 0.90 ^a	67.63 $\pm$ 2.76 ^b

^a The calculation was based on the theoretical redox reaction from Eq. 2.1.;

^b The calculation was based on the reaction:



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Chapter 3

PYRITE-INDUCED SULFATE AND IRON OXIDE SUPPORTS
ANAEROBIC OXIDATION OF METHANE FOR ANAEROBIC
METHANOTROPHIC CONSORTIA

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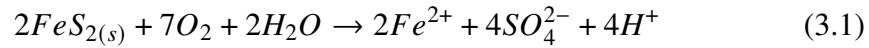
This chapter is in preparation for publication.

3.1 Abstract

Pyrite is the most abundant iron-sulfur mineral in ocean sediments. Its potential contribution to the anaerobic oxidation of methane (AOM) mediated by a symbiotic partnership between an anaerobic methane-oxidizing (ANME-2c) archaea and a sulfate reducing bacteria (SRB) was investigated. Here the reaction of anaerobic pyrite oxidation with anoxic seawater in dark was confirmed, which produced sulfate and iron oxides, particularly magnetite. This was verified through geochemical measurements and mineralogical analysis, including Mössbauer spectroscopy and X-ray diffraction. Both sulfate and iron oxide mineral were found enabling to power the AOM, as determined by the single cell biosynthetic activity measurement via FISH-nanoSIMS (fluorescence *in situ* hybridization and nanoscale secondary ion mass spectrometry). This study offers a new perspective on the energy sources for microbial anaerobic oxidation of methane by linking the anaerobic pyrite oxidation process, which may represent an important yet underrated mechanism supporting the enigmatic life forms of the deep biosphere.

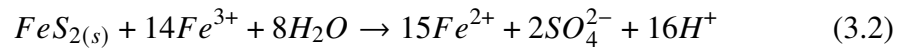
3.2 Introduction

Pyrite (FeS_2) is the most abundant authigenic iron-sulfur mineral found in sediments (Rickard et al., 2017). Its transformation plays a crucial role in Earth's iron, sulfur, and carbon cycles, as well as in determining the Earth's surface redox state (Rickard & Luther, 2007). The fate of pyrite, particularly through the pyrite oxidation process, has been extensively discussed over the past decades, with oxygen identified as a major oxidant (Chandra & Gerson, 2010; Nordstrom & Southam, 2018; Rimstidt & Vaughan, 2003; Williamson & Rimstidt, 1994; Zhang et al., 2016). Among the various mechanisms, microbial oxidation of pyrite plays a significant role, which is dependent on aerobic respiration by chemolithoautotrophic bacteria capable of oxidizing sulfide and ferrous iron (Fe^{2+}) leached from the pyrite dissolution, serving as the electron donors for sulfide-oxidizing bacteria (Okibe & Johnson, 2004; Sasaki et al., 1998) and iron-oxidizing bacteria (Bacelar-Nicolau & Johnson, 1999; Hiltunen et al., 1981; Kocaman et al., 2016), respectively. The overall stoichiometry of pyrite oxidation with oxygen can be described by the reaction in Eq 3.1.

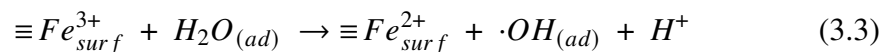


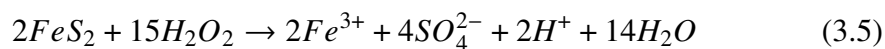
Taken together with the abiotic aerobic oxidation of pyrite, the equation is believed to be a major source of sulfate input both in modern (Raymond & Oh, 2009) and ancient environments (Crowe et al., 2013; Konhauser et al., 2011; Stüeken et al., 2012).

In addition to oxygen, ferric iron (Fe^{3+}) is another important oxidant enabling the process of pyrite oxidation anaerobically (shown below in Eq 3.2).



Importantly, under Fe^{3+} -free conditions, the non-stoichiometric defect sites (i.e., the dangling bonds between S-Fe and S-S bonds) on the pyrite surface can initiate a series of reactions with H_2O due to a reduction in the energy requirements for the chemisorption of H_2O molecule. As a result of pyrite oxidation, several products are generated, including hydrogen peroxide and sulfate (Eqs. 3.3, 3.4 and 3.5) (Borda et al., 2001, 2003; Gil-Lozano et al., 2017; Gil-Lozano et al., 2014; Lefticariu et al., 2007; McKibben & Barnes, 1986; Wang et al., 2012).





Pyrite oxidation, as described in Eq. 3.5, generates oxidizing equivalents in the form of Fe^{3+} and SO_4^{2-} , which can be coupled with the oxidation of CH_4 to CO_2 through anaerobic methanotrophic activity. This transformation of pyrite is thought to play a significant role in sulfate-driven anaerobic oxidation of methane (AOM), commonly observed in methane seep sediments (Ruban et al., 2025), where hydrogen sulfide produced can facilitate the precipitation of pyrite. The connection between pyrite oxidation and methanotrophy can be an integral part of a cryptic sulfur cycle in deep marine sediments, potentially supporting the enigmatic life forms within the deep biosphere. However, this link has received limited discussion.

Building on these concepts, this study emphasized the potential role of pyrite in the AOM system, particularly in the absence of external sulfate or other electron acceptors. The primary objective was to investigate the reaction of pyrite with seawater at cold temperature in darkness, and to explore how these reactions affect the activity of methane oxidation. This was achieved through a combination of short- and long-term incubations, geochemical measurements, mineralogical characterization, and single-cell activity determination, utilizing the high sensitivity and spatial resolution of nanometer-scale secondary ion mass spectrometry (nanoSIMS) in conjunction with fluorescence in situ hybridization (FISH).

3.3 Results

Reaction of pyrite with seawater

Short-term assays were conducted to test two sizes of pyrite particles: 100 nm and 40 μm , using sulfate-free anoxic artificial water (ASW). Overall, hydrogen peroxide (H_2O_2) and sulfate were produced, which showed a clear size-dependent trend with over a 5-fold difference between the two sizes of pyrite particles (Figure 3.1a). Specifically, for the pyrite nanoparticles, H_2O_2 was generated immediately at the beginning of the incubation, within the first minute after mixing of pyrite with ASW (approximately 6 mmol g^{-1} FeS_2). This was followed by a rapid decrease in concentration to around 1.5 mmol g^{-1} FeS_2 , with a gradual decline observed over time (Figure 3.1a). In contrast, for micron size pyrite, a spike of H_2O_2 production was not detected. Instead, the concentration initially increased from approximately

0.1 to 0.2 mmol H₂O₂ g⁻¹ FeS₂ during the first 30 min, before decreasing to below 0.05 mmol H₂O₂ g⁻¹ FeS₂ thereafter (Figure 3.1a).

Likewise, sulfate was generated within the first minute of this assay (Figure 3.1b). Pyrite nanoparticles exhibited over a five-fold greater capability for sulfate production compared to the micron-sized pyrite particles. For the 100 nm pyrite, sulfate concentrations increased significantly in the first 10 min, corresponding to the consumption of H₂O₂ shown in Figure 3.1a. Subsequently, the sulfate concentration stabilized until the end of the assay. In contrast, the sulfate concentration for the 40 μ m pyrite did not show any clear changes over time (Figure 3.1b). We also attempted to determine the presence of hydroxyl radicals ($\cdot OH$) in this assay, but did not observe their production at detectable levels (data not shown, see *Methods*).

Long-term microcosm experiment of pyrite-induced AOM

Long-term incubations were established to investigate whether the aforementioned reaction could support the anaerobic methanotrophic (AOM) activity. Accordingly, an ANME-2c/SRB-dominated sediment-free enrichment (as determined by both 16S rRNA amplicon sequencing and FISH, see *Methods*) was amended with pyrite nanoparticles due to their higher reactivity. Multiple conditions were manipulated, including two independent sets of pyrite/live cells incubations, a canonical sulfate-AOM condition serving as a positive control, and several different negative controls, including cultures with pyrite and killed cells, cultures without any cells, cultures without pyrite addition, and cultures lacking both sulfate and pyrite (for details, see Table 3.1).

To track the activity of AOM over time, ¹³C-labeled CH₄ was amended, which proved to be a precise method (Scheller et al., 2016). In the AOM reaction, CO₂ is the end product from the oxidation of methane (shown as DIC (dissolved inorganic carbon), primarily in the form of bicarbonate due to the pH in the cultures). Consequently, the ¹³C-labeled DIC could be quantified and interpreted as an indicator of AOM activity. As shown in Figure 3.2a, both independent replicating sets of pyrite incubations with live ANME/SRB enrichments demonstrated a clear AOM activity over time, with rates of $0.35 \pm 0.06 \mu\text{mol CH}_4 \text{ d}^{-1}$ for the Pyrite rep-1 set and $0.28 \pm 0.07 \mu\text{mol CH}_4 \text{ d}^{-1}$ for the Pyrite rep-2 set. These rates were approximately one third of the rate in the canonical sulfate-AOM, which was $0.99 \pm 0.06 \mu\text{mol CH}_4 \text{ d}^{-1}$ (calculated over the first 32 days; see Figure 3.2a). Notably, there was no significant increase in DIC production observed in any of the negative controls.

In parallel, sulfate concentrations were monitored over the first 32 days of incubation (Figure 3.2b). The sulfate-AOM condition showed clear sulfate consumption, corresponding to the previously mentioned AOM activity. The Pyrite rep-1 set initially accumulated slight amounts of sulfate during the first 22 days, followed by a decline. No significant changes in sulfate levels were observed in the other control conditions. Additionally, sulfide (HS^-) and iron (Fe^{2+} and Fe^{3+}) concentrations were monitored throughout the incubation period. Detectable concentrations of sulfide were not observed in either the pyrite or control conditions (Figure 3.2c), nor were iron ions detected in any of the conditions (data not shown, see *Methods*).

Anabolic activity of ANME/SRB growing with pyrite

High-resolution biosynthetic activity was analyzed for phylogenetically ANME-2c archaea and their partner SRB using FISH-based microbial identification (ANME-2c-760 oligonucleotide probe and Desulfosarcina/Desulfococcus group probe) (McGlynn et al., 2015; Yu et al., 2021). A total of 8,764 FISH-identified cells across 59 consortia were quantified, comprising 4,499 annotated as ANME-2c cells and 4,265 as SRB (Figure 3.3, Table 3.2). In comparison to the unlabeled control, a significant enrichment of N-15 atoms was observed in the two independent sets of pyrite incubations, where a clear distribution of iron-sulfur mineral particles was located at the aggregate surface (Figure 3.7). Both ANME-2c and SRB cells showed an obvious anabolic activity. Specifically, the ^{15}N fractional abundance (^{15}F) of ANME-2c in the Pyrite rep-1 set was measured at 3.73 ± 2.21 , which was approximately one-third of the average activity observed in the canonical sulfate-AOM positive control (Table 3.2). This finding was consistent with the rates of the methane oxidation indicated by the concentrations of newly formed DIC (Figure 3.2). A slightly higher enrichment was noted in the Pyrite rep-2 set, with a ^{15}F of 4.38 ± 3.95 (^{15}F), compared to the Pyrite rep-1. This increase can be attributed to a longer incubation (208 days vs. 99 days). Interestingly, despite the nearly doubled incubation duration in the Pyrite rep-2 set, the enrichment did not reach twice the level of that in the Pyrite rep-1 set (mean: 4.38 vs. 3.73).

In comparison to ANME-2c activity, SRB exhibited significantly lower anabolic activity as determined by FISH-nanoSIMS. On average, SRB cells showed enrichment in ^{15}N at levels of 1.24 ± 0.68 in the Pyrite rep-1 set and 0.88 ± 0.67 in the Pyrite rep-2 set. This finding contrasts with the results for ANME-2c (Table 3.2). Notably, a comparable enrichment level was determined with a ^{15}F ratio of ANME-2c to SRB as 1.33 ± 0.25 (Table 3.2) in the sulfate-AOM condition,

which was consistent to previous reports indicating that ANME and SRB showed similar levels of anabolic activity when utilizing sulfate and methane as substrates (McGlynn et al., 2015; Scheller et al., 2016). However, a significant disparity was found in the higher anabolic activity of ANME-2c compared to SRB under both pyrite-methane conditions, with values of 3.27 ± 1.20 for the Pyrite rep-1 and 4.92 ± 3.23 for the Pyrite rep-2 (Table 3.2). This suggests the potential involvement of additional electron acceptors functioning alongside sulfate.

Observation of damaged ANME cells

While living in the presence of pyrite nanoparticles, some ANME-2c archaeal cells were occasionally found to be inactive, as indicated by either the absence of FISH probing or a lack of enrichment in ^{15}N atoms, as demonstrated in Figures 3.3 and 3.4. To assess the anabolic activity, we calculated the average activity across each fully active cluster (see *Methods* for details). This observation led to the hypothesis that the observed inactivity may be related to the pyrite nanoparticles used in the incubations, which were positioned outside the ANME-2c/SRB consortia. Accordingly, when examining the relationship between the averaged cluster activity and their distance to the boundary, we found that the activity of individual clusters was not correlated with their proximity to the edges of aggregates (Figure 3.4). However, upon investigating the probability distribution function of these clusters relative to their distance to the boundary, we observed a higher probability for active ANME-2c clusters located approximately $4\ \mu\text{m}$ from the boundary after 99 days of exposure to pyrite (Pyrite rep-1 set). This distance decreased to less than $2\ \mu\text{m}$ after 208 days of incubation with pyrite (Pyrite rep-2 set) (Figure 3.5).

Furthermore, we analyzed the relationship between the averaged cluster activity and their distance to the pyrite particle interface (Figure 3.6). A clear decreasing trend in probability density was observed as the distance to the pyrite particles increased, particularly evident in Figure 3.6a). This trend may suggest a favorable role of pyrite particles in enhancing the activity of the ANME-SRB consortia.

Mineralogy change of pyrite

The composition of pyrite mineral was determined using Mössbauer spectroscopy and X-ray Diffraction (XRD). Mössbauer spectroscopy revealed that magnetite (Fe_3O_4) was generated as the primary byproduct alongside pyrite after the long-term incubations (e.g., samples from the Pyrite rep-1 set) (Figure 3.8). The conversion of pyrite to magnetite was also reported previously (Brothers et al., 1996; Thorpe et al.,

1984). This finding was consistent with the results obtained from XRD analysis (Figure 3.9). Notably, other forms of iron oxide, such as hematite (Fe_2O_3), and iron sulfur minerals, including pyrrhotite (Fe_{1-x}S) and elemental sulfur, were not detected at significant levels.

3.4 Discussion

This study for the first time to investigate the potential role of pyrite, the most abundant iron-sulfur mineral in the seabed sediments, in powering the anaerobic oxidation of methane in the absence of external electron acceptors, a long-standing question found in the context of deep methane seep sediments Holmkvist et al., 2011. Robust AOM activity was observed during long-term incubations with pyrite, alongside multiple controls (Table 3.1). Initially, the possibility of a pure chemical reaction between pyrite with methane was excluded under the “No cells” condition. Subsequently, the putative reaction catalyzed by cellular materials was ruled out using the “Killed cells” condition. The baseline AOM activity for the ANME-2c/SRB enrichment was found to be negligible, as almost no activity was detected in the presence of live cells without either sulfate or pyrite. Furthermore, we successfully replicated similar results from two independent sets of incubations involving live cells and pyrite nanoparticles (Pyrite rep-1 and -2) (Figure 3.2).

From the perspective of the pyrite reaction with water, as described in equations 3.3, 3.4 and 3.5, sulfate emerged as one of the final products, with its production demonstrating a size-dependent effect (Figure 3.1). Nanoparticles are expected to have a greater surface area-to-volume ratio compared to micron-sized particles, which increases the likelihood of exposing sulfur-deficient defect sites at the mineral-water interface (Gil-Lozano et al., 2014). Consequently, sulfate reasonably serves as the electron acceptor for AOM, consistent with the results from FISH-nanoSIMS, where SRB were anabolically active (Figure 3.3, Table 3.2). It is important to note that the pyrite nanoparticles were thoroughly rinsed by HCl and water (see *Methods*) before being added to the AOM incubations. This step effectively ruled out any potential contaminants, particularly sulfate-related compounds that may have been sorbed onto the pyrite surface. Additionally, in parallel with sulfate production, H_2O_2 was also quantified in the short-term assay. An evident increase in sulfate yield was concomitant to a consumption of H_2O_2 in the first 5 minutes (Figure 3.1), matching the prediction of the reaction (Eq. 3.5). Notably, we did not detect hydroxyl radicals ($\cdot\text{OH}$), likely due to their short lifetime and highly reactivity in liquid environments. This finding aligns with the observation that no

detectable iron species or sulfide (HS^-) was present in the incubations.

Fe(III)-minerals, most likely in the form of iron oxides, could serve as additional electron acceptors alongside sulfate, contributing to AOM activity during the long-term incubations. Several lines of evidence support this hypothesis. First, higher ratios of ANME-2c to SRB anabolic activity were observed from nanoSIMS in both the Pyrite rep-1 and rep-2 sets (Table 3.2). Previous studies, including the present one, indicated that a roughly 1:1 of ANME-2c to SRB activity would be expected if sulfate was the sole electron acceptor (McGlynn et al., 2015; Scheller et al., 2016), which was not the case when pyrite was added. This suggests that ANME-2c may preferentially utilize other electron acceptors over SRB. Second, in-liquid Fe^{3+} should not serve as the acceptor for AOM. Fe^{3+} ion can diffuse, and its lower reduction potential compared to sulfate would short-circuit the utilization of sulfate (Scheller et al., 2016), leading to a lack of anabolic activity in SRB cells. Additionally, the putative $\cdot\text{OH}$ and H_2O_2 are strong oxidants that would chemically react with iron ions prior to the reduction of Fe^{3+} by methane. Third, a tendency of active ANME-2c clusters closer to the aggregate boundary as well as to the pyrite mineral surface was observed (Figure 3.5 and Figure 3.6). This indicates a potentially more favorable distance for direct interspecies electron transfer (DIET) between ANME-2c and the mineral, which is essential for AOM activity (McGlynn et al., 2015). Lastly, there is no evidence to suggest that $\cdot\text{OH}$ and/or H_2O_2 can serve as electron acceptors for ANME-mediated AOM. Their strong oxidative capability might lead to cell damage rather than facilitating positive interactions in the culture. This damage could stem from high concentrations used in laboratory settings, while lower concentrations resulting from native pyrite reactions *in situ* may fall within a more favorable physiological range. Notably, putative peroxide oxidases have been identified in ANME genomes from previous metagenomic surveys (Chen et al., 2023), leaving this possibility open for further exploration.

This study offers a new perspective, suggesting that pyrite was not as “inert” as previously thought, which could, in turn, support methane oxidizing archaea in the absence of canonical electron acceptors. The generation of sulfate presents another intriguing question, particularly in the context of the early reductive ocean (Crowe et al., 2014). Previous research has demonstrated that pyrite nanoparticles are continuously produced and supplied by deep-sea hydrothermal vents (Findlay et al., 2019; Gartman et al., 2014; Yücel et al., 2011). Under the extreme environment of high temperatures, absence of light, and relatively low oxygen concentrations,

these newly generated pyrite nanoparticles could serve as non-negligible source of sulfate production. This topic is important yet remains understudied within the global biogeochemical sulfur cycle.

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Author contributions

Y.G. conceived the project; V.J.O supervised the project; Y.G. and V.J.O designed the methodology; Y.G. performed the experiments; K.H., P.G., Y.G. and B.F. performed the Mössbauer spectroscopy measurements and analysis; R.D. and Y.G. performed magnetometer detection; G.W. provided the ANME-SRB enrichment; Y.G. generated the original draft of the manuscript, and all authors provided advice and contributed to the final drafting of the manuscript.

Competing interests

The authors declare no competing interests.

3.5 Materials and Methods

Artificial seawater composition

Artificial seawater (ASW) (final concentration) was used in the study modified from reference (Wegener et al., 2016). The composition of the medium was: NaCl 451.2 mM, Na₂SO₄ 10.0 mM (if required), MgCl₂ 27.9 mM, HEPES (pH 7.5) 25 mM, NH₄Cl 2.0 mM, KCl 8.0 mM, NaHCO₃ 5.0 mM, CaCl₂ 1.5 mM, K₂HPO₄ 1.0 mM, SeO₃²⁻ 0.01 μ M, WO₄²⁻ 0.0067 μ M, 0.1% trace element solution, containing per liter: nitrilotriacetic acid 150 mg, MnCl₂ · 4H₂O 610 mg, CoCl₂ · 6H₂O 420 mg, ZnCl₂ 90 mg, CuCl₂ · 2H₂O 7 mg, AlCl₃ 6 mg, H₃BO₃ 10 mg, Na₂MoO₄ · 2H₂O 20 mg, SrCl₂ · 6H₂O 10 mg, NaBr 10 mg, KI 70 mg, FeCl₃ · 6H₂O 500 mg, NiCl₂ · 6H₂O 25 mg. Fresh ASW was make anoxic by N₂ flushing for at least 30 min.

Short-term bench assays of anoxic pyrite reaction

Two categories of pyrite particles were used, micron-size (-325 mesh (44 μ m), CAS #778117, Sigma-Aldrich) and nanometer-size (<100 nm, NRE-5119, Nano Research Elements, Singapore). All experiment were operated strictly in the anaerobic chamber (O₂: *ca.* 20 ppm), or in stoppered vials prior to being moved out.

Pre-weighed pyrite particles were in 2-ml eppendorf tubes, and suspended in the ASW (without sulfate) aforementioned. After a quick vortex and centrifugation (16,000 g, 1 min), 120 μ l of supernatant was sampled for downstream geochemical measurements (sulfur compounds and hydrogen peroxide, see below). Then tubes were vortexed and incubated. After each interval of 5 min, liquid samples were extracted by repeating the cycle of centrifugation and vortex. The assays were done within 1 hr in triplicates at room temperature in dark.

Long-term incubation of anaerobic methane oxidizing enrichment with pyrite

Sediment-free ANME-SRB enrichment (named as Caldera enrichment) was used in this study, which was originally from the coastal hydrocarbon seeps off the Mediterranean island Elba and was a gift from Prof. Gunter Wegener (Wegener et al., 2016). 16S rRNA amplicon sequencing verified that there were dominated by ANME-2c and ANME-2a arhaea speceis.

Different conditions were set by mixing the AOM enrichment and pyrite particles as details according to Table 3.1 in serum vials with stoppers. Pyrite nanoparticles were first washed by 1N HCl overnight, and then raised by pure water several times (both 1N HCl and pure water were first sonicated in order to remove

air from it). The head space was manipulated by over-pressured methane (with 5% of C-13 labeled). N-15 labeled ammonium was the only nitrogen source and used as the isotopic proxy for anabolic activity measurement by FISH-nanoSIMS. All incubations were kept anaerobically at 25 °C in dark. Along with the course of incubation, liquid samples were retrieved for geochemistry measurements. Enrichment biomass was withdrawn at the end of incubations and fixed by 2% PFA for FISH-nanoSIMS.

Geochemistry measurements

Hydrogen sulfide produced by sulfate reduction was detected in technical triplicate by the methylene blue method Cline, 1969. Quantification was carried out in a plate reader (TECAN Sunrise) by monitoring the absorbance at 670 nm.

Sulfate and thiosulfate were measured via parallel Dionex Integrion HPIC (ThermoFisher, Waltham, MA, USA) ion chromatography systems with anion and cation columns running in parallel housed at the Caltech Environmental Analysis Center. The ion chromatography system was run as described previously (Green-Saxena et al., 2014; Speth et al., 2022) with the following modifications. A Dionex AS-DV autosampler loaded samples diluted 1:50 in 18 MΩ water to serial anion and cation columns and detectors, which are maintained at 30 °C. Anions were resolved by a 2×250 mm Dionex IonPac AS19-4 μm analytical column protected by a 2×50 mm Dionex IonPac AG19-4 μm guard column (ThermoFisher, Waltham, MA, USA). A potassium hydroxide eluent generator cartridge generated a hydroxide gradient that was pumped at 0.25 ml min⁻¹. The gradient was constant at 10 mM for 5 min, increased linearly to 48.5 mM at 27 min, then increased linearly to 50 mM at 40 min. A Dionex AERS 500 suppressor provided suppressed conductivity detection running on recycle mode with an applied current of 31 mA. Cations were resolved by a 2×250 mm Dionex IonPac CS16-4 μm analytical column protected by a 2×50 mm Dionex IonPac CG16-4 μm guard column. A methanesulfonic acid eluent generator cartridge generated a methanesulfonic acid gradient that was pumped at 0.16 ml min⁻¹. The gradient was constant at 10 mM for 5 min, nonlinearly increased to 20 mM at 20 min (Chromeleon curve 7, concave up), and nonlinearly increased to 40 mM at 40 min (Chromeleon curve 1, concave down). A Dionex CERS 2 mm suppressor provided suppressed conductivity detection with an applied voltage of 4.2 V. Chromatographic peaks were integrated by Chromeleon 7.2.9 using the Cobra algorithm, and were correlated to concentration by running known standards and generating 4-point calibration curves.

Hydrogen peroxide was determined spectrophotometrically using potassium titanium(IV) oxalate modified from (Sellers, 1980). Briefly, hydrogen peroxide reacts readily with titanium(IV) to form titanium(IV)-peroxide complex, which is yellow-orange in color and can be read at a wavelength of 400 nm.

Quantification of newly formed DIC based on $^{13}\text{CH}_4$ was done by a GC-IR-MS GasBench II (Thermo Scientific). Gasbench data analysis was using the approach described in (Scheller et al., 2016). Gas samples were quantified by a GC-TCD equipped with a HP-MOLSIV column (SN US3351826H, Agilent Technologies).

Ferrozine based protocol was used for the measurement of iron ions in the solution Riemer et al., 2004. In detail, 0.1% w/vol ferrozine solution was made in 50% w/vol acidified ammonium acetate. First, aqueous Fe^{2+} concentrations was detected by 1:1 by volume mixing liquid samples with ferrozine solution at the wavelength of 562 nm. Then, HAHC sloution was made (10% w/vol hydroxylamine hydrochloride in 1M HCl), which was used to reduce the putative proportion of Fe^{3+} down to Fe^{2+} . The reactant was subsequently measured by ferrozine same as aforementioned. This was interpreted as total Fe, and thus Fe^{3+} concentrations.

Hydroxyl radical measurement on EPR

Free hydroxyl radicals were measured from the short-term bench assay by Electron Paramagnetic Resonance (EPR) spectroscopy. BMPO (5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide) (ALX-430-141, CAS# 387334-31-8, Enzo Life Sciences, USA) was used as a trapping agent to stabilize the free radicals for a better measurement (Zhao et al., 2001). An extension of lifetime to around 30 min could be expected. In this study, every sample was extracted, and immediately reacted with BMPO and went to detection.

In detail, first made up a solution of 250 mM BMPO in water. Prepared the reaction mixture to a total reaction volume of 150 μl . Added 120 μl of distilled water to an Eppendorf tube. Added 20 μl of your 250 mM BMPO solution, and followed by 20 μl of liquid sample. Mixed the reactants and quickly transferred the solution to a quartz flat cell. An EMX X-band continuous-wave (CW) EPR spectrometer was used (Electron Paramagnetic Resonance (EPR) Facility, Caltech) with 14" Bruker EPR Magnet. Parameters were used as followed: room temperature, microwave power of 22mW, microwave frequency of 9.84 GHz, sweep width of 100.0 G, Center Field of 3510.10 G, and Sweep Time of 40.0 s.

X-ray powder diffraction (XRPD)

Mineral particle samples were retrieved from culture vials, and dried by speed vacuum. The dried mineral powders were readily brought back to the anaerobic chamber for downstream mineralogy detection. Mineral particle samples were first characterized with X-ray powder diffraction (XRPD). Powders were analyzed with a Rigaku SmartLab powder diffractometer at the X-Ray Crystallography Facility in the Beckman Institute at the California Institute of Technology. A Cu K α source at 50 kV and 40 mA was used. Scans were run from 20° to 70° 2 θ at a scanning speed of 0.02° 2 θ /s. Rigaku SmartLab Studio II was used to fit and identify peaks and the 2020 Crystallography Open Database was used for phase identification.

Mössbauer spectroscopy

Mössbauer spectra were acquired in transmission geometry at room temperature. A constant-acceleration spectrometer equipped with a $^{57}\text{Co}(\text{Rh})$ source (initial activity 25 mCi, $t_{1/2} \approx 272$ days; activity ≈ 23 mCi at measurement) was used. Powder samples (around 150 μm) with a sample loading of about 20 mg/cm 2 were sandwiched between two layers of Kapton tape to protect against air and moisture. They were further enclosed between two 2 mm-thick Pb foils with a 9.5 mm aperture to suppress background γ -rays. Spectra were collected continuously over a ± 12 mm/s Doppler velocity range for approximately 24 h. The velocity scale was calibrated against a 30 μm α -Fe foil measured at room temperature. Data were fitted to Lorentzian line shapes using MossA (Prescher et al., 2012). From these fits we extracted isomer shifts, quadrupole splittings, and magnetic hyperfine fields.

Magnetometer measurement

Rock magnetic analyses and tests were made on a 2G DC-SQUID magnetometer with the RAPID setup (Kirschvink et al., 2008). The background field in the shield room that houses the magnetometer is 150 nT. About XX mg of dried sample was loaded into quartz tubes, then loaded onto the magnetometer for a series of rock magnetic tests (Biasi et al., 2021). These tests include progressive anhysteretic remanent magnetization (ARM) experiment, isothermal remanent magnetization (IRM) acquisition (5.7 to 1000 mT) and demagnetization (1.6 to 384 mT) experiment, and modified Lowrie-Fuller tests (Johnson et al., 1975; Lowrie & Fuller, 1971).

Sample preparation for aggregate embedding, sectioning, FISH, and nanoSIMS

Sample preparation was carried out following protocol as described previously (McGlynn et al., 2015). Briefly, PFA-fixed consortia were detached from the sediment particles via sonication on ice with microtip probe (Branson). Aggregates were Percoll separated and concentrated by centrifugation, immobilized in molten noble agar (3%). The biomass in agar blocks were then dehydrated via a series of ethanol concentrations of 50%, 75%, 90%, 100%, 100%, and 100%. The dehydrated blocks were embedded in glycol methacrylate (Heraeus Kulzer-Technovit 8100). Sections of *ca.* 3 μm thickness were cut and stretched on a water droplet on a polylysine coated slide with teflon wells (Tekdon Inc) and analyzed by FISH. Images of the FISH-stained consortia were collected, and the location of these consortia were mapped for subsequent nanoSIMS analysis as described below.

FISH and probes used in this study

The FISH hybridization followed the protocol as described previously (McGlynn 2015). The samples were hybridized in hybridization buffer containing 35% formamide and incubated at 46 °C for 2 hrs, followed by a wash step at 48 °C for 15 min. The FISH probes were used in this study (5 ng/ μl for each):

ANME-2c-760 (Alexa488): 5' to 3' = GTGCTCCCCCGCCAATTCCT (Knittel et al., 2005);

ANME-2a-647 (Cy3): 5' to 3' = GGTCGCACCGTGTCTGACACCT (Knittel et al., 2005);

DSS658 (Alexa647), dual labeled: 5' to 3' = TCCACTTCCCTCTCCCAT (Lösekann et al., 2007; Manz et al., 1998).

nanoSIMS measurements

Sections with aggregates and cells were first coated with 20-nm-thick Au (High Resolution Sputter Coater 208HRD, Cressington). NanoSIMS measurements were performed on a CAMECA nanoSIMS 50L in the Center for Microanalysis at the California Institute of Technology. Gold-coated samples were presputtered with a 160-pA primary Cs^+ ion beam with aperture diaphragm D1 = 1 until the $^{14}\text{N}^{12}\text{C}^-$ ion counts stabilized. Data were collected using a 1.8-pA or 3.6-pA beam with D1 = 3 or D1 = 2 and entrance slit (ES) = 2. Masses were collected corresponding to the $^{14}\text{N}^{12}\text{C}^-$, $^{15}\text{N}^{12}\text{C}^-$, $^{32}\text{S}^-$ and $^{56}\text{Fe}^-$ ions, using a tuning protocol as described previously (Chadwick et al., 2019). Acquisitions were collected from diverse-size

square raster areas, with 512×512 pixels, and 1 to 4 planes were collected per area. Dwell time settings resulted in acquisition times 30 min to 1 hr per plane depending on the size of the raster.

nanoSIMS data analysis

NanoSIMS data processing and analysis were done in Matlab (R2024b) and R (R4.4.2 version). NanoSIMS.im data files were initially processed using the Look @ NanoSIMS Matlab GUI (Polerecky et al., 2012) to align planes and export raw data. To align isotopic enrichment data of nanoSIMS with the taxonomic information in the space resolution, the processed nanoSIMS raw $^{14}\text{N}^{12}\text{C}^-$, and $^{15}\text{N}^{12}\text{C}^-$ images were used for the region of interest (ROI) drawing by hand combining with FISH images as reference. This was performed in the software Fresco (Adobe) on tablet. Regions of acquisitions that contained ANME-SRB cells were outlined based on the manual cell ROIs, and the elemental information was assigned to each ROI, which generated the single-cell level isotopic enrichment.

For ANME-2c averaged single cluster activity analysis, groups of ANME-2c cells belonging to separate clusters were recognized based on both FISH staining and ^{15}N atom enrichment from nanoSIMS. This was rooted on two aspects of knowledge: only ANME-2c cells grew as clusters; FISH staining was based on 16S rRNA, which was expected to be degraded if cells were inactive. Accordingly, clusters that were either stained by ANME-2c FISH probes or enriched with ^{15}N were regarded as active clusters, and the rest of clusters were inactive. ^{15}N fractional abundance was calculated by averaging each whole cluster area. The aggregate boundary was also recognized manually.

DNA extraction and iTag community analysis

DNA was extracted from the sediments stored after 4-month incubation using DNeasy PowerSoil Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Illumina iTag 16S rRNA gene sequencing protocol was followed as described in the study (Yu et al., 2021). Briefly, PCR amplification, barcoding and sequencing of the 16S rRNA hypervariable region (V4-V5) was performed using PCR primers 515f and 906r and amplification conditions as previously described (Yu et al., 2018). Sequencing data was processed using QIIME v1.8.0 (74), clustered at 99% sequencing identity using UCLUST v7.0.1001 (Edgar, 2010), and the taxonomic identity of the most abundant sequence in each cluster was assigned using a custom SILVA database modified from SILVA Ref NR 99 Database Release 115

(Mason et al., 2015; Quast et al., 2013). iTag community analysis was performed using dada2 and R.

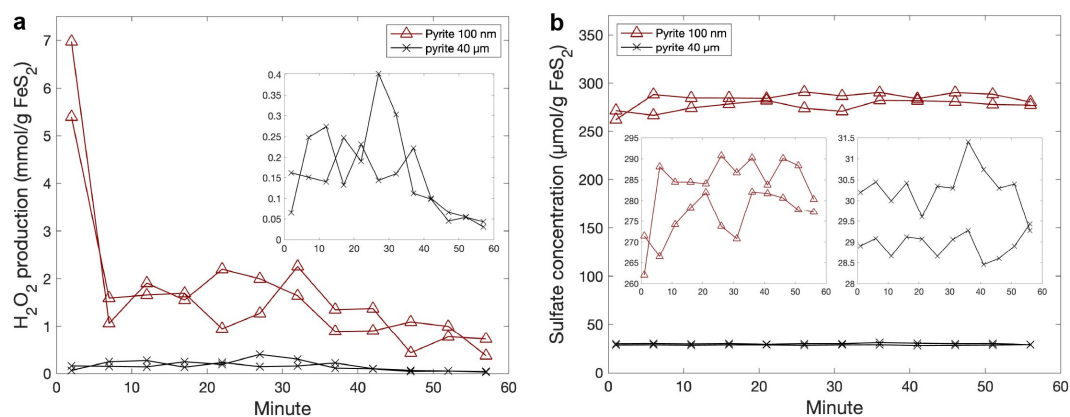


Figure 3.1: Concentrations of hydrogen peroxide (a) and sulfate (b) generated from short-term assays of pyrite and ASW reaction within 1 hr in dark. The inserted plots were the zoomed-in illustrations. The assays were done in biological replicates.

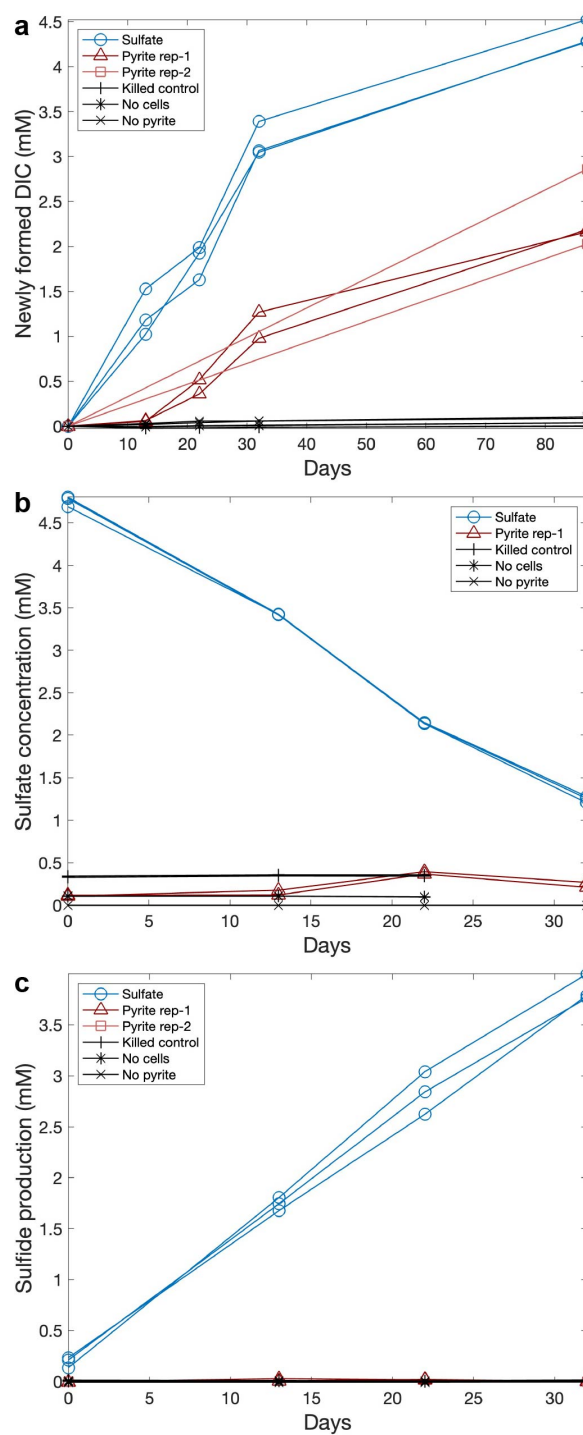


Figure 3.2: The course of AOM activity in the long-term incubations. **a**, The newly generated DIC concentration over time measured via ^{13}C labeled methane oxidation. **b, c**, The sulfate and sulfide concentration over time.

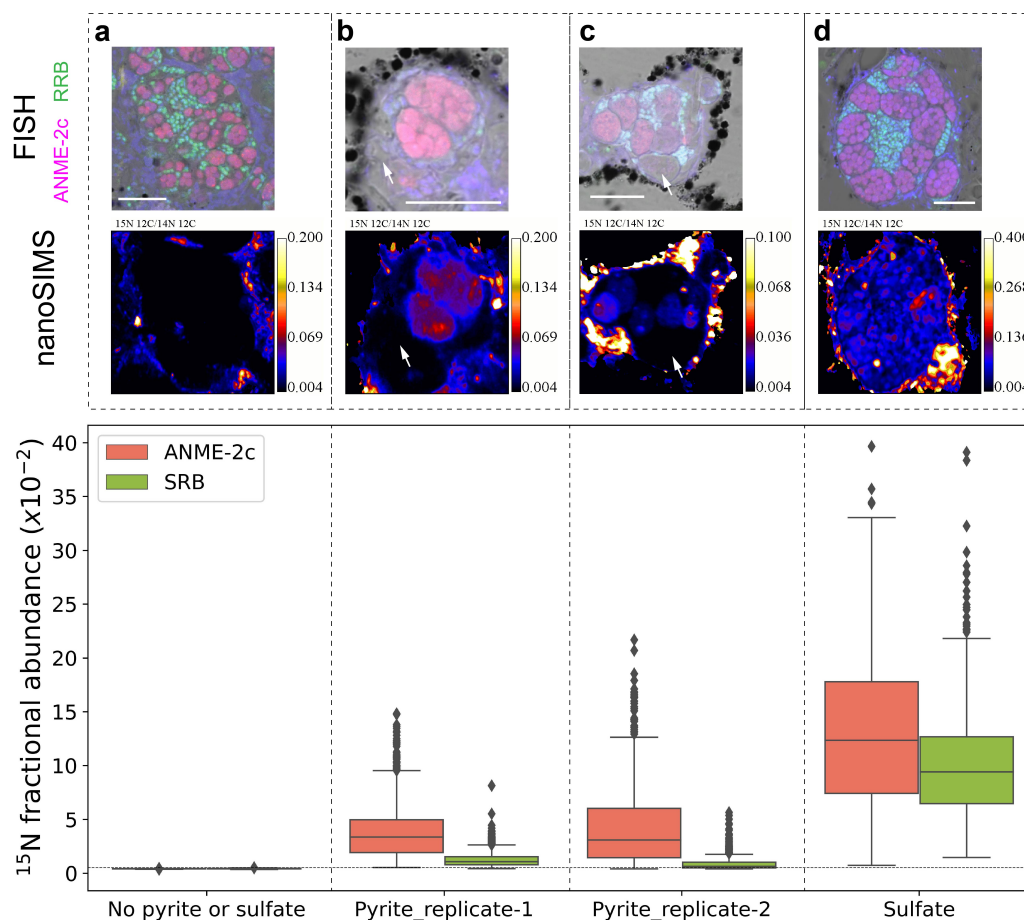


Figure 3.3: Anabolic activity summarized from paired FISH–nanoSIMS experiments for ANME-2c lineage and its syntrophic SRB. Representative FISH–nanoSIMS images from long-term microcosms. Panel **a**, the nanoSIMS result from control condition; **b**, the pyrite incubation replicate-1 condition; **c**, the pyrite incubation replicate-1 condition; **d**, the positive control (canonical AOM condition with sulfate). In each panel, the top row shows FISH pictures for ANME-2c (red) and their syntrophic SRB partners (green) composited together with bright field images; the middle shows a representative nanoSIMS image for each condition; the bottom shows all the results collected from single cells analyzed in this study is presented. See Table 3.2 for detail. The white arrows marked in **b**, **c** represented damaged cluster of cells. Scale bar: 10 μm .

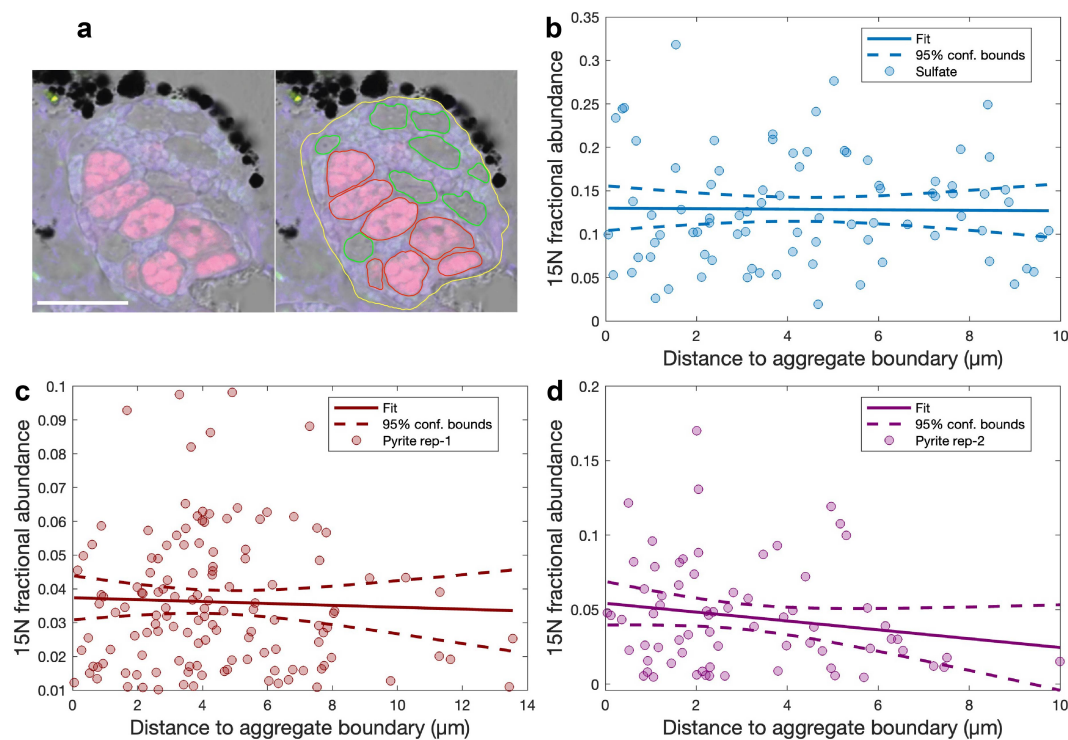


Figure 3.4: The relationship of the averaged anabolic activity at single cluster level against the distance to the aggregate boundary. **a**, Left: an example of an aggregate shown as the original FISH image (ANME-2c, pink; black dots were iron-sulfur mineral particles); right: segmented ANME-2c cell clusters where active clusters were marked by red contours, inactive marked by green and the boundary of the aggregate marked by yellow. See *Methods* for details. Scale bar: 10 μm . **b**, **c**, **d**, Activity of single archaeal clusters plotted against distance to the aggregate boundary (**b**, sulfate condition, $n = 83$, $R^2 = 0.000196$; **c**, pyrite replicate-1 set, $n = 133$, $R^2 = 0.00148$; **d**, pyrite replicate-2 set, $n = 70$, $R^2 = 0.0323$).

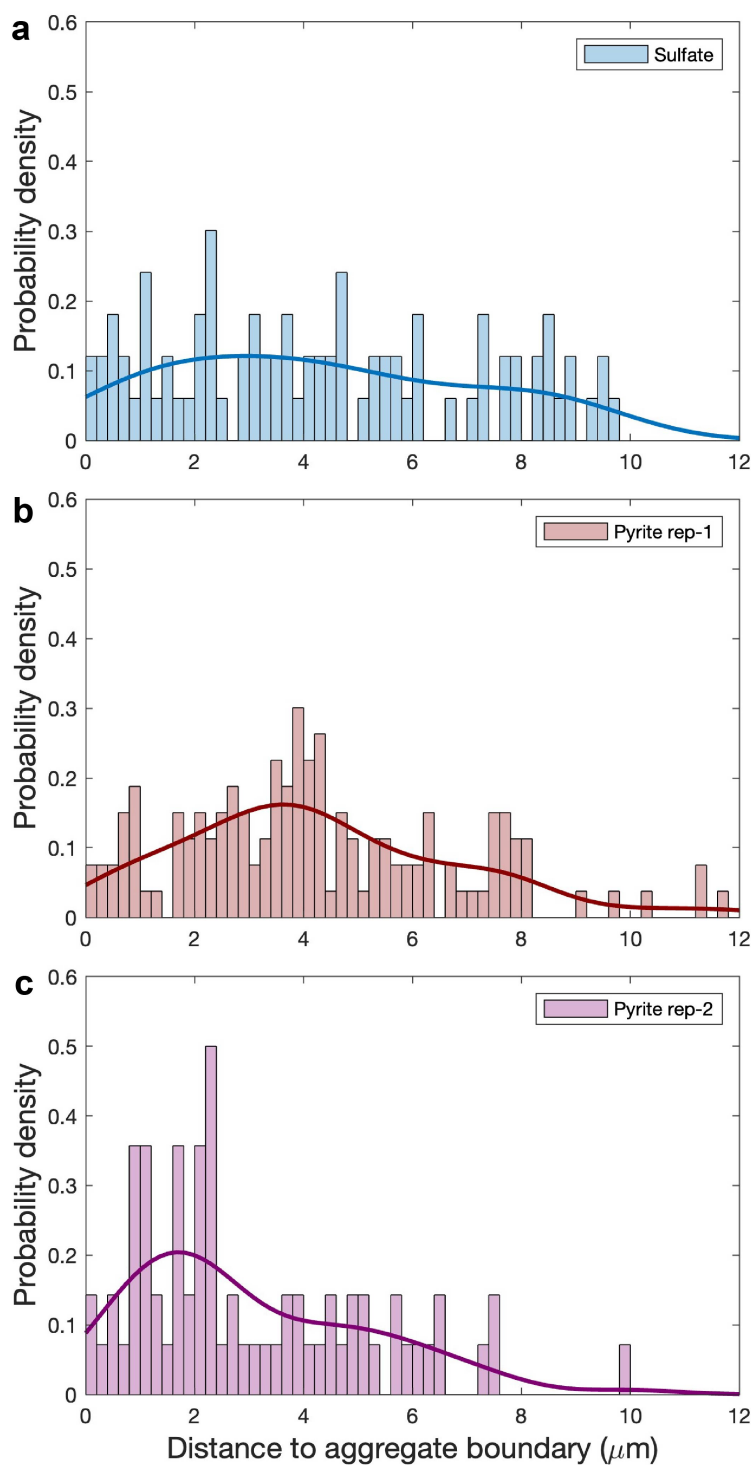


Figure 3.5: The probability distribution function of active ANME-2c clusters against distance to the aggregate boundary. **a**, Sulfate condition; **b**, pyrite replicate-1 set; **c**, pyrite replicate-2 set.

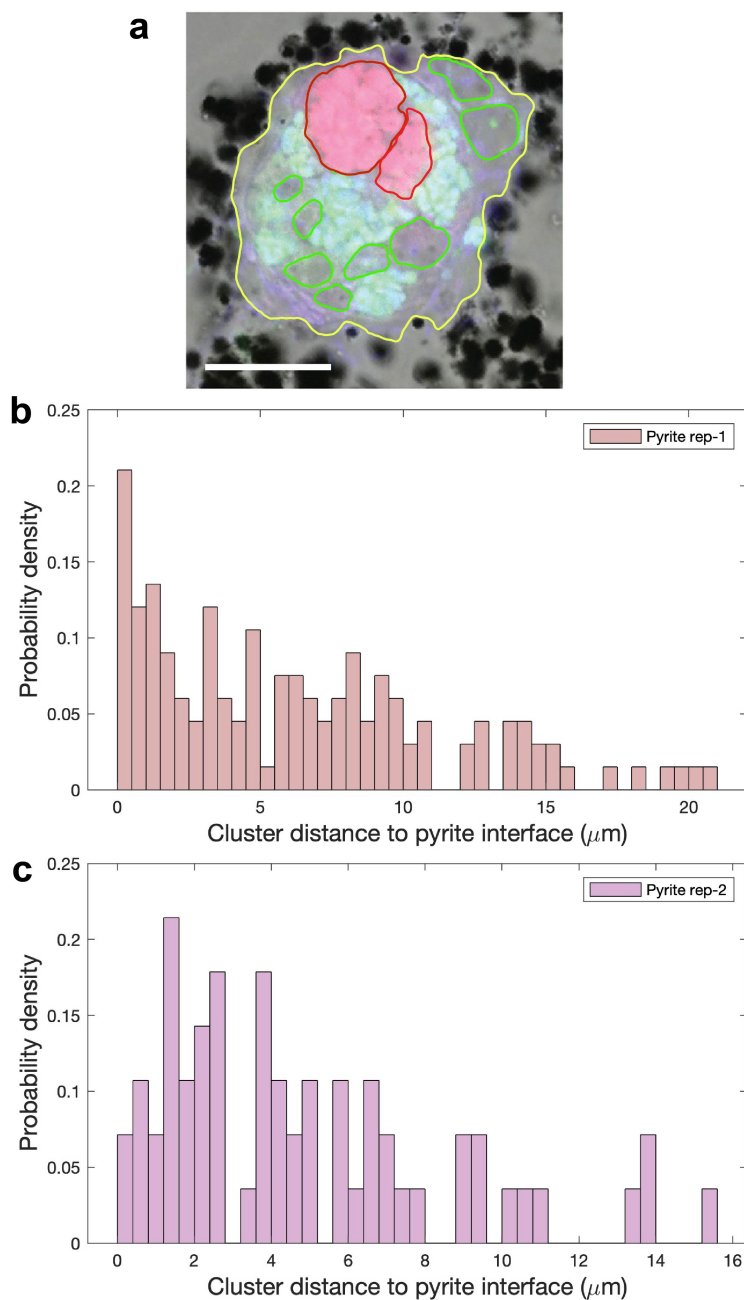


Figure 3.6: The probability distribution function of active ANME-2c clusters against distance to the pyrite particles. **a**, An example of an aggregate shown as the FISH image (ANME-2c, pink; black dots were iron-sulfur mineral particles), where segmented active ANME-2c cell clusters were marked by red contours, inactive marked by green and the interface of pyrite particles marked by yellow. See *Methods* for details. Scale bar: 10 μm . ; **b**, pyrite replicate-1 set; **c**, pyrite replicate-2 set.

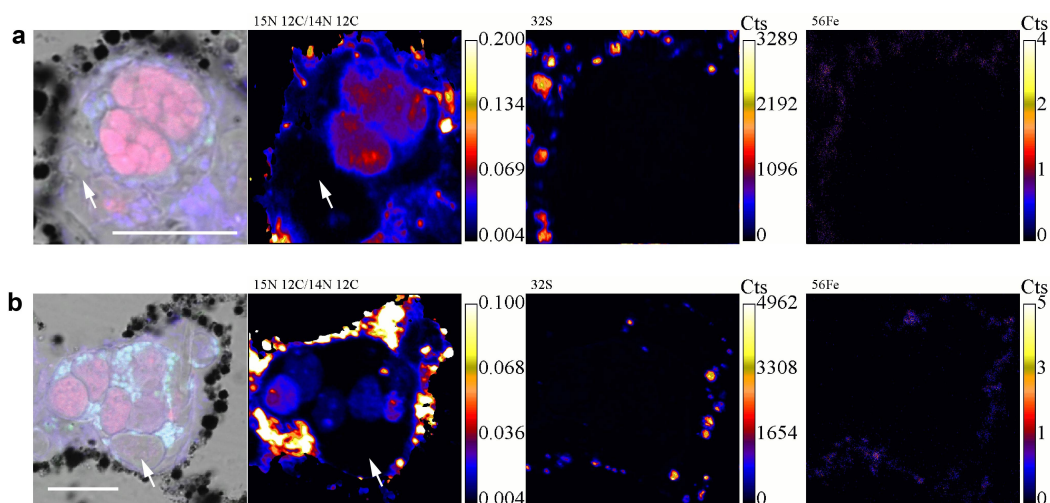


Figure 3.7: Other representatives of paired FISH–nanoSIMS results with Fe and S signals for ANME-2c lineage and its syntrophic SRB. In FISH pictures composited together with bright field images, red for ANME-2c and green for their syntrophic SRB partners. The white arrows marked represented damaged cluster of cells. Scale bar: 10 μm .

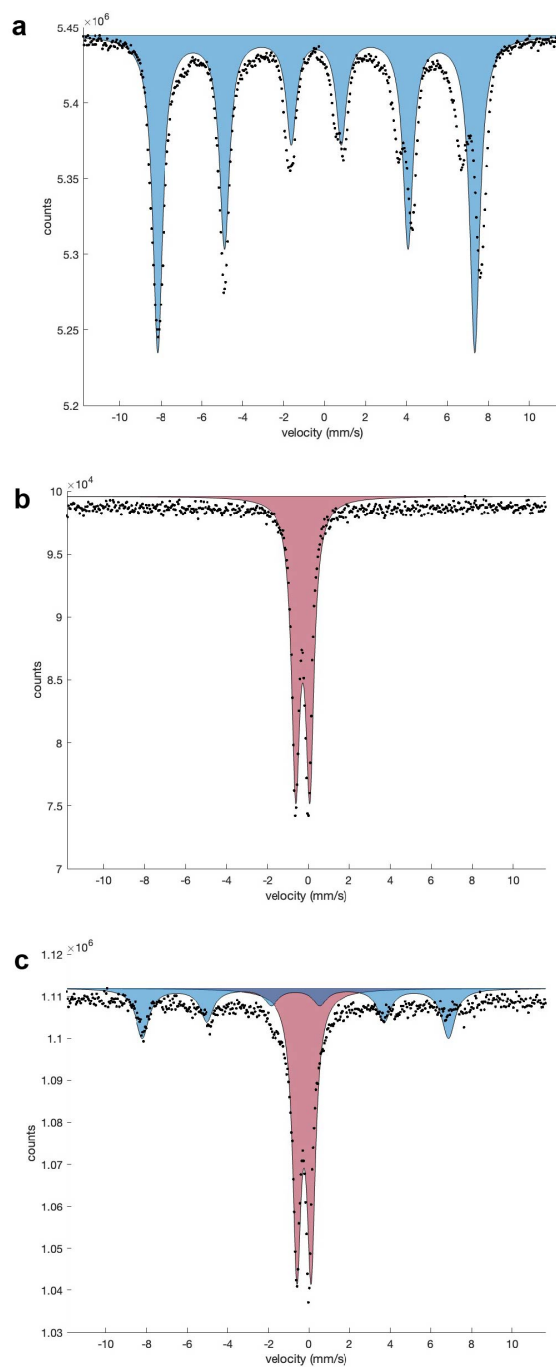


Figure 3.8: Representative Mössbauer spectroscopy collected from this study. **a**, Magnetite standard; **b**, pyrite standard; **c**, mineral sample collected from pyrite incubation replicate-1.

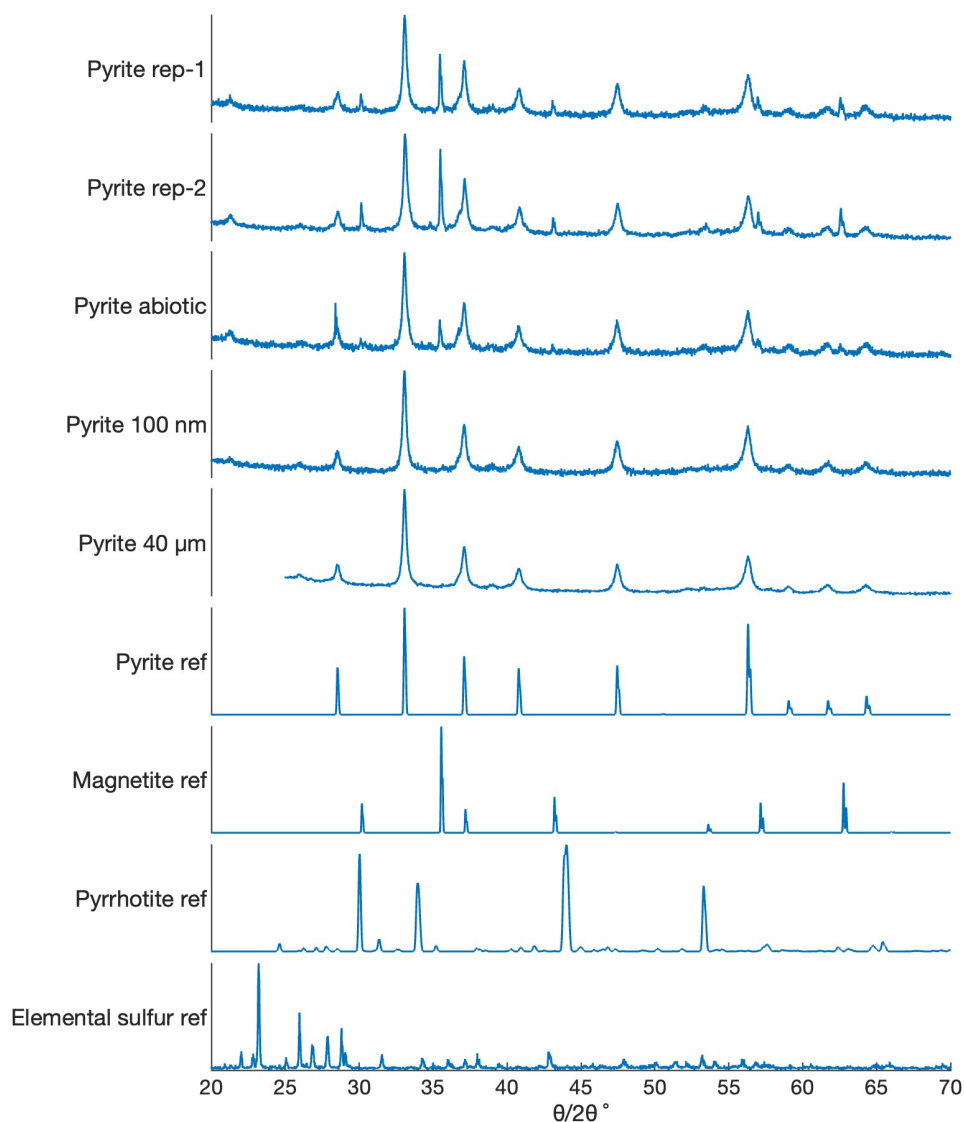


Figure 3.9: XRD spectra of samples in this study. Mineral particle samples included conditions of pyrite incubation replicate-1, replicate-2, pyrite abiotic control, fresh pyrite nanoparticle (100 nm) before use, fresh pyrite particle (sigma, 40 μm) before use, pyrite standard (RRUFF R100166), magnetite standard (RRUFF R061111), pyrrhotite standard (RRUFF R061127), and elemental sulfur standard (RRUFF R050006).

Table 3.1: Summary of microcosm incubations and their conditions developed in this study

Condition	Sulfate	Pyrite rep-1	Pyrite rep-2 ^a	Killed control	No cells	No pyrite
Biological replicates	3	2	2	2	2	2
Biomass	Live	Live	Live	Dead	n ^b	Live
Pyrite	n	y ^c	y	y	y	n
Sulfate	y	n	n	n	n	n
CH ₄	y	y	y	y	y	y
nanoSIMS	y (99 days)	y (99 days)	y (208 days)	n	n	y (99 days)

^a A parallel incubation replicate where conditions were the same as pyrite rep-1 group;
^{b, c} y and n represents “yes” (addition) and “no” (none, absent).

Table 3.2: ^{15}N enrichment of ANME-SRB consortia at single cell level from FISH-nanoSIMS analysis

		Unlabeled control	Pyrite rep-1 ^a	Pyrite rep-2 ^b	Sulfate
ANME-2c	^{15}F ^c no. of cells	0.40 ± 0.014 ^d (998)	3.73 ± 2.21 (2000)	4.38 ± 3.95 (583)	13.07 ± 7.20 (918)
SRB	^{15}F no. of cells	0.42 ± 0.028 (1020)	1.24 ± 0.68 (1020)	0.88 ± 0.67 (798)	9.95 ± 4.79 (1427)
	$\frac{^{15}F_{(ANME)}}{^{15}F_{(SRB)}}$	0.92 ± 0.10	3.27 ± 1.20	4.92 ± 3.23	1.33 ± 0.25
	no. of aggregates	15	21	13	10

^a nanoSIMS measurement after 99 days of incubation together with control and sulfate conditions;

^b nanoSIMS measurement after 208 days of incubation;

^c ^{15}F : ^{15}N fractional abundance, $^{15}N / (^{15}N + ^{14}N) \times 100\%$;

^d mean $\pm$ standard deviation;

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*Chapter 4*RECORDING AND *IN SITU* READOUT OF SINGLE CELL
SPATIOTEMPORAL ACTIVITY OF AS-YET-UNCULTURED
ANAEROBIC METHANOTROPHIC CONSORTIAYongzhao Guo[†], Justus W. Fink, Victoria J. Orphan[†]Division of Geological and Planetary Sciences, California Institute of Technology,
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4.1 Abstract

Tracking the dynamics of microbial metabolic activity at the level of individual cells over time within their native spatial context remains an understudied challenge in environmental microbiology. The complexity of environmental matrices necessitates novel approaches to overcome this limitation. In this study, we describe a pulse-chase pipeline that allows microbes to record anabolic activity information by probing multiple heavy isotopes at distinct periods of growth. This information can subsequently be read out from single cells *in situ* using FISH-nanoSIMS (fluorescence *in situ* hybridization and nanoscale secondary ion mass spectrometry) measurements on end-point samples. Using this approach as a proof of principle, we investigated a well-known yet uncultured microbial consortium composed of methane-oxidizing archaea (ANME) and sulfate-reducing bacteria (SRB). We successfully resolved cells exhibiting significant changes in their cellular activity over time, referred to as “naughty cells”. Our results indicated that these naughty cells tended to be located in close proximity to their partners and within the periphery of aggregates. The mechanisms underlying these spatial patterns may be attributed to energy metabolism and the availability of nutrients. The capability of this approach to track spatiotemporal activity *in situ* demonstrates great potential for application across diverse environmental microbial systems.

4.2 Introduction

Microbial metabolic activity, both spatially and temporally, is fundamentally important in microbiology. The advancement of state-of-the-art technologies has enabled us to investigate microbial processes *in situ* at the single-cell level more effectively. An increasing number of studies have been developed to capture the spatial distribution of metabolic molecules, such as transcriptomes (mRNA) (Chen et al., 2015; Dar et al., 2021; Eng et al., 2019; Sarfatis et al., 2025; Shah et al., 2018; M. Zhang et al., 2021) and proteomes (proteins) (Guo et al., 2025; Takei et al., 2025; Z. Zhang et al., 2025), across various biological cells from different metabolic perspectives. In addition to these approaches, the non-toxic nature of stable isotopes, combined with the high sensitivity and spatial resolution of nanometer-scale secondary ion mass spectrometry (nanoSIMS) and fluorescence in situ hybridization (FISH), holds great potential for quantitatively studying microbial phylogeny and their processes in bulk natural samples (Orphan & House, 2009). For instance, the N-15 atom tracer was frequently employed in methane seep sediments to investigate nitrogen cycling and active microbial metabolism (Dekas et al., 2009; Kopf et al., 2015; McGlynn et al., 2015; Orphan et al., 2009; Scheller et al., 2016). Significant progress has been made in understanding spatial metabolism; however, studies addressing the temporal context of metabolic dynamics have been largely overlooked.

Efforts have been made to address the topic. A straightforward approach involves harvesting samples across multiple time points throughout the course of experiments. The underlying assumption is that biological cells are homogeneous, and thus, the subpopulation extracted at an earlier time point can adequately represent the entire population. However, this assumption is clearly undermined in natural environmental microbial communities, which are known to be heterogeneous in space (Orphan et al., 2004). Furthermore, bulk measurements inevitably disrupt the native biological structure and result in the loss *in situ* spatial information. To overcome these limitations, studies have been reported that manipulate either genetic or cellular molecules by creating or adding a time tag, allowing for subsequent decoding of information (Chadly et al., 2024; Choi et al., 2022; Chow et al., 2021; Frieda et al., 2017; Islam et al., 2024). However, due to the complexity of environmental samples and the inherent challenges of studying unculturable organisms, all reported approaches failed in resolving native spatiotemporal activity effectively.

Building on these, we focused on the anaerobic oxidation of methane (AOM)

microbial consortia, a well-studied yet uncultured environmental microbial system. This system typically consists of syntrophic species, including anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria (SRB), that thrive on the edge of thermodynamic constraints through the reaction of methane with sulfate (Knittel & Boetius, 2009; Orphan et al., 2001).



We then leveraged the concept of pulse-chase workflow and grounded in FISH-nanoSIMS to conduct a proof-of-concept experiment using multiple isotope-labeled substrates (ammonium-15N, bicarbonate-13C, water-17O, and water-18O) at different time intervals. The time-resolved anabolic activities, represented by particular heavy isotopes, enabled us to read out the *in situ* spatiotemporal activity of single cells within the AOM consortia.

4.3 Results and Discussion

Recording of temporal anabolic activity via pulse-chase pipeline

To ultimately decipher the temporal activities over time while maintaining the *in situ* spatial context, we developed a pulse-chase protocol based on the robust approach of stable isotopic probing combined with FISH-nanoSIMS (McGlynn et al., 2015). The pulse-chase pipeline relies on the concept that a stable, heavy isotopically labeled nutrient is initially introduced during the pulse stage, followed by the introduction of the same nutrient compound with the same isotope but unlabeled during the chase phase (Figure 4.1a). The information regarding past anabolic activity is recorded in the matrix of heavy isotopic enrichment, which can be preserved at a considerable level over subsequent time periods. Meanwhile, another heavy stable isotope is introduced in conjunction with the chase phase of the previous element (Figure 4.1a). At the end of the incubations, microbes (specifically ANME-2b and syntrophic SRB) can be harvested. Notably, every single cell within the aggregates in the sediments contains multiple heavy stable isotopes, each representing distinct anabolic activities retrospectively during different growth periods, as determined through end-point measurements.

Readout of “naughty cells” over time

The dynamics of single cell activities over time were tracked and further read out through measurements obtained using FISH-nanoSIMS. The *Multi – iso* dataset represented a culture where microbial cells were routinely fed with multiple nutrient isotopes simultaneously. This approach enabled an assessment of the correlations

between anabolic activities with different element proxies, which manifested as a set of distributions. Within these distributions, the activity of each cell could be similarly explained by all the isotopes. Accordingly, cells that exhibited distinct isotopic information in cellular biomass from the *pulse – chase* experiment, lying beyond this distribution, can be interpreted as having undergone significant changes in anabolic activities over time during different growth periods represented by isotopes. We categorized these cells as “naughty cells”, while those exhibiting unchanged activities were labeled as “conservatives” (Figures 4.2 and 4.3) (see *Methods* for detailed description).

High-resolution biosynthetic activity was resolved for phylogenetically classified ANME-2b archaea and their partner SRB using FISH-based microbial identification (ANME-2b-729 oligonucleotide probe and Desulfosarcina/Desulfococcus group probe). A total of 14,221 FISH-identified cells within 49 consortia were categorized as part of the *multi – iso* dataset, while 10,284 cells within 27 aggregates were analyzed in the *pulse – chase* experiment (Table 4.2). Both the *multi – iso* and *pulse – chase* incubations demonstrated AOM activities (Table 4.1), enabling us to assess the anabolic activity of single cells in both spatial and temporal contexts.

From an aggregate-level perspective, an average of $29.6 \pm 20.1\%$ of naughty cells were identified per aggregate across the entire cell population (Table 4.3), with all aggregates containing naughty cells. A representative aggregate showcasing these “naughty cells” is illustrated Figure 4.4. We further investigated two parameters to characterize the spatial morphological intermixing degree of syntrophic ANME-2b and SRB species: J values and Moran’s I index (see *Methods* for detailed description). These metrics were proved suitable quantitative measures to describe aggregate spatial mixing within the AOM consortia systems (McGlynn et al., 2015). Despite this conformational variability, the degree of mixing between both species did not affect the percentage of naughty cells per aggregate (Figure 4.5), suggesting that the presence of naughty cells was independent of the aggregate morphologies.

Spatial patterns of “naughty cells” within aggregates

Statistically, approximately 16% and 28% of ANME-2b cells exhibited significant changes in anabolism when comparing activities during the N-15 period with those during the C-13 period, and from the C-13 to the O-17 period, respectively (Table 4.4). Concurrently, around 37% and 30% of syntrophic SRB cells showed significant changes in their activities during the N-15 and C-13 periods, respectively, compared

to the preceding periods (Table 4.4). Notably, about half of these cells showed activities that were either higher or lower than in the previous time intervals (Tables 4.4 and 4.5). In particular, we identified the neighborhood interface between ANME and SRB species, where pairs of these syntrophic species were recognized as direct spatial contacts. This analysis revealed a disproportionately greater portion of naughty cells localized within the neighborhood, such as ANME-2b during the N-15 vs. C-13 period (Table 4.5). Furthermore, around 73% of neighboring ANME-2b naughty cells were paired with SRB naughty cells simultaneously, which was similarly observed for SRB cells (66%) under the C-13 vs. O-17 condition (Table 4.5).

Inspired by this observation, we calculated the probability density distribution (PDF) of naughty cell counts based on their spatial positioning within the AOM consortia, utilizing Kernel fitting (Bowman & Azzalini, 1997). A higher probability was observed for ANME-2b naughty cells occurring in close proximity to their syntrophic partners as well as near the boundary of the consortia during the N-15 vs. C-13 condition. In contrast, a higher probability for SRB naughty cells was only noted near the aggregate periphery (Figure 4.6). This observation was not applicable under the C-13 vs. O-17 condition, where there was a tendency for naughty cells to occur near the interface of syntrophic partners or the aggregate boundary (Figure 4.8). However, a deeper investigation indicated that ANME-2b naughty cells exhibiting higher activities during the C-13 over O-17 period had a greater probability of being closer to their partners and likely to the aggregate boundary (Figure 4.9).

Spatial patterns of cellular activity for “naughty cells”

Semi-quantitative activities for “naughty cells” were determined, where a higher absolute value of normalized activity represented a more dramatic change in the biosynthetic activity. Upon examining the ^{15}N - or ^{13}C -based activity patterns of individual ANME-2b and SRB cells within the AOM consortia, no clear trend emerged indicating that the activity of naughty cell was related to proximity to their nearest partners or the aggregate boundary (R^2 values < 0.02) (Figures 4.10 and 4.11). However, it was more common to observe naughty cells exhibiting the most dramatic activity changes located near their partners or the aggregate boundary. This was particularly evident for ANME-2b and SRB under the N-15 vs. C-13 condition, as well as for SRB during the C-13 vs. O-17 period (Figures 4.12 and 4.13).

Energy and nutrient availability for “naughty cells”

The availability of energy and nutrients plays a crucial role in influencing the dynamics of anabolic activities among microbes within the AOM consortia over time. According to the sulfate-related AOM reaction (Equation 4.1), methane acts as the terminal electron donor, which is oxidized by ANME-2b archaea to produce bicarbonate (Knittel & Boetius, 2009). As a gas, methane can freely diffuse through the aggregates, allowing inner ANME cells to access it easily. Increasing evidence points to the importance of direct interspecies electron transfer (DIET), which facilitates electron flow and supports syntrophic energy metabolism within the AOM consortia (McGlynn et al., 2015; Wegener et al., 2015). Simulations predict that an energy loss occurs over distances due to factors such as ohmic resistance and activation loss (He et al., 2021). A hybrid approach that combines DIET with the diffusive exchange of electron donors may offer energetic advantages for syntrophic consortia (Chadwick et al., 2022; He et al., 2021). These factors may help explain the high likelihood of ANME-2b cells being classified as “naughty” in the regions close to their sulfate-reducing bacterial partners.

In contrast, proximity to the archaeal-bacterial interface does not confer an advantage for syntrophic SRB cells to be considered “naughty”, unlike their ANME counterparts. The availability of sulfate ions is essential for the energy metabolism of SRB, where inward diffusion plays a critical role. This observation aligns with the tendency for naughty SRB cells to be located near the aggregate boundary. Moreover, both ANME-2b and SRB naughty cells are more often found at the periphery of aggregates, likely due to the diffusion of nutrients from the surrounding environment.

Temporal dynamics of “naughty cells”

The consistency of the same naughty cells was examined across different time periods. Only a subset of microbial cells showed a continuous state of being naughty in their anabolic activity during both the C-13 and N-15 periods. For example, on average, approximately 23% of ANME-2b cells and around 38% of SRB naughty cells identified as “naughty” during the C-13 period were also found to be naughty in the subsequent N-15 period (Table 4.5). Among these naughty cells, 60% of ANME-2b and 7% of SRB naughty cells demonstrated a consistent increase in anabolic activity during both the C-13 and N-15 periods. However, it is important to note that these findings may not be fully representative, as 63 of the 91 ANME cells and 14 of the 17 SRB cells were sourced from a single aggregate (Table

4.5). This indicates that most ANME-2b and SRB cells tended to exhibit naughty behavior only within specific time window, rather than maintaining a constant state of naughtiness throughout or being consistently in the same “naughty” state.

4.4 Conclusion

This study introduced the concept of a pulse-chase experiment by leveraging the advantage of FISH-nanoSIMS, successfully resolving cellular anabolic activity both spatially at the single cell level and temporally with a resolution of two weeks in uncultured anaerobic methanotrophic consortia from bulk methane seep sediments. The dynamics of cellular activity over time demonstrated a dependence on geometric locations within the aggregates, revealing hotspots in the neighborhood regions between the ANME-SRB partnerships and at the periphery of the aggregates. These spatial patterns may be rooted in the mechanisms of electron transfer across the consortia (DIET) and the availability of nutrients essential for energy and biosynthesis metabolism.

The combination of pulse-chase and multi-iso experiments have proved to be an effective bulk protocol to record and readout microbial spatiotemporal activity. This approach holds great potential for application across a broad range of microbial systems, including microbial biofilms and bulk environmental microbial assemblages containing yet uncultured microbes. Notably, this protocol can be expanded to probe a more diverse array of species by taking advantage of the well-developed multiplexed fluorescence imaging (Chen et al., 2015; Eng et al., 2019). Furthermore, the temporal resolution can be manipulated by varying the number of stable isotopic proxies or adjusting the duration of incubations, provided that the requirement for enriching heavy isotopes in cellular biomass is met. Additionally, the pulse-chase pipeline can be utilized to investigate microbial communities with distinct isotopes, which could then be integrated with SIP-sequencing protocols if spatial resolution is less of a focus. Temporally resolved cellular activity represents a critical dimension of metabolic information, and this study could serve as an inspiration for exploring a central yet often understudied topic in environmental microbiology.

4.5 Materials and Methods

Sediment collection and processing

Methane seep sediments covered with white microbial mats were collected by push core (PC6) in the dive AD4912 on 27 May 2017 by DSV Alvin, launched from R/V Atlantis on research cruise AT37-13 in Jaco Scar, off Costa Rica, at 1,811-m water depth (lat 9.1163, long 284.8372). The sediment core was sliced into 3-cm-thick horizons shipboard which were stored individually in separate Whirl-Pak bags and kept anaerobically at 4 °C in sealed Mylar bags sparged with Ar until they were shipped to the laboratory. Sediments (serial number #10073-76) from Costa Rica were particularly used in this study.

Medium composition

Artificial seawater (ASW) (final concentration) was used in the study modified from reference (Yu et al., 2021). The composition of the medium was: NaCl 457 mM, Na₂SO₄ 10.0 mM, MgCl₂ 47 mM, HEPES (pH 7.5) 25 mM, NH₄Cl 2.0 mM, KCl 7.0 mM, NaHCO₃ 5.0 mM, CaCl₂ 1.0 mM, K₂HPO₄ 1.0 mM, SeO₃²⁻ 0.01 μM, WO₄²⁻ 0.007 μM, 0.1% trace element solution, containing per liter: nitrilotriacetic acid 150 mg, MnCl₂ · 4H₂O 610 mg, CoCl₂ · 6H₂O 420 mg, ZnCl₂ 90 mg, CuCl₂ · 2H₂O 7 mg, AlCl₃ 6 mg, H₃BO₃ 10 mg, Na₂MoO₄ · 2H₂O 20 mg, SrCl₂ · 6H₂O 10 mg, NaBr 10 mg, KI 70 mg, FeCl₃ · 6H₂O 500 mg, NiCl₂ · 6H₂O 25 mg.

Pulse-chase microcosm development

All manipulations of the sediment incubation were done anaerobically. Sediments to be used were first divided into two 100-ml serial vials and maintained with sulfate/methane prior to establishment of the formal microcosm experiments.

The schematic of pulse-chase incubation is described in Figure 4.1.

In general, 4-fold concentrated ASW (4×ASW) without ammonium, sulfate, bicarbonate or monohydrogen phosphate was used to better amend incubations together with water-dissolved heavy isotopically labeled nutrients and critical compounds sulfate and phosphate to the final concentration as mentioned above. 10-ml serum vials were used throughout this study. 1.0 ml of settled sediment was inoculated together with a total medium volume of 2.0 ml. All the incubations were maintained with 2 bar over-pressurized methane anaerobically at 4 °C in dark.

In detail, at the beginning (T0), water-17O (90 atom% 17O, Cambridge Isotope

Laboratories, Catalog # OLM-782-90-1) was first used with a final concentration of 18‰ of the total water pool in the bottles, i.e., 18‰ of ^{17}O labeling strength. After 2 weeks (14 days, as T1), liquid sample was withdrawn for further geochemical determination. Before switching to next isotope, to remove potential residual water- ^{17}O from the previous period, sediments in the vials were washed by 1×ASW for 5 times of the loop of re-suspension and centrifugation.

For duration between T2 and T3 (2 weeks), heavy isotopic compound bicarbonate- ^{13}C (99 atom‰ ^{13}C , Cambridge Isotope Laboratories, Catalog # CLM-441-5) was amended to a final concentration of 5mM and labeling strength of 99%. Additionally, water- ^{18}O (97 atom‰ ^{18}O , Sigma-Aldrich, Catalog #329878) was used (final concentration and labeling strength: 19.4‰) as a probe to calibrate and analyze the measured result of ^{17}O from nanoSIMS. Liquid sample was taken at T3 same as T1. Sediments were rinsed again similar to T1 to eliminate residual bicarbonate- ^{13}C .

For duration between T4 and T5 (2 weeks), N-15 labeled ammonium (99 atom‰ ^{15}N , Cambridge Isotope Laboratories, Catalog #NLM-467-10) was amended as the only nitrogen source (2 mM, 99% labeling strength). Same as C-13 duration, water- ^{18}O (97 atom‰ ^{18}O , Sigma-Aldrich, Catalog #329878) was continued to be used (final concentration and labeling strength: 19.4‰). The reason to use ammonium as the final isotopic tracer was because of its tendency to be sorbed onto mineral surface.

At T5, sediment slurry was extracted and fixed in 2% paraformaldehyde (PFA) solution at 4 °C overnight. The fixed material was for downstream FISH-nanoSIMS analysis.

Multi-iso microcosm setup

In parallel with the pulse-chase microcosm setup, we developed another set of incubations with multiple heavy isotopes at one time. For set-1, the four heavy isotopic compounds (ammonium- ^{15}N , bicarbonate- ^{13}C , water- ^{17}O , and water- ^{18}O) were amended all together. For set-2, only ammonium- ^{15}N and bicarbonate- ^{13}C were added. The concentrations and labeling strength were the same as pulse-chase setup. Sediment slurry was withdrawn after 14, 35 and 77 days, and fixed by 2% PFA as mentioned above. Liquid samples were collected for sulfide production over time.

Hydrogen sulfide measurement

Hydrogen sulfide produced by sulfate reduction was detected in technical triplicate by the methylene blue method (Cline, 1969). Quantification was carried out in a plate reader (TECAN Sunrise) by monitoring the absorbance at 670 nm.

Sample preparation for aggregate embedding, sectioning, FISH, and nanoSIMS

Sample preparation was carried out following protocol as described previously (McGlynn et al., 2015). Briefly, PFA-fixed consortia were detached from the sediment particles via sonication on ice with microtip probe (Branson). Aggregates were Percoll separated and concentrated by centrifugation, immobilized in molten noble agar (3%). The biomass in agar blocks were then dehydrated via a series of ethanol concentrations of 50%, 75%, 90%, 100%, 100%, and 100%. The dehydrated blocks were embedded in glycol methacrylate (Heraeus Kulzer-Technovit 8100). Sections of *ca.* 1 μ m thickness were cut and stretched on a water droplet on a polylysine coated slide with teflon wells (Tekdon Inc) and analyzed by FISH. Images of the FISH-stained consortia were collected, and the location of these consortia were mapped for subsequent nanoSIMS analysis as described below.

FISH and probes used in this study

The FISH hybridization followed the protocol as described previously (Scheller et al., 2016). The samples were hybridized in hybridization buffer containing 35% formamide and incubated at 46 °C for 2 hrs, followed by a wash step at 48 °C for 15 min. The FISH probes were used in this study (5 ng/ μ l for each): ARCH915 (Alexa488), dual labeled: 5' to 3' = GTGCTCCCCCGCCAATTCCT (Amann et al., 1990);

ANME-2b-729 (Cy3), dual labeled: 5' to 3' = CGTTCTCGTAGGGCGCCT (McGlynn et al., 2015);

DSS658 (Alexa647), dual labeled: 5' to 3' = TCCACTTCCCTCTCCCAT (Lösekann et al., 2007; Manz et al., 1998).

nanoSIMS measurements

Sections with aggregates and cells were first coated with 20-nm-thick Au (High Resolution Sputter Coater 208HRD, Cressington). NanoSIMS measurements were performed on a CAMECA nanoSIMS 50L in the Center for Microanalysis at the California Institute of Technology. Gold-coated samples were presputtered with a 130-pA primary Cs⁺ ion beam with aperture diaphragm D1 = 1 until the ¹⁴N¹²C⁻

ion counts stabilized. Data were collected using a 5-pA or 10-pA beam with D1 = 2 and entrance slit (ES) = 2. Masses were collected corresponding to the $^{12}\text{C}^-$, $^{13}\text{C}^-$, $^{16}\text{O}^-$, $^{17}\text{O}^-$, $^{18}\text{O}^-$, $^{14}\text{N}^{12}\text{C}^-$, and $^{15}\text{N}^{12}\text{C}^-$ ions, using a tuning protocol as described previously (Chadwick et al., 2019). Acquisitions were collected from diverse-size square raster areas, with 512×512 pixels, and 3 to 4 planes were collected per area. Dwell time settings resulted in acquisition times from 1 min to 2 hrs per plane depending on the size of the raster.

nanoSIMS data analysis

NanoSIMS data processing and analysis were done in Matlab (R2024b) and R (R4.4.2 version). NanoSIMS.im data files were initially processed using the Look @ NanoSIMS Matlab GUI (Polerecky et al., 2012) to align planes and export raw data. To align isotopic enrichment data of nanoSIMS with the taxonomic information in the space resolution, the processed nanoSIMS raw $^{14}\text{N}^{12}\text{C}^-$, and $^{15}\text{N}^{12}\text{C}^-$ images were used for the region of interest (ROI) drawing by hand combining with FISH images as reference. This was performed in the software Fresco (Adobe) on tablet. Regions of acquisitions that contained ANME-SRB cells were outlined based on the manual cell ROIs, and the elemental information was assigned to each ROI, which generated the single-cell level isotopic enrichment.

Self curing “dilution effect” in pulse-chase experiment

The net accumulation of individual isotopes over each period of incubation was reflected from the nanoSIMS detection, $Enrichment_{detected}$ in the pulse-chase experiment.

$$Enrichment_{detected} = Enrichment_{real} - Assimilation_{light-iso}$$

where $Enrichment_{real}$ is the actual anabolism while fed by specific isotopes, which was affected by the “dilution effect” from the assimilation of light isotopes (unlabeled nutrients) in the following chase periods of time ($Assimilation_{light-iso}$).

The “dilution effect” can be self cured. To solve this issue, we amended a parallel experiment with multiple labeled nutrients fed at one time (hereafter *multi-iso* setup, see *Methods*, Figure 4.1b). In this setup, the relationship between these heavy isotopes can be resolved simply by nanoSIMS. Difference between labeled and unlabeled nutrients could be ignored considering the short incubation duration (2 weeks) and viable concentrations amended in the culture. Thereby, the amounts of light isotopes assimilated in the chase phases (the term $Assimilation_{light-iso}$) can

be extrapolated from the unknown yet real isotopic information. For example, in this study, water-17O was pulsed in the first 2 weeks, and then replaced with water-18O in the following 4 weeks. At nanoSIMS, the unaffected O-18 atom enrichment directly from nanoSIMS can be used to estimate how much oxygen atom was uptaken and to what degree O-17 was “diluted” by other oxygen isotopes in the following 4 weeks. Likewise, C-13 was pulsed in the middle 2 weeks, and chased by unlabeled-C in the last 2 weeks, which ultimately “diluted” C-13 enrichment. Unlike oxygen element with 3 stable isotopes, C-13 is the only stable heavy carbon isotope. Alternatively, we know that ammonium-15N was used in the last 2 weeks, which would be appropriately used to extrapolate the amount of unlabeled-C in the same duration of incubation based on the cellular stoichiometry relationship between different elements, i.e., N-15 and C-13 assimilation, when microbes were growing.

Apart from the “dilution effect” from the chasing times, we investigated the potential contribution of the light isotopes from the AOM reaction (Equation 4.1) due to the utilization of unlabeled CH₄ and SO₄²⁻ as the electron donor and acceptor for the system. The contribution of unlabeled H₂O to the total H₂O pool in the medium was estimated to be less than 0.02%, which was neglected in this study (Section 4.5). In contrast, the newly generated unlabeled bicarbonate from the AOM reaction can hardly be ignored considering its production at the same order of magnitude as that of the original bicarbonate concentration in the medium. The expected carbon assimilation was thus corrected by combining the catabolic activities, reflected by the sulfide production over time in the incubations (Table 4.1).

“Dilution effect” in pulse-chase experiment and its correction by *multi-iso* setup

The analysis was based on the principle of mass balance.

$$(m_{cell_0} + m_{13C})F_{13C_{real}} + m_{C(N)}F_{13C_{nat}} = (m_{cell_0} + m_{13C} + m_{C(N)})F_{13C_{det}} \quad (4.2)$$

$$F_{13C_{real}} = \frac{m_{C(N)}}{m_{cell_0} + m_{13C}}(F_{C_{det}} - F_{C_{nat}}) + F_{C_{det}} \quad (4.3)$$

where m_{cell_0} is the initial total C mass in cells of interest before C-13 addition, $F_{C_{nat}}$ is the C-13 natural abundance, m_{13C} is the amount (mass) of total C atom uptaken during C-13 duration time, $F_{13C_{real}}$ is the real C-13 fractional abundance (C-13 enrichment) in the C-13 weeks which is the values to be determined here, $m_{C(N)}$ is the C mass assimilated during N-15 period of time, and $F_{C_{det}}$ is the C-13 fractional abundance obtained from nanoSIMS measurement.

In equation 4.2, $m_{C(N)}$ is the C mass assimilated during N-15 period of time, and the denominator part, $m_{cell_0} + m_{13C}$, is the total C mass just before N-15 addition. Then the critical question can be re-phrased as how much $m_{C(N)}$ is. In the pulse-chase pipeline, unlabeled bicarbonate was used as the only C source in N-15 addition time, but it cannot be resolved by nanoSIMS, while N-15 enrichment (fractional abundance) can be determined. For the same species of cells, there must be a stoichiometry between different elemental components, i.e., C and N. Based on this, we can extrapolate the assimilation of C atom from the amount of N up-taken during the same growth time.

$$\frac{^{13}C}{^{12}C} = f\left(\frac{^{15}N}{^{14}N}\right) \quad (4.4)$$

$$\frac{m_{C(N)}}{m_{cell_0} + m_{^{13}C}} \approx \frac{^{13}C}{^{12}C} = f\left(\frac{^{15}N}{^{14}N}\right) \quad (4.5)$$

To figure out their relationship, we conducted an incubation where all the C source to be assimilated was C-13 labeled, which simultaneously was together with N-15 (ammonium) as the only N source (see Methods above). Importantly, here it is fair to assume that the C assimilation is independent of unlabeled (C-12) or labeled (C-13). The resulting C-13 from this setup (in 4.4) can be simply applied to the situation of pulse-chase N-15 period of time. Therefore, the term $\frac{^{13}C}{^{12}C}$ could be approximating to $\frac{m_{C(N)}}{m_{cell_0} + m_{^{13}C}}$, which were supposed to be in a function with $\frac{^{15}N}{^{14}N}$ (as shown in Equation 4.5).

Likewise, in the case of O-17 and O-18,

$$(m_{cell_0} + m_{^{17}O})F_{^{17}O_{real}} + 0.8 \times m_{^{18}O}F_{^{17}O_{nat}} = (m_{cell_0} + m_{^{17}O} + m_{^{18}O})F_{^{17}O_{det}} \quad (4.6)$$

$$F_{^{17}O_{real}} = \frac{m_{^{18}O}}{m_{cell_0} + m_{^{17}O}}(F_{^{17}O_{det}} - 0.8 \times F_{^{17}O_{nat}}) + F_{^{17}O_{det}} \quad (4.7)$$

$$\frac{m_{18O}}{m_{cell_0} + m_{17O}} \approx f \left(\frac{18O_{det}}{16O + 17O} \right) \quad (4.8)$$

where m_{cell_0} is the initial mass of cells of interest, $F_{17O_{nat}}$ is the O-17 natural abundance, m_{17O} is the amount (mass) of O-17 atom uptaken during O-17 time, m_{18O} is the amount (mass) of O-18 atom uptaken during O-18 time, $F_{17O_{real}}$ is the real O-17 fractional abundance (O-17 enrichment) during O-17 weeks, and $F_{18O_{det}}$ is the O-18 fractional abundance obtained from nanoSIMS measurement.

Calibration of O-17 and O-18 assimilation by the dilution from AOM reaction

One mole of H₂O is produced alongside with the reaction of one mole of methane and one mole of sulfate from the AOM reaction (Equation 4.1). The dilution of unlabeled H₂O was estimated here. Take water molar volume of 18.016 ml mol⁻¹ (5 °C) (Jones et al., 2023) and incubation volume of 2 ml. Combined with the catabolic activities in Table 4.1, around 14 μ mol of unlabeled H₂O was contributed to 1.1×10^5 μ mol of water pool in the medium, that is around 0.013 %.

Calibration of C-13 assimilation by the dilution from AOM reaction

As the carbon source for ANME-SRB cells, apart from the initial addition, the bicarbonate generated from the oxidation of methane (as shown in Equation 4.1) will contribute to the bicarbonate pool in the medium, which potentially affects the C-13 enrichment from the nanoSIMS measurement. In this study, unlabeled methane was used throughout all the experiments. Its effect was calibrated.

Since the C-13 labeling strength changed over time with the production of unlabeled bicarbonate from unlabeled methane, several parameters are considered.

$$Mixing\ Ratio_{13C} = \frac{bicarb_{13C_{int}}}{bicarb_{12C} + bicarb_{13C_{int}}} = \frac{bicarb_{13C_{int}}}{r_{cat} \cdot t + bicarb_{13C_{int}}} \quad (4.9)$$

$$[13C\ atom]_{det} = \int_0^t \frac{bicarb_{13C_{int}}}{r_{cat} \cdot t + bicarb_{13C_{int}}} \cdot r_{ana} dt \quad (4.10)$$

$$[12C\ atom]_{predict} = \int_0^t \frac{r_{cat} \cdot t}{r_{cat} \cdot t + bicarb_{13C_{int}}} \cdot r_{ana} dt \quad (4.11)$$

$$F_{13C_det} = \frac{[13C \text{ atom}]_{det}}{[12C \text{ atom}]_{det} + [13C \text{ atom}]_{det}} \quad (4.12)$$

$$r_{ana} \Rightarrow F_{13C_calib} = \frac{[13C \text{ atom}]_{det} + [12C \text{ atom}]_{predict}}{[12C \text{ atom}]_{det} + [13C \text{ atom}]_{det}} \quad (4.13)$$

where *Mixing Ratio*_{13C} is the portion of C-13 bicarbonate in the bulk medium, *bicarb*_{13C_{int}} is the initial concentration of C-13 bicarbonate in the culture, *r_{cat}* is the catabolic rate of cells ($\mu\text{mol d}^{-1} \text{ cm}_{sed}^{-3}$), *r_{ana}* is the anabolic rate of cells ($\mu\text{mol d}^{-1} \text{ cm}_{sed}^{-3}$), $[13C \text{ atom}]_{det}$ is the mole of C-13 atom assimilated into the cellular biomass, $[12C \text{ atom}]_{predict}$ is the mole of C-12 atom predicted to be incorporated into biomass together with 13C over a period of time *t*, *F*_{13C_{det}} is the raw C-13 fractional abundance based on data detected from nanoSIMS, and *F*_{13C_{calib}} is the real C-13 fractional abundance, i.e., anabolic activity in term of C-13, after calibration. It is assumed here that the anabolism is much smaller than the catabolism considering the slow growth rate for ANME-SRB aggregates.

For calibration, catabolic activity data in Table 4.1 were used. Conversion factors were used as follows: 1.694 for C-13 stage in pulse-chase dataset, 1.447 for multi-iso [N, C] dataset, and 2.838 for multi-iso [N, C, O] dataset.

Outlier boundary and naughty cells from pulse-chase experiment

Linear regression combined with its residual information was used to compute the outlier boundary. In detail, first the *multi – iso* dataset was fitted by linear regression with the purpose of find the residuals of the regression. That is, the Euclidean “width”, i.e., the orthogonal distance from points to the regressed line, of the *multi – iso* dataset distribution along the linear regression was regarded as the “outlier boundary”. Next, 2 residuals (2 standard deviations (2ε)) away from the regression were used as the threshold “width” and applied to the *pulse – chase* dataset. In this way, points that were located outside were “naughty cells” of interest, where their anabolic activities changed significantly in a specific time comparing to the previous period of time.

Explanation on 2D sectioned slices and 3D whole aggregates

In this study, two dimensional (2D) slices were measured on nanoSIMS. This is rooted in the nature of isotopic ion acquisition on nanoSIMS, where a flat surface

will end up with a good resolution. The relationship between cellular activity and the relative locations within aggregates were analyzed. As mentioned in (McGlynn et al., 2015), though information might be missing from the effects of the layers above and below the slice of detection, the basic assumption is that the 2D geometry is able to represent for 3D spatial arrangement statistically. All the measurements of distance mentioned in this study are the best approximations to the situation of 3D aggregates.

Neighboring cell recognition

The recognition of neighborhood interface between ANME and SRB species was performed by importing 2D coordinates (x, y) of the regions of interest (ROIs) centroids to the R statistical package and then processed with the spdep package (Bivand & Piras, 2015; Bivand et al., 2013). The construction algorithm of Spheres of Influence neighborhoods was used in this study, which was shown working great in ANME-SRB consortia (McGlynn et al., 2015), and the resulting data were further corrected manually.

Metrics for degree of mixing

Two independent parameters were investigated for the purpose of illustrating the spatial conformations within aggregates, Moran's I (Moran, 1950) and the matrix of degree of mixing (J metric). J is a quantitative metric first developed by (McGlynn et al., 2015), which was proved to be a robust parameter to describe the spatial degree of mixing between ANME and SRB cells.

In Moran's I algorithm, an identity value of 1 or 0 was assigned to each ROI. In our case, the phylogenetic affiliation of the ROIs was considered, and 1 was for ANME and 0 for SRB (McGlynn et al., 2015).

$$I = \frac{n}{\sum_{ij} w_{ij}} \frac{\sum_{ij} w_{ij} (x_i - \bar{x})(x_j - \bar{x})}{\sum_i (x_i - \bar{x})^2} \quad (4.14)$$

$$J_{aa} = \sum_{ij} w_{ij} f_{aa}(x_i, x_j) \quad (4.15)$$

$$J_{bb} = \sum_{ij} w_{ij} f_{bb}(x_i, x_j) \quad (4.16)$$

$$J_{ab} = \sum_{ij} w_{ij} f_{ab}(x_i, x_j) \quad (4.17)$$

$$J = \frac{\frac{J_{aa}}{\binom{n_a}{2}} + \frac{J_{bb}}{\binom{n_b}{2}}}{2 \frac{J_{ab}}{n_a n_b}} \quad (4.18)$$

In those equations, the weight function w_{ij} is $1/r_{ij}^2$, where r_{ij} is the distance between ROI i and j (in micron). The functions f_{aa} , f_{bb} and f_{ab} are logical operators which return 1 if the subscript case is met. If we assume a is as ANME and b as SRB, when ROI i and j are both ANME cells, then f_{aa} equals to 1, and f_{bb} and f_{ab} are 0. $\binom{n_a}{2}$ is the total number of connections between two ANME ROIs. Likewise, $\binom{n_b}{2}$ is the total number of connections between two SRB ROIs, and $n_a n_b$ is the total number of connections between ANME and SRB.

Normalization of cellular activity for “naughty cells”

A semi-quantitative cellular activity was determined by normalizing the residuals of “naughty cells” away from the regression line over 2 residuals (2 standard deviations (2ε) in *multi – iso* dataset regression) away from the regression. The normalized results were presented as ratios greater than 1 or smaller than -1, where positive values were significantly higher activities in N-15 over C-13 time or in C-13 over O-17, and vice versa.

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Author contributions

Y.G. and V.J.O devised the study, Y.G. conducted the experiments, Y.G. and J.W.F conducted the data analysis, Y.G. generated the draft, and all authors contributed to data interpretation and writing of the manuscript.

Competing interests

The authors declare no competing interests.

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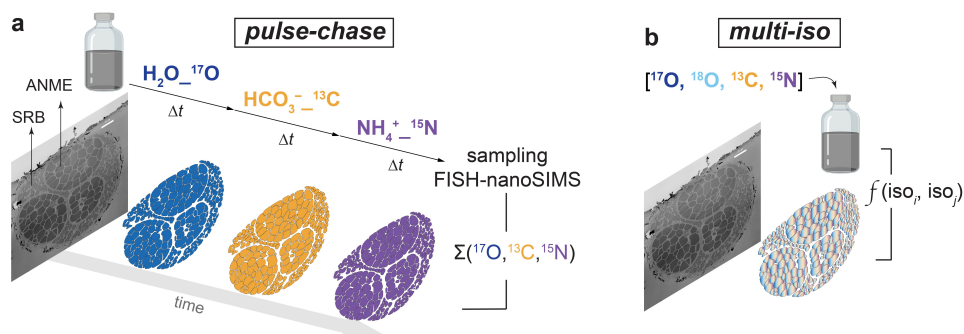


Figure 4.1: Multiple isotopic labeling by pulse-chase demonstration of recording and *in situ* readout of cellular anabolism over time. **a**, the detailed schematics of pulse-chase experiment, where individual heavy stable isotopes, O-17, C-13 and N-15 atoms, are incorporated into cellular biomass via anabolic activity at distinct periods of time, respectively. At the end, the history activities at each stage can be extracted. **b**, The illustration of multi-iso setup in parallel with the pulse-chase experiment. Multiple isotopes are amended simultaneously at one time in order to correct the “dilution effect” and be used to seek for the “dynamic cells”.

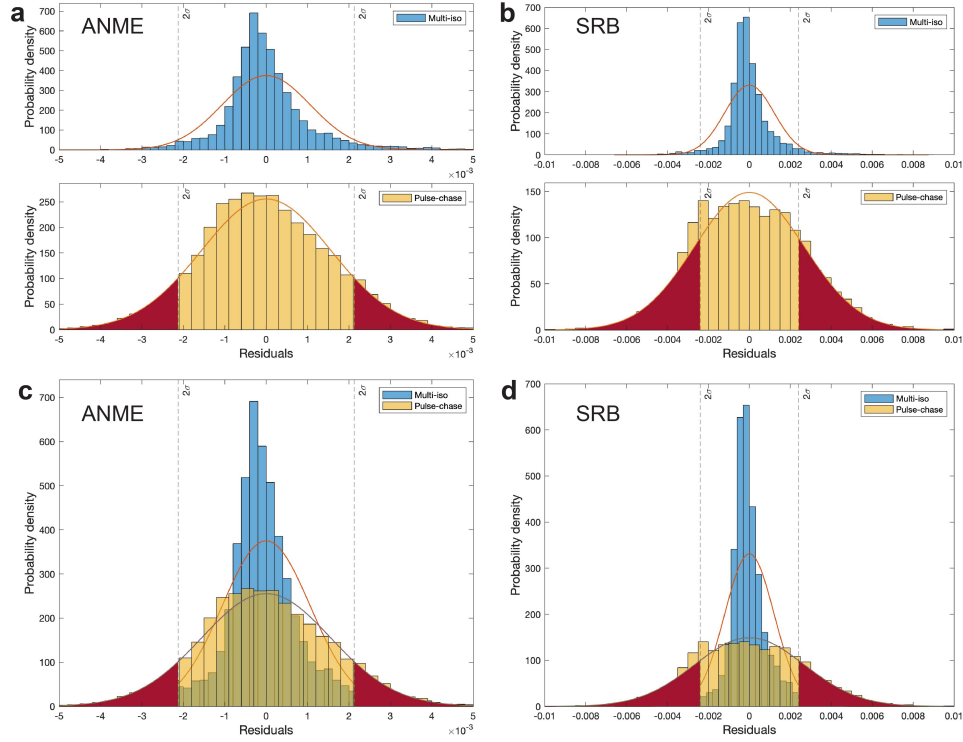


Figure 4.2: The thresholding of “naughty cells”. **a,b**, The blue histogram was the probability density distribution (PDF) of *multi – iso* [N, C] dataset, and the yellow histogram was the PDF of *pulse – chase* N-15 vs. C-13 data. The red curve lines were the stimulated PDF distribution. **c,d**, The merged plots from **a,b**, where the red parts were the points of “naughty cells”.

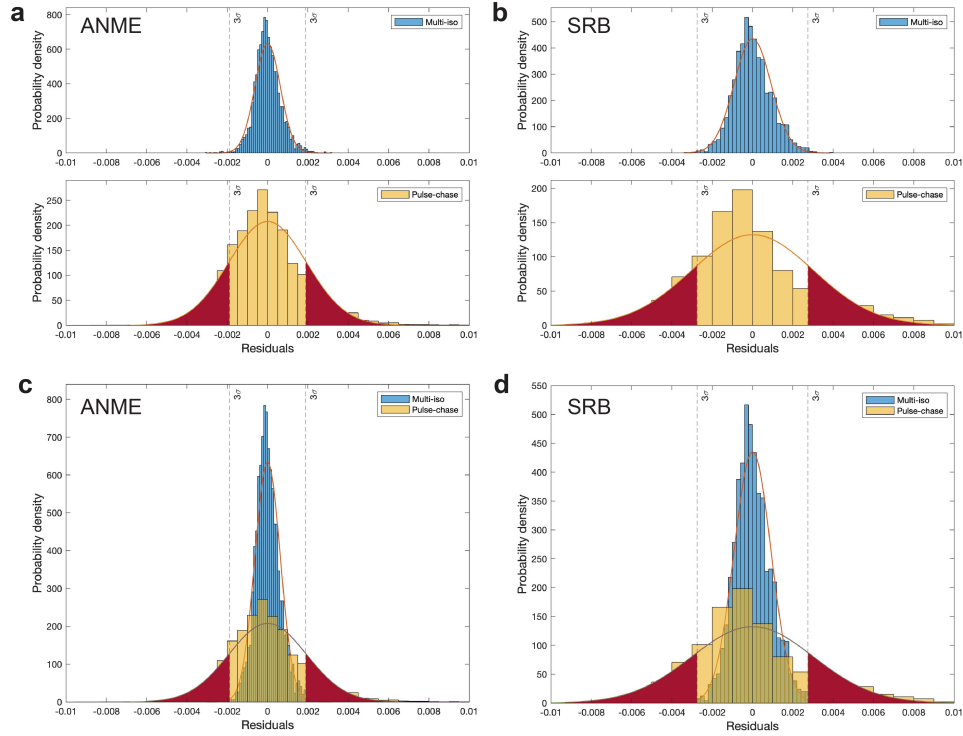


Figure 4.3: The thresholding of “naughty cells”. **a,b**, The blue histogram was the probability density distribution (PDF) of *multi – iso* [N, C, O] dataset, and the yellow histogram was the PDF of *pulse – chase* C-13 vs. O-17 data. The red curve lines were the stimulated PDF distribution. **c,d**, The merged plots from **a,b**, where the red parts were the points of “naughty cells”.

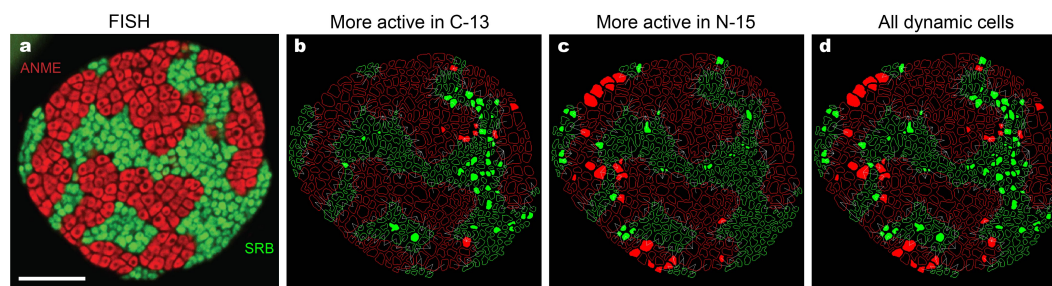


Figure 4.4: A representative example of an ANME-SRB aggregate marked by “naughty cells”. **a**, The FISH image of the example aggregate with ANME archaea (red) and SRB bacteria (green). **b**, The marked cells with significantly higher activities in C-13 over N-15 period of time. **c**, The marked cells with significantly higher activities in N-15 over C-13 period of time. **d**, All the cells with a significant change in their activities over time marked in this example aggregate. Closed cells represented the “naughty” cells. The white lines showed the identification of cells that share a border with a syntrophic partner (archaea adjacent to bacteria).

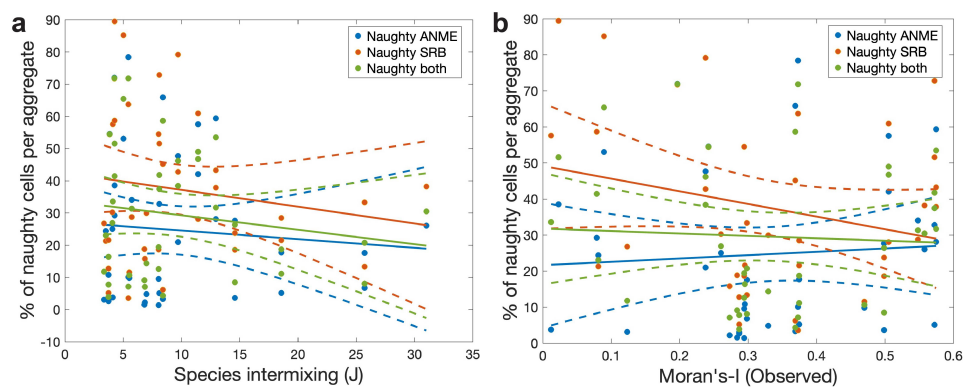


Figure 4.5: Percentage of naughty cells per aggregate plotted against the join index (J) (a) and Moran's I (b) of spatial intermixing between ANME-2b and syntrophic SRB partners, where lower J and Moran's I values represent greater mixing.

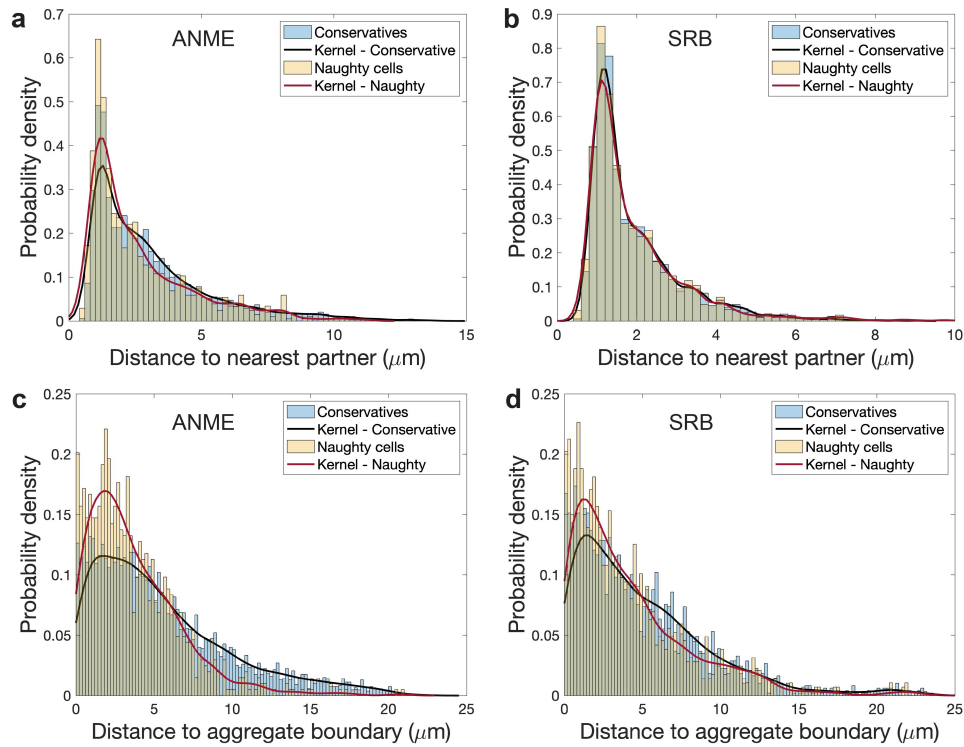


Figure 4.6: The spatial distribution of naughty cells (N-15 vs. C-13) and conservative cells within the AOM consortia. The probability density function of the distance between ANME-2b (a, c) or SRB (b, d) cells and their nearest syntrophic partners (a, b) or the aggregate boundary (c, d). The cyan histogram was the distribution of conservative cells and its Kernel distribution was shown in the black line. The yellow histograms represented the distribution of naughty cells and the Kernel density was plotted in red line.

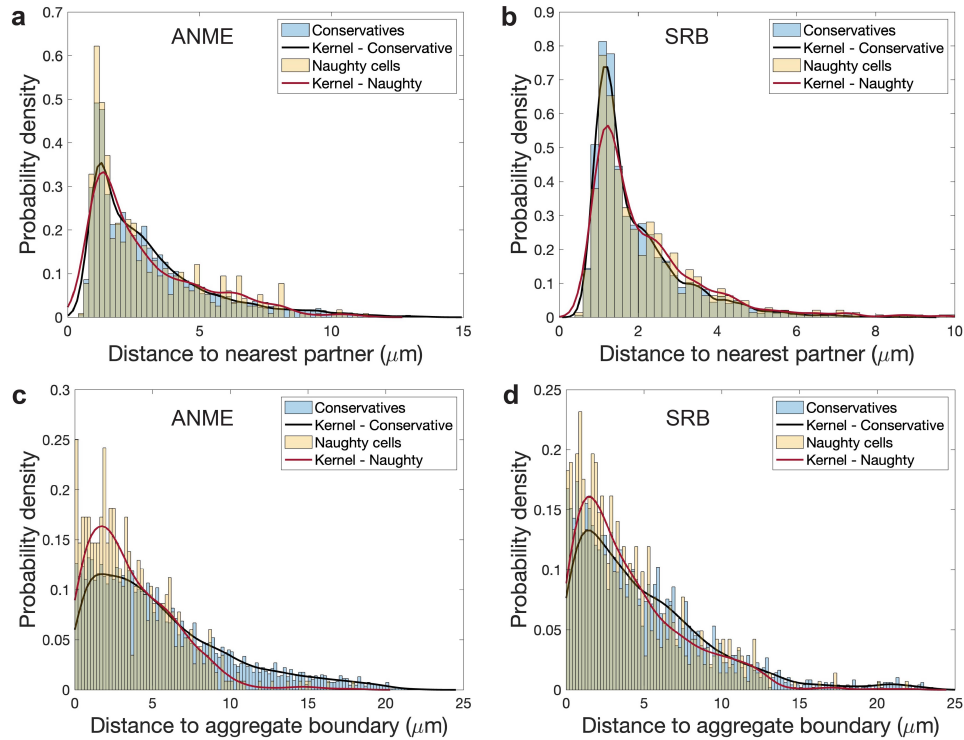


Figure 4.7: The spatial distribution of naughty cells with a significantly higher anabolic activity in N-15 than C-13 within the AOM consortia. The probability density function of the distance between ANME-2b (**a**, **c**) or SRB (**b**, **d**) cells and their nearest syntrophic partners (**a**, **b**) or the aggregate boundary (**c**, **d**). The cyan histogram was the distribution of conservative cells and its Kernel distribution was shown in the black line. The yellow histograms represented the distribution of naughty cells and the Kernel density was plotted in red line.

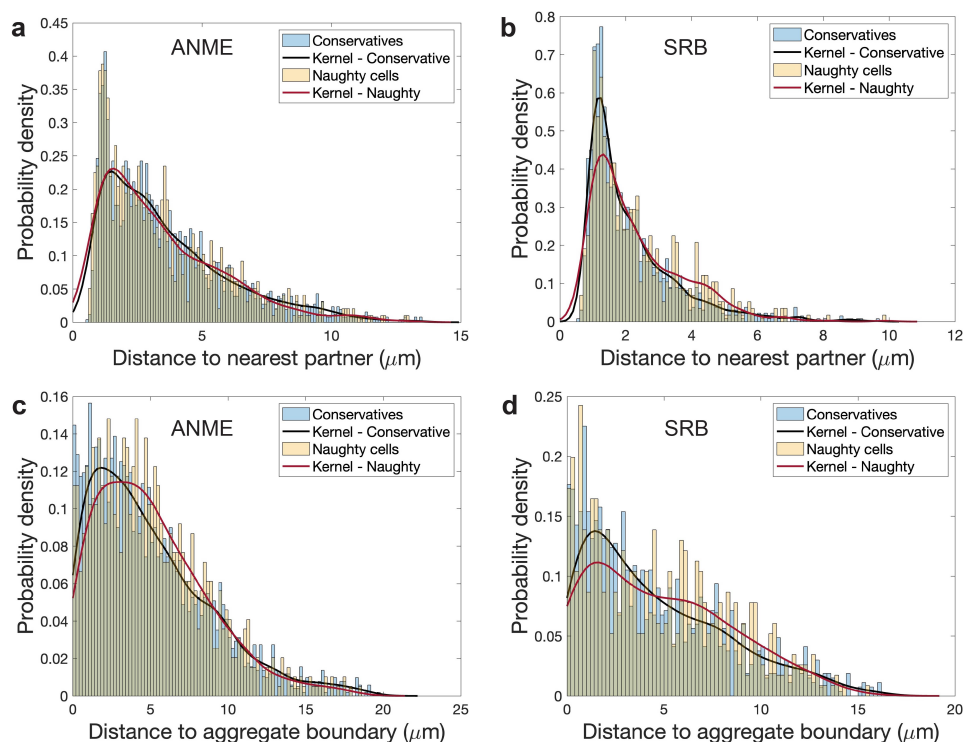


Figure 4.8: The spatial distribution of naughty cells (C-13 vs. O-17) and conservative cells within the AOM consortia. The probability density function of the distance between ANME-2b (**a, c**) or SRB (**b, d**) cells and their nearest syntrophic partners (**a, b**) or the aggregate boundary (**c, d**). The cyan histogram was the distribution of conservative cells and its Kernel distribution was shown in the black line. The yellow histograms represented the distribution of naughty cells and the Kernel density was plotted in red line.

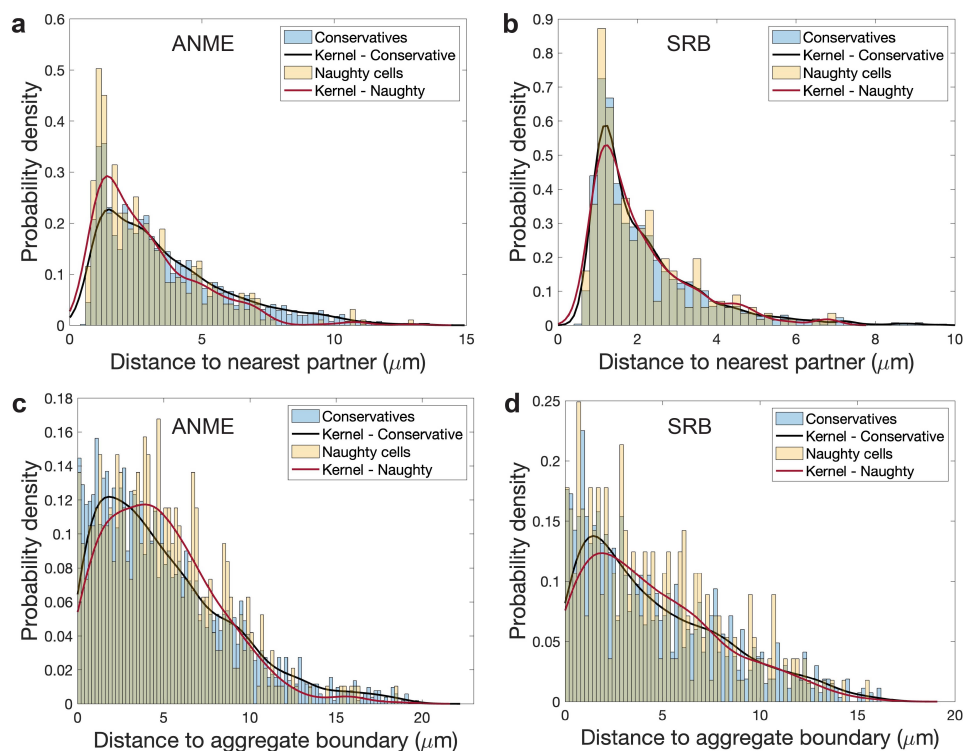


Figure 4.9: The spatial distribution of naughty cells with a significantly higher anabolic activity in C-13 than O-17 within the AOM consortia. The probability density function of the distance between ANME-2b (**a**, **c**) or SRB (**b**, **d**) cells and their nearest syntrophic partners (**a**, **b**) or the aggregate boundary (**c**, **d**). The cyan histogram was the distribution of conservative cells and its Kernel distribution was shown in the black line. The yellow histograms represented the distribution of naughty cells and the Kernel density was plotted in red line.

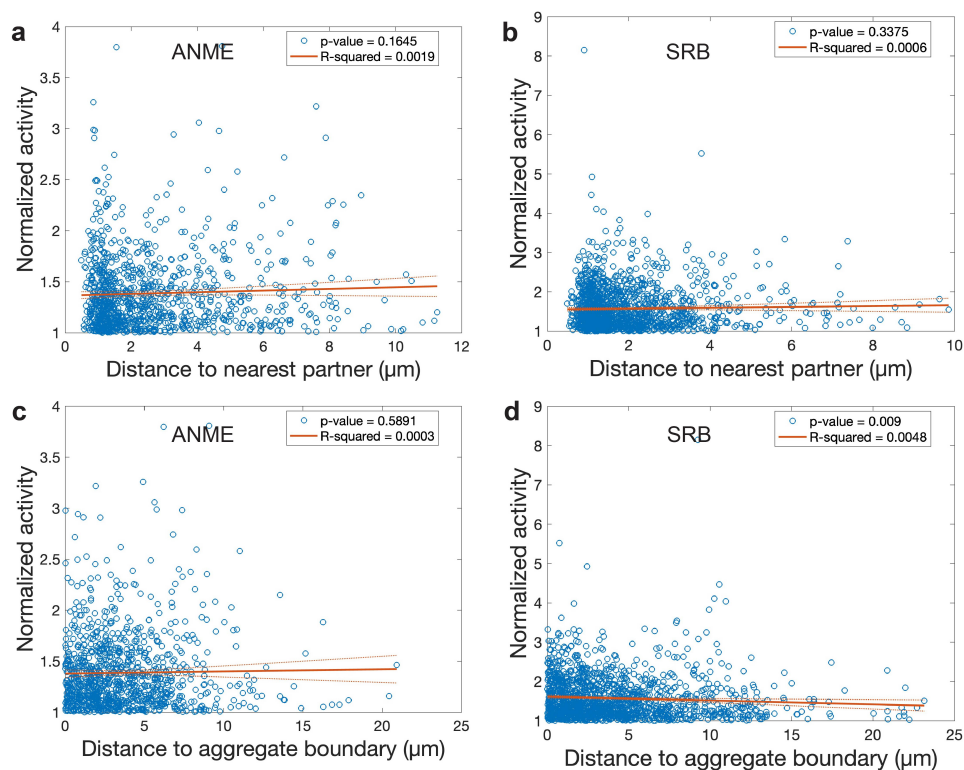


Figure 4.10: The relationships between normalized activity (N-15 vs. C-13) of naughty cells and their distance to their partners or aggregate boundary in AOM consortia. Blue dots were the naughty cells. Red line was the linear regression with dotted lines as 95% confidence boundaries. p -value and R-squared values were marked in the legend. All of the data were treated with absolute conversion.

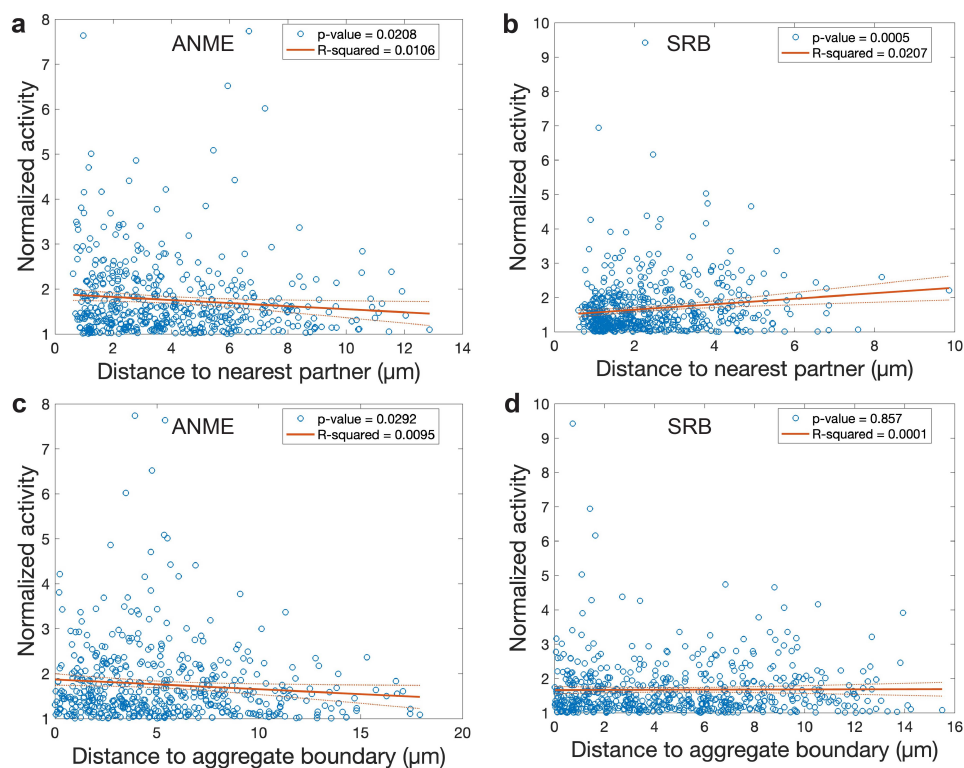


Figure 4.11: The relationships between normalized activity (C-13 vs. O-17) of naughty cells and their distance to their partners or aggregate boundary in AOM consortia. Blue dots were the naughty cells. Red line was the linear regression with dotted lines as 95% confidence boundaries. p -value and R-squared values were marked in the legend. All of the data were treated with absolute conversion.

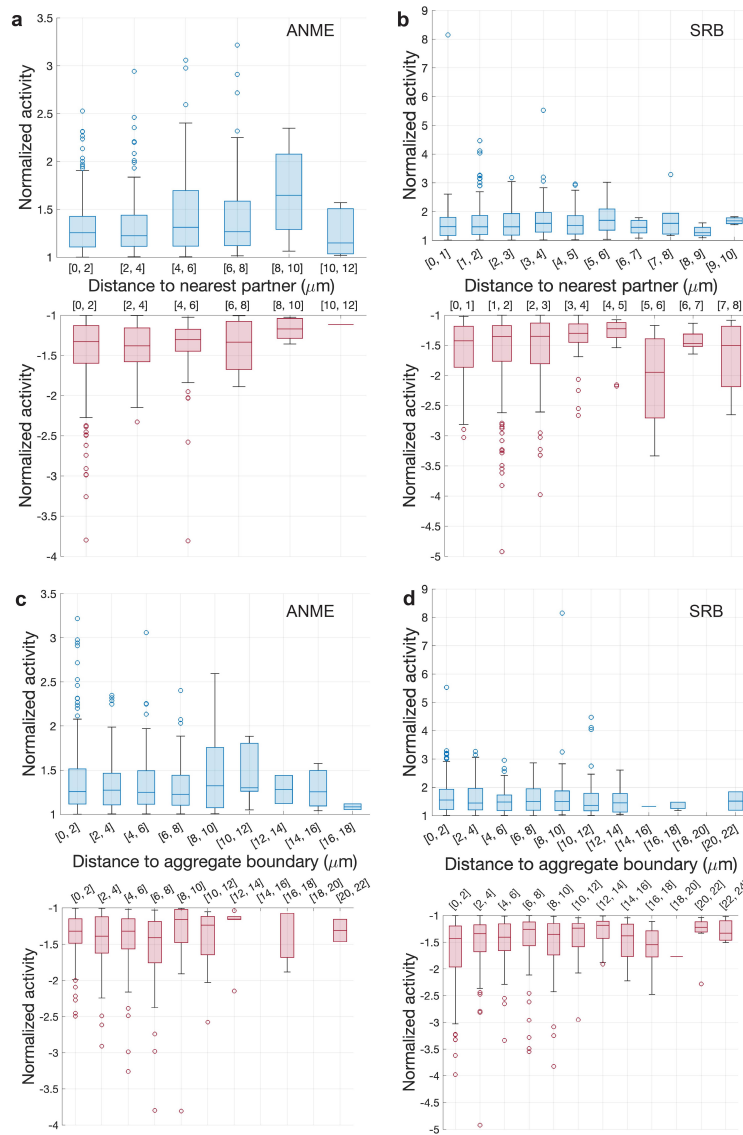


Figure 4.12: The relationships between normalized activity (binned and quantiled) (N-15 vs. C-13) of naughty cells and their distance to their partners or aggregate boundary in AOM consortia. Blue dots were the naughty cells. Red line was the linear regression with dotted lines as 95% confidence boundaries. p -value and R-squared values were marked in the legend. The blue dots and bins represented the naughty cells with significantly higher activities in N-15 over C-13 period; in contrast, the pink were naughty cells with significantly lower activities in N-15 over C-13 period.

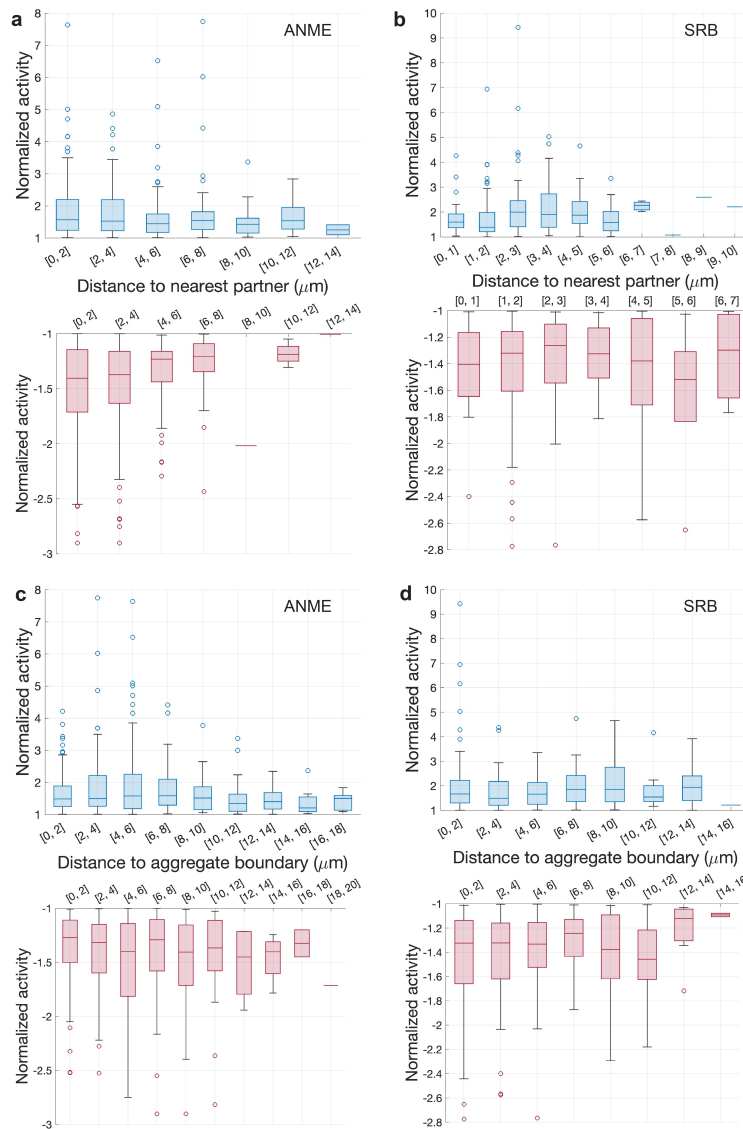


Figure 4.13: The relationships between normalized activity (binned and quantiled) (C-13 vs. O-17) of naughty cells and their distance to their partners or aggregate boundary in AOM consortia. Blue dots were the naughty cells. Red line was the linear regression with dotted lines as 95% confidence boundaries. p -value and R-squared values were marked in the legend. The blue dots and bins represented the naughty cells with significantly higher activities in C-13 over O-17 period; in contrast, the pink were naughty cells with significantly lower activities in C-13 over O-17 period.

Table 4.1: Sulfide production rate in the experiments

Experiment	Pulse-chase ^a			Multi-iso ^b	
	O-17	C-13	N-15	[N, C] ^c	[N, C, O] ^d
$\mu\text{mol } HS^- \text{ d}^{-1} \text{ cm}^{-3}_{sed}$	0.94 ± 0.03^e	1.18 ± 0.02	0.84 ± 0.01	1.01 ± 0.01	0.67 ± 0.01

^a The pulse-chase incubation setup where individual heavy isotopes were amended separately over the course of experiment;

^b The Multi-iso experiment with heavy isotopes simultaneously;

^c Multi-iso experiment with N-15 and C-13;

^d Multi-iso experiment with N-15, C-13, O-17 and O-18;

^e Sulfide production rates, which can be approximately equal to the rate of bicarbonate production from the methane oxidation based on the Equation 4.1;

^f Results in technical replicates.

Table 4.2: Summary of single cells and aggregates analyzed in this study

Experiment	[N, C] ^a		[N, C, O] ^b	
	Pulse-chase	Multi-iso	Pulse-chase	Multi-iso
no. of ANME-2b cells	6,402	5,130	3536	3,695
no. of SRB cells	3,882	2,688	1909	2,708
no. of aggregates	27	33	11	16

^a The datasets used in cellular activity change between N-15 and C-13 periods;

^b The datasets used in cellular activity change between C-13 and O-17 periods.

Table 4.3: Summary of aggregate spatial morphological patterns and percentage of naughty cells in each aggregate

Aggregate ID	J value ^a	Moran's I ^b	Period	% naughty _c ANME	% naughty _d SRB	% naughty ^e
PC_2nd_new_sp2	11.436	0.505	[N, C]	57.5	28.1	46.7
PC_2nd_new_sp3	8.041	0.294	[N, C]	1.4	54.5	19.4
PC_2nd_new_sp4	5.459	0.373	[N, C]	78.4	63.7	71.8
PC_2nd_new_sp5	25.715	0.298	[N, C]	6.8	13.3	8.1
PC_2nd_new_sp6	8.099	0.572	[N, C]	5.1	72.8	37.4
PC_2nd_new_sp7	9.709	0.238	[N, C]	21.0	79.2	38.4
PC_2nd_new_sp8	18.581	0.375	[N, C]	5.2	28.5	11.1
PC_2nd_new_sp9	8.409	0.369	[N, C]	3.3	6.2	4.3
PC_2nd_new_sp11	14.578	0.499	[N, C]	3.6	18.6	8.5
PC_2nd_new_sp12	3.754	0.287	[N, C]	3.0	12.7	7.8
PC_2nd_new_sp13	12.953	0.575	[N, C]	59.4	43.2	53.5
PC_2nd_2d_ovn_1-4	6.989	0.329	[N, C]	4.8	30.0	14.3
PC_2nd_2d_ovn_5	3.367	0.123	[N, C]	3.1	26.8	11.8
PC_2nd_2d_ovn_6	5.018	0.090	[N, C]	53.1	85.2	65.4
PC_2nd_2d_ovn_7	5.535	0.470	[N, C]	9.8	11.5	10.6
PC_2nd_2d_ovn_8	4.240	0.197	[N, C]	72.0	71.7	71.9
PC_2nd_2d_ovn_9	31.013	0.558	[N, C]	26.1	38.2	30.5
PC_2nd_2d_ovn_10	4.119	0.012	[N, C]	3.8	57.6	33.6
PC_2nd_2d_ovn_11	4.264	0.079	[N, C]	29.3	58.7	41.4
PC_2nd_2d_ovn_12	4.077	0.260	[N, C]	25.0	30.2	26.9
PC_2nd_2d_ovn_13	6.829	0.284	[N, C]	1.6	18.8	9.1
PC_2nd_2d_ovn_14	6.872	0.273	[N, C]	2.2	15.8	7.1
PC_2nd_2d_ovn_15	5.741	0.548	[N, C]	34.1	28.7	31.4
PC_2nd_2d_ovn_16	3.522	0.081	[N, C]	24.4	21.3	23.1
PC_2nd_2d_ovn_17	4.269	0.023	[N, C]	38.6	89.5	51.6
PC_2nd_2d_sp2	3.837	0.242	[N, C]	54.5	54.4	54.5
PC_2nd_2d_sp8	3.746	0.295	[N, C]	10.8	21.6	16.4
PC_2nd_new_sp2	11.436	0.505	[C, O]	42.1	61.0	49.0
PC_2nd_new_sp3	8.041	0.294	[C, O]	9.5	18.6	12.6
PC_2nd_new_sp4	5.459	0.373	[C, O]	10.1	3.5	7.1
PC_2nd_new_sp5	25.715	0.298	[C, O]	17.6	33.3	20.8
PC_2nd_new_sp6	8.099	0.572	[C, O]	32.8	51.6	41.8
PC_2nd_new_sp7	9.709	0.238	[C, O]	47.7	42.8	46.2
PC_2nd_new_sp8	18.581	0.375	[C, O]	17.7	21.5	18.7
PC_2nd_new_sp9	8.409	0.369	[C, O]	65.9	45.1	58.6
PC_2nd_new_sp11	14.578	0.499	[C, O]	27.7	23.8	26.4
PC_2nd_new_sp12	3.754	0.287	[C, O]	2.7	5.2	3.9
PC_2nd_new_sp13	12.953	0.575	[C, O]	28.1	37.8	31.7

^a The parameter J representing the mixing degree of cells within aggregates in space. Find more details in Section 4.5;

^b Moran's I: another parameter representing the mixing degree of cells within aggregates in space. Find more details in Section 4.5;

^c The ratio of ANME naughty cells in the ANME population in each aggregate;

^d The ratio of SRB naughty cells in the SRB population in each aggregate;

^e The ratio of all naughty cells in the whole population in each aggregate;

Table 4.4: Summary of naughty cells resolved from pulse-chase experiment

		ANME-2b ^a	SRB ^b
Naughty cells N-15 vs. C-13 ^c	no. of more active ^d	579	712
	% of more active	9.04%	18.34%
	no. of less active ^e	440	723
	% of less active	6.87%	18.62%
Naughty cells C-13 vs. O-17 ^c	no. of more active	477	281
	% of more active	13.49%	14.72%
	no. of less active	502	296
	% of less active	14.20%	15.51%

^a FISH targeted by ANME-2b-729 oligonucleotide probe;

^b FISH targeted by Desulfosarcina/Desulfococcus group probe (DSS658);

^c The comparison of single cell activities between N-15 over C-13 period, or C-13 over O-17 period;

^d Naughty cells showing higher activities in N-15 over C-13, or higher in C-13 over O-17;

^e Naughty cells showing lower activities in N-15 vs. C-13, or lower in C-13 vs. O-17.

Table 4.5: Positioning of naughty cells in the neighboring region with partners

Periods	ANME-2b			SRB	
	N-15 vs. C-13	C-13 vs. O-17		N-15 vs. C-13	C-13 vs. O-17
no. of naughty cells ^a	1,019 (16%)	979 (28%)		1,435 (37%)	577 (30%)
neighboring ^b	458 (45%)	282 (29%)		813 (57%)	253 (44%)
in pair ^c	333 (73%)	165 (59%)		352 (43%)	167 (66%)
naughty in N-15 & C-13 ^d	152 (31%)	152 (16%)		246 (34%)	246 (43%)
more active in N-15 & C-13 ^e	91 (60%)			17 (7%)	

^a The number of naughty cells; the percentage represented the ratio of naughty cells in the whole population;
^b The number of naughty cells which were directly linked to their partners in neighboring space; the percentage represented the ratio of neighboring naughty cells in the whole population of naughty cells;
^c The number and percent of naughty cells shown to be both involved in the neighboring region;
^d The number and percent of the same naughty cells consistent in both times of N-15 and C-13;
^e The number and percent of the same naughty cells showing more active in both times of N-15 and C-13.

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*Chapter 5***DECIPHERING NATIVE DIVISION FOR YET UNCULTURED
ANAEROBIC METHANE OXIDIZING (ANME) ARCHAEA BY
RECONSTRUCTION OF WHOLE-CELL DIVISION PLANES**

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This chapter is in progress.

5.1 Abstract

To explore the growth of the natural microorganisms in their native context is challenging due to the complexity of both the surrounding environment and the intrinsic properties that render many microbes unculturable. This study addresses this question by leveraging an innovative approach that combines serial block-face electron microscopy with deep learning imaging analysis to investigate the division process in uncultured anaerobic methane-oxidizing archaea (ANME). The protocol is founded on achieving high spatial resolution (a few nanometers per pixel) and reconstructing 3D representations of cells collected from their natural habitat. This resolution enables us to identify division planes within the cells, which reflect the actual developmental stages during cell division cycles. By computing the completeness of these division planes, we can gather temporal information about the division process. Analyzing hundreds of cells will provide clear insights not only into the spatial development but also into the dynamic changes in the division turnover rate.

5.2 Main

Growth is one of the most fundamental topics of prokaryotic cell biology, with the division process playing a crucial role in population proliferation. Current approaches to monitor and quantify cell division are largely restricted to cultured model microorganisms through direct visualization (Bolhaqueiro et al., 2018; Cotton et al., 2024; Denoth-Lippuner et al., 2021; Gahlmann & Moerner, 2014; Jeckel & Drescher, 2021). Consequently, very little is known about uncultured microbes, which constitute the vast majority of microbial diversity in nature (Lloyd et al., 2018; Rappé & Giovannoni, 2003). Capturing division information from these organisms while preserving their native context necessitates the development of new methodological strategies capable of addressing the challenges posed by their slow growth rates and the complexity and opaqueness of bulk environments.

In this study, we leveraged the serial-block face electron microscopy technique (Deerinck et al., 2010) combined with deep learning-based computational imaging analysis to investigate three-dimensional (3D) cells of interest and identify the whole-cell division planes. These planes can reasonably serve as a proxy for the cellular division process (Figure 5.1).

Based on this approach, we investigated the microbial consortia involved in the anaerobic oxidation of methane (AOM), a well-studied yet uncultured environmental microbial system typically consisting syntrophic species, anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria (SRB) (Knittel & Boetius, 2009). These organisms generally grow slowly with an estimated generation time of several months (Orphan et al., 2009; Scheller et al., 2016). Using this protocol, we successfully collected 10 high-quality aggregates, most of which had their 3D volumes effectively captured by SBEM (Table 5.1). Specifically, a high resolution was achieved, with a lateral pixel size ranging from 2 to 5 nm and a vertical slice thickness of 70 or 80 nm (defining the vertical resolution) (Table 5.1). This high resolution allowed for better downstream imaging analysis. Given the limitations in resolution for sub-cellular structure analysis within the SRB bacterial space, the focus of this study was primarily on the ANME archaea.

Direct connection of ANME to syntrophic partners

An example of this can be seen in aggregate E_01 (Table 5.1), where a total of 459 high-quality 3D ANME cells were successfully reconstructed, presenting a highly segregated broccoli-like structure, characteristic of typical sarcina spatial

arrangements (Figure 5.2). Statistically, each single 3D cell comprised 28 ± 5 vertical stacks, with a volume of $5.91 \pm 1.54 \mu\text{m}^3$. These cells showed a wide range of distances to the aggregate boundary; interestingly, nearly all ANME cells were found to be in direct contact with their syntrophic SRB cells, with only a few exceptions (10 out of 459) still maintaining a proximity within a $0.5 \mu\text{m}$ range.

Illustration of division plane in ANME cells

Sectioning a closed 3D ball-like cell will generate a closed 2D area. Likewise, cutting through a 2D division plane (such as a wall-like septum within a cell) results in a 1D line within the cellular area. As illustrated in the example cell #493 (Figure 5.2), the white lines present in each slice of the cells are expected to be part of the division plane (highlighted in pink). By reconstructing all the components belonging to cell #493, these pink lines formed a 2D wall, representing the whole-cell division plane (Figure 5.2, bottom). Notably, there is an incomplete section in the division plane of the cell. Predicting this incomplete part may provide insights into the completeness of the division plane, reflecting the growth stage of the particular cell.

Prediction of incomplete division plane

Our segmented data encompasses not only the full cell body but also the portion of the wall that develops during mitosis, which we refer to as the “wall bud”. This can be observed in Figure 5.3, where arrows indicate a typical portion of the data slice. Any cell containing a wall bud in 3D is actively undergoing division, allowing us to predict the complete division wall from the current new wall stage. Based on our data observations, we assume that the new wall effectively functions as a plane bisecting the mother cell into two daughter cells, each approximately equal in volume, as corroborated by our analysis. To determine this plane of division, we fit a plane equation $aX + bY + cZ = 0$ to the set of points $X_i, Y_i, Z_i, i = 1, 2 \dots N$ given by the segmented wall bud. A least square error is minimized using linear regression and the plane parameters (a, b, c, d) computed. Only the portion of this plane inside the segmented cell forms the dividing wall which is obtained by taking the intersection of the plane with the cell body. This full wall prediction, along with the current wall bud, is then used to determine the extent of division wall development in the mother cell, which is reported as a percentage of the total wall size.

Remodeling the development of division plane

Next, we attempted to model the development of the division plane alongside the process of cellular division in ANME. We hypothesized a universal principle of division that applies consistently across all ANME cells. In this context, all reconstructed division planes were extracted and rebuilt, arranged from the least to the most complete. This sequence reflects a temporal progression from the initiation of division to its conclusion.

From the conceptual model of the division plane development in ANME cells (Figure 5.5), it was observed that the wall typically started growth from one side of the cell, developing linearly until it reached the central position of the proposed whole dividing wall. Interestingly, upon passing the center, ANME cells showed a distinct development pattern characterized by a “fan” movement that circled around the center point as the dividing wall continued to grow. Due to measurement limitations, it was challenging to determine whether the “fan” development originated from both sides or solely from one side.

Resolving the temporal pattern of the dividing process

A probability density function representing the completion percentage of the dividing wall in the aggregate E_01 was established (Figure 5.6), indicating the dynamic development of division planes over time. ANME cells within this aggregate displayed a bimodal distribution concerning completeness. Statistically, a higher probability at specific completion statuses, such as 20% completion, represents a greater likelihood of being captured during sample preparation. This observation may be linked to the wall turnover rate, where a higher probability of wall completion corresponds to a lower turnover rate. As illustrated in Figure 5.6, the two peaks in this bimodal distribution represented two distinct dividing states characterized by the slowest turnover rates. Additionally, alternative explanations exist. For instance, the probability density distribution may depend on unresolved intercellular relationships among cells in space. Factors such as cohorts interfering with one another and the availability of nutrients could influence the development of the dividing wall, indicating the need for further investigation in future studies.

Spatial patterns of the dividing wall orientation

The orientations of the dividing walls were then examined in detail. To achieve this, all dividing walls were displayed in their native locations within the aggregate E_01, with their completeness represented by color (Figure 5.7). The primary focus

of this analysis was to understand the orientation, which determines the direction and pattern of future cell growth. In particular, for two adjacent cells presumed to originate from the same mother cell, their dividing walls were observed to either align in parallel on the same plane or intersect perpendicularly (Figure 5.7). Analyzing the mechanisms that govern these orientations is currently in progress, with the aim of modeling and predicting the growth of ANME cells within the aggregates.

5.3 Conclusion

In this report, we optimized a cutting-edge protocol that combines serial block-face scanning electron microscopy (SEM) with machine learning imaging analysis, applied to an example aggregate. As a result, 3D structures of ANME cells were successfully reconstructed while preserving their native spatial context within the aggregate. Notably, the dividing planes within the cells were identified, serving as a proxy to represent the ANME cellular division process. Future work will primarily focus on understanding how the walls develop over time and in space, as well as investigating how ANME cells grow and evolve within their native aggregate. Additionally, exploring the real 3D distance between ANME cells and their partner SRB cells is essential. Direct contact between these syntrophic partners may significantly influence inter- and intra-species interactions within the ANME-SRB consortia.

5.4 Materials and Methods

Sediment collection and processing

Methane seep sediments covered with white microbial mats were collected by push core (PC6) in the dive AD4912 on 27 May 2017 by DSV Alvin, launched from R/V Atlantis on research cruise AT37-13 in Jaco Scar, off Costa Rica, at 1,811-m water depth (lat 9.1163, long 284.8372). The sediment core was sliced into 3-cm-thick horizons shipboard which were stored individually in separate Whirl-Pak bags and kept anaerobically at 4 °C in sealed Mylar bags sparged with Ar until they were shipped to the laboratory. Sediments (serial number #10073-76) from Costa Rica were particularly maintained and used in this study.

Artificial seawater composition

Artificial seawater (ASW) (final concentration) was used in the study modified from reference (Yu et al., 2021). The composition of the medium was: NaCl 457 mM, Na₂SO₄ 10.0 mM, MgCl₂ 47 mM, HEPES (pH 7.5) 25 mM, NH₄Cl 2.0 mM, KCl 7.0 mM, NaHCO₃ 5.0 mM, CaCl₂ 1.0 mM, K₂HPO₄ 1.0 mM, SeO₃²⁻ 0.01 μM, WO₄²⁻ 0.007 μM, 0.1% trace element solution, containing per liter: nitrilotriacetic acid 150 mg, MnCl₂ · 4H₂O 610 mg, CoCl₂ · 6H₂O 420 mg, ZnCl₂ 90 mg, CuCl₂ · 2H₂O 7 mg, AlCl₃ 6 mg, H₃BO₃ 10 mg, Na₂MoO₄ · 2H₂O 20 mg, SrCl₂ · 6H₂O 10 mg, NaBr 10 mg, KI 70 mg, FeCl₃ · 6H₂O 500 mg, NiCl₂ · 6H₂O 25 mg.

Fixation and concentration of consortia in sediment

Sample preparation was modified from the protocol described previously (McGlynn et al., 2018). Briefly, sediment slurry was withdrawn from incubation bottles using N₂ flushed needle. After quick spinning and removal of supernatant, first fixed the sediment pellet in paraformaldehyde solution on ice for 2h (2% paraformaldehyde, 7.25 mM HEPES, pH 7.5, 5 g l⁻¹ NaCl). Then fixed the sediment pellet in glutaraldehyde solution in 4°C fridge overnight (2.5% glutaraldehyde, 7.25 mM HEPES, pH 7.5, 5 g l⁻¹ NaCl). Washed the fixed sediment for 3 times with 3×PBS. Aggregates were Percoll separated and concentrated by centrifugation, immobilized in molten noble agar (3%). The immobilized agar blocks were suspended in 3×PBS and stored in 4°C fridge for downstream preparation.

Staining and embedding microbial consortia for SBEM

The staining and embedding protocol was modified from the previous pipeline (Deerinck et al., 2010; Gillies et al., 2017). The microbial consortia in the agar blocks were subjected to a heavy-metal staining protocol to achieve conductivity and contrast (Deerinck et al., 2010). Fresh staining solution was made right before use which 3% potassium ferrocyanide in 0.3 M cacodylate buffer with 4 mM calcium chloride was combined with an equal volume of 4% aqueous osmium tetroxide (EMS). The agar blocks sat in this solution for 1 h on ice.

Then fresh TCH (thiocarbohydrazide) solution was prepared by adding 0.1 g thiocarbohydrazide (Ted Pella) to 10 ml double-distilled water (ddH₂O) in a 60°C oven for 1 hr and agitating periodically. The solution was filtered through a 0.22- μ m Millipore syringe filter. At the end of the osmium tetroxide incubation, the samples were washed with ddH₂O at room temperature 5 times for 3 min each time (*ca.* 15 min total) and then incubated in the filtered TCH solution for 20 min at room temperature. The samples were then rinsed again 5 times for 3 min each in ddH₂O at room temperature and thereafter placed in 2% osmium tetroxide (not osmium ferrocyanide) in ddH₂O for 30 min at room temperature. Following this second exposure to osmium, the cells were washed 5 times for 3 min each time at room temperature in ddH₂O and then placed in 1% uranyl acetate (aqueous) and left in a refrigerator (4°C) overnight. The next day, *en bloc* staining with Walton's lead aspartate staining was performed by dissolving 0.998 g of L-aspartic acid (Sigma-Aldrich) in 250 ml of ddH₂O. Lead nitrate (0.066 g) was then added to 10 ml of aspartic acid solution, and the pH was adjusted to 5.5 with 1N KOH. The lead aspartate solution was placed in a 60°C oven for 30 min. The cells were washed 5 times for 3 min each time in ddH₂O at room temperature, placed in the lead aspartate solution, and then returned to the oven for 30 min. The cells were then washed 5 times for 3 min each in room temperature ddH₂O and dehydrated using ice-cold solutions of freshly prepared 20%, 50%, 70%, 90%, and 100% (two separate additions of 100%) anhydrous ethanol for 5 min each, placed in anhydrous ice-cold acetone, and left at room temperature for 10 min.

Durcupan ACM resin (EMS) was formulated by weight as 11.4 g part A, 10 g part B, 0.3 g part C, and 0.05 to 0.1 g part D, yielding a hard resin when polymerized. The resin was mixed thoroughly. Samples were placed into 25% Durcupan-acetone for 2 h, into 50% Durcupan-acetone for 2 h, and then into 75% Durcupan-acetone for 2 h. The cells were placed in 100% Durcupan overnight and then into fresh

100% Durcupan for 2 h. The agar blocks were then embedded in a thin layer of fresh resin in an aluminum weigh boat and placed in a 60°C oven for 48 h.

SBEM analysis of microbial consortia

Microbial consortia were cut out of the Durcupan resin blocks, precision trimmed with a glass knife, mounted on aluminum pins, and imaged using a Zeiss Merlin compact scanning electron microscope fitted with a serial block face sectioning system (3View; Gatan, Inc.). Specimens were imaged at 3 keV in a 30-Pa pressure chamber to reduce specimen charging, using 80-nm cutting intervals.

Computational imaging analysis

Serial block face electron microscopy images containing aggregates of cells were segmented using the jcell (Guerrero Peña et al., 2020) and cellpose (Stringer et al., 2021) deep learning tools. Segmentation of aggregates to separate them from other spurious parts present in the image stacks and subsequent segmentation of cells solely within aggregates were done using fine tuned models. Pretrained models from the tools were not sufficient to create quality segmentations as they over segmented cells containing partial walls or were not built for electron microscopy signals.

Data Preparation

We manually discarded slices which didn't contain the aggregate of interest leaving 200 slices for segmentation. These were downsampled to a more manageable size of 1,200×1,200 pixels for training a model. The highly noisy images were enhanced to improve contrast via local normalization and signal noise was greatly reduced which helped obtain better segmentations when compared to segmentations after training with plain raw noisy data.

Image Annotation

We performed manual annotations of stacks slices delineating aggregates and then ANME cells within aggregates for training and generating newly tuned models. We manually masked aggregates in 21 images, 10.5% of all images, to train an aggregate segmentation model. ANME cells were annotated in two rounds. A few annotations, 10 images in total, were performed on the first round to quickly train a model to test if the tools would have capabilities of properly segmenting cells containing partial walls. Once confirmed, we then combined manually selected true-positive segmented cells obtained using the initial model with manually traced remaining false-negative cells in 29 images, 14.5% of total available images, amounting to

3,063 training cells, 18.7% of all 16,369 cell slices segmented in the 200 image slices. We manually corrected the automatic stitching of cell slices to form true 3D cells (see below) using the Trackmate tool in ImageJ/Fiji. Using Trackmate's tracking editing tool we were able to reassign segmented cell slices to the correct 3D cells and dispose those not pertaining to a valid cell. This was crucial to form the 3D cells we were interested in analyzing. We also manually traced the incomplete walls (bud walls) inside cells so they can be jointly segmented with the cells and provide data to calculate the dividing wall completion ratio whenever present.

2D and 3D Segmentation

The 2D annotations described above were sufficient to create robust segmentation models for our stack slices. We fine-tuned the super generalist cyto3 model of Cellpose so the newly obtained models would segment the aggregate regions of interest and they would no longer divide cells containing partial growing walls while at the same time picking only ANME cells within aggregates and no SRB cells. The JCell models trained from scratch performed equally well after visually comparing results but it was the only tool capable of also segmenting the partial walls (wall buds) inside dividing cells.

All 200 aggregate regions present in the center of the images were segmented and then refined to remove odd contour shapes and enforce convexity on segmentation masks conforming with our visual inspection of data and segmentation results. To diminish discontinuities and large changes of aggregate shape on adjacent slices we performed a median mask computation for every segmented aggregate taking into account the neighboring four segmented slices. This approach effectively eliminated ill shapes and promoted a smoother transition along the aggregate depth.

To build true 3D cells from all 2D segmentations we used the Trackmate tool in ImageJ/Fiji. This allowed us to automatically stitch all 16,369 segmented 2D cells masks along the Z depth of the aggregate to form the 3D cells we are interested in analyzing. After manually correcting the 3D cell compositions, as outlined in the Image Annotation paragraph, we computed the dividing wall completion ratios for each cell. Segmentation given by the tuned JCell model provided the dividing wall mask, when present, for each cell slice. We fitted a parabola to a single pixel wide line representing this wall so we could compute the entire wall and thus the remaining part of the wall to be build inside the cell and their ration. Doing so for all slices of the cell then gives the total wall completion ratio.

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Author contributions

Y.G. and V.J.O conceived the project; V.J.O supervised the project; Y.G. and V.J.O designed the methodology; A.C. and Y.G. performed the imaging analysis; G.L.C. and S.E.M. collected and prepared the microbial consortia samples; T.J.D., A.M. and M.H.E. performed the SBEM detection and collected the data; Y.G. generated the original draft of the manuscript, and all authors provided advice and contributed to the final drafting of the manuscript.

Competing interests

The authors declare no competing interests.

Table 5.1: Summary of the AOM consortia collected in this study

Aggregate	no. of slices	Pixel size (nm/pixel) ^a	Slice thickness (nm) ^b
A_01	504	4.718	70
A_02	360	4.718	70
A_03	201	4.718	70
B_01	350	4.718	70
C_01	185	4.718	70
C_02	358	4.718	70
C_03	151	4.718	70
D_01		3.445	70
D_02		2.484	70
E_01	200	3.549	80

^a The lateral resolution of raw data;

^b The vertical resolution.

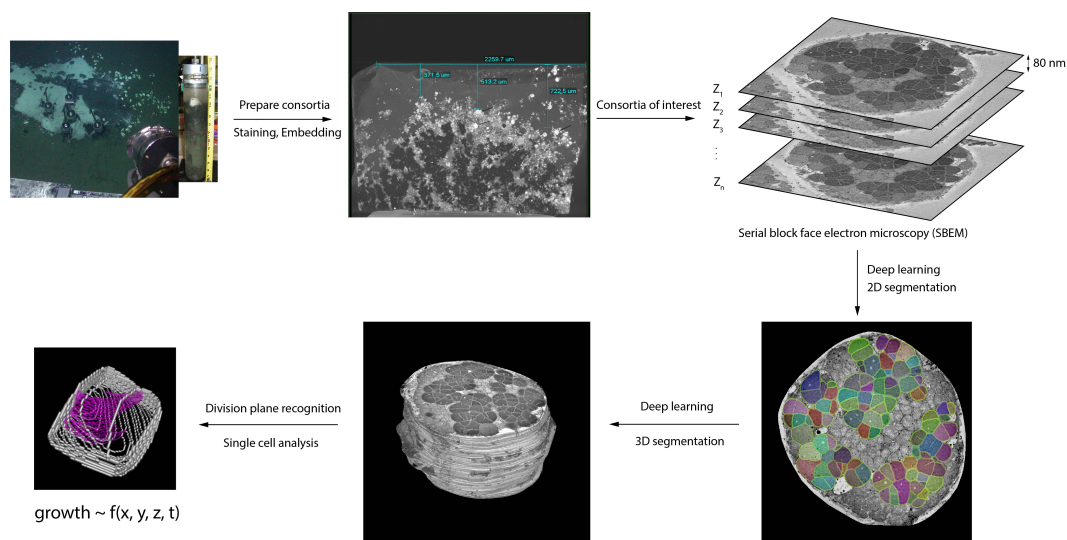


Figure 5.1: The schematic of whole-cell division plane reconstruction based on serial block face electron microscopy (SBEM) platform and deep learning computational imaging analysis for yet uncultured ANME archaeal cells from bulk seep methane sediments.

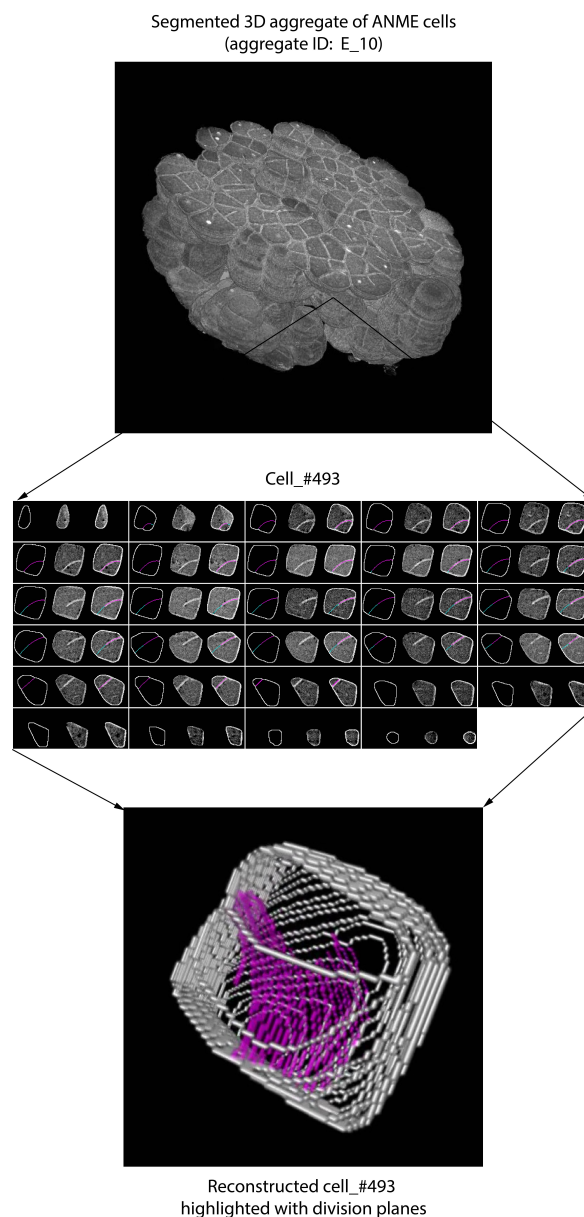


Figure 5.2: An example of reconstructed cell (ID #493) in the aggregate E_10. **Top**, the aggregate of ANME cells after 3D segmentation. **Middle**, the illustration of each 2D segmentation slice in an example cell #493, where each slice was shown by 3 separate images, which are (left) mask image after subtraction of background, (middle) the original 2D segmentation, and (right) the division plane sectioned lines in each slice. Pink showed the recognized division planes, and cyan was the predicted incomplete part of division plane in each slice. **Bottom**, the presentation of cell #493 reconstructed from all the slices showed above, where pink was the division plane and white was the cell contour.

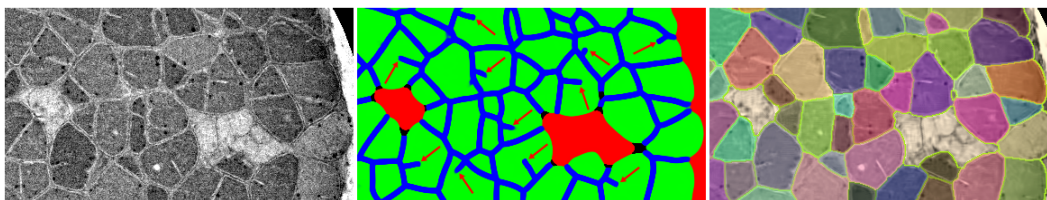


Figure 5.3: A montage showing the recognition of dividing walls in cells. **Left**, the original SEM image; **Middle**, the recognition of dividing walls pointed by red arrows; **Right**, the 2D segmentation.

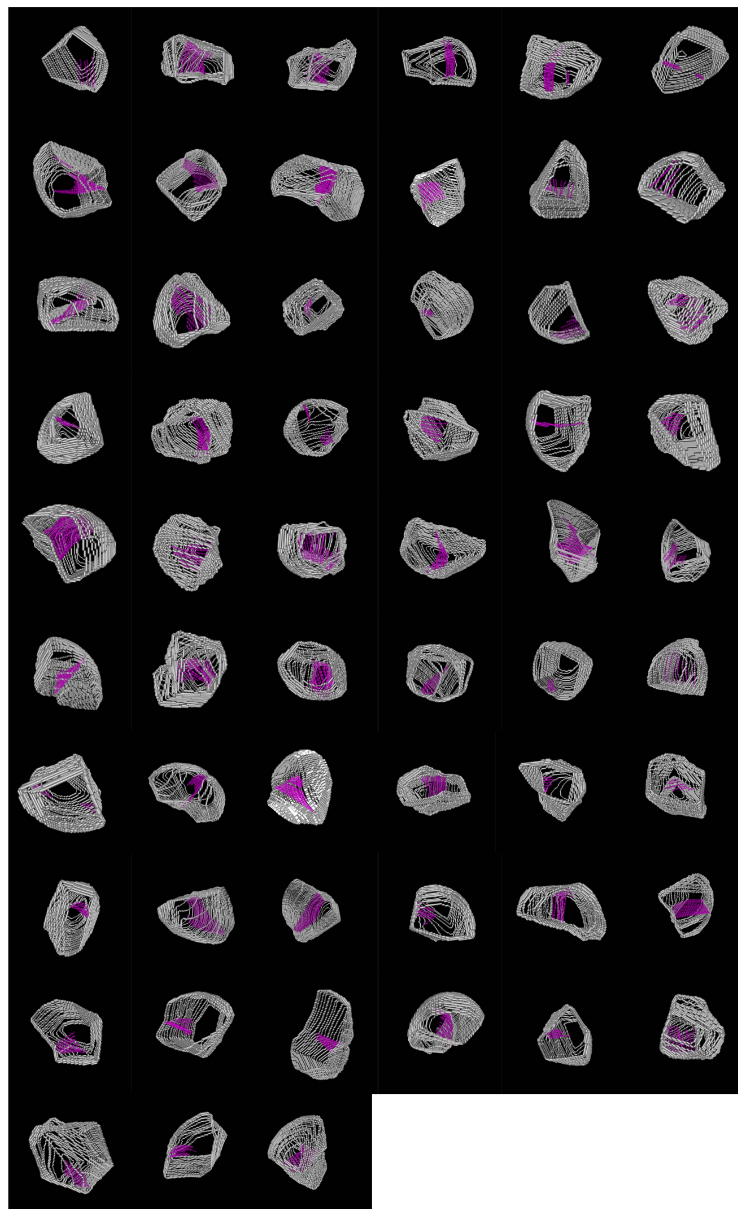


Figure 5.4: A collection of parts of 3D reconstructed cells, where pink represents the division planes and white is the cellular boundary.

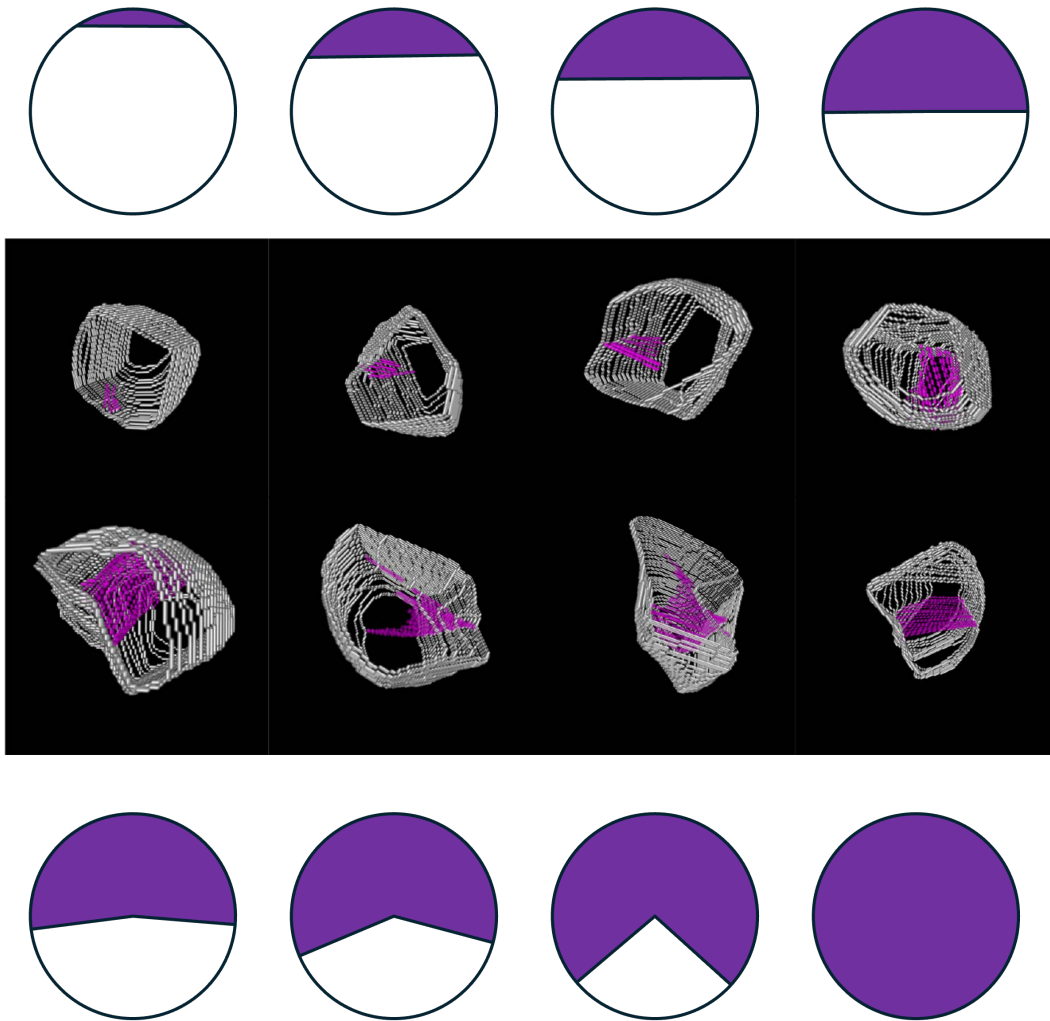


Figure 5.5: A conceptual model of the dividing wall development in ANME cells. Images in the middle panels are those reconstructed 3D cells with dividing planes (pink) and the cellular boundary (white). The circles above or below those 3D cells represented the conceptual dividing walls, where the pink areas showed the completed parts in the walls.

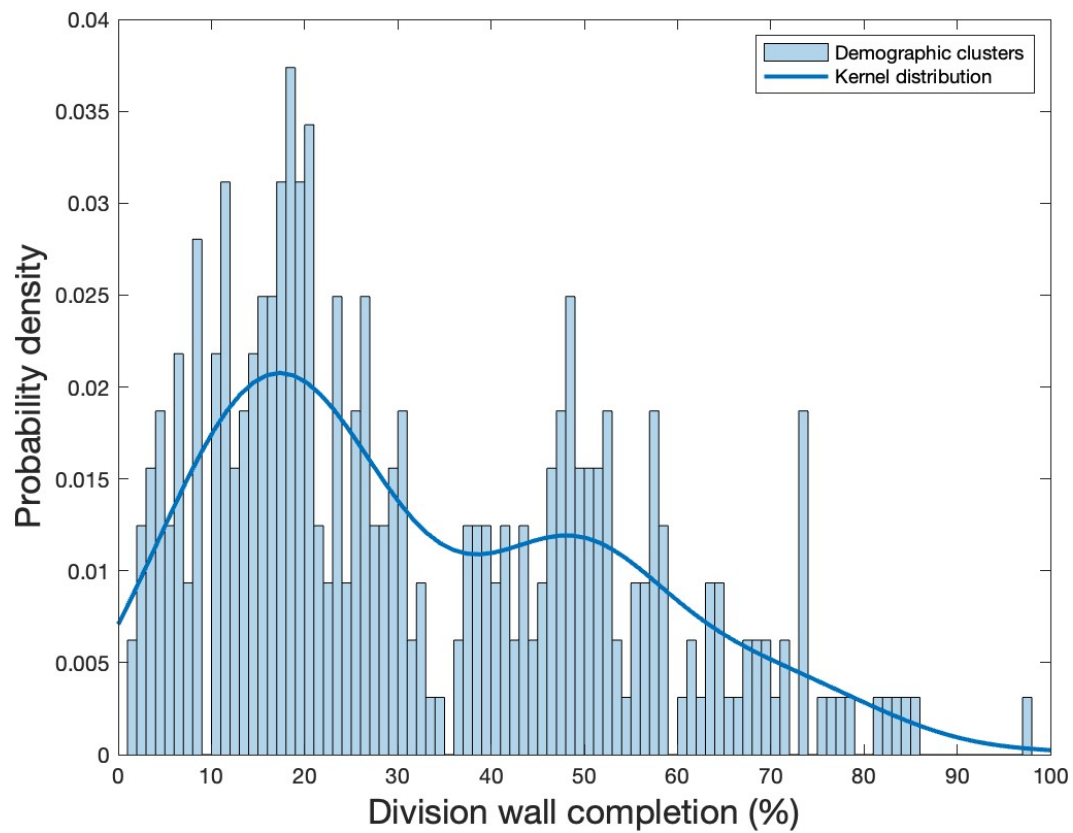


Figure 5.6: The probability distribution of the dividing wall completion in aggregate E_01. The fitting curve was shown as a result from Kernel distribution prediction.

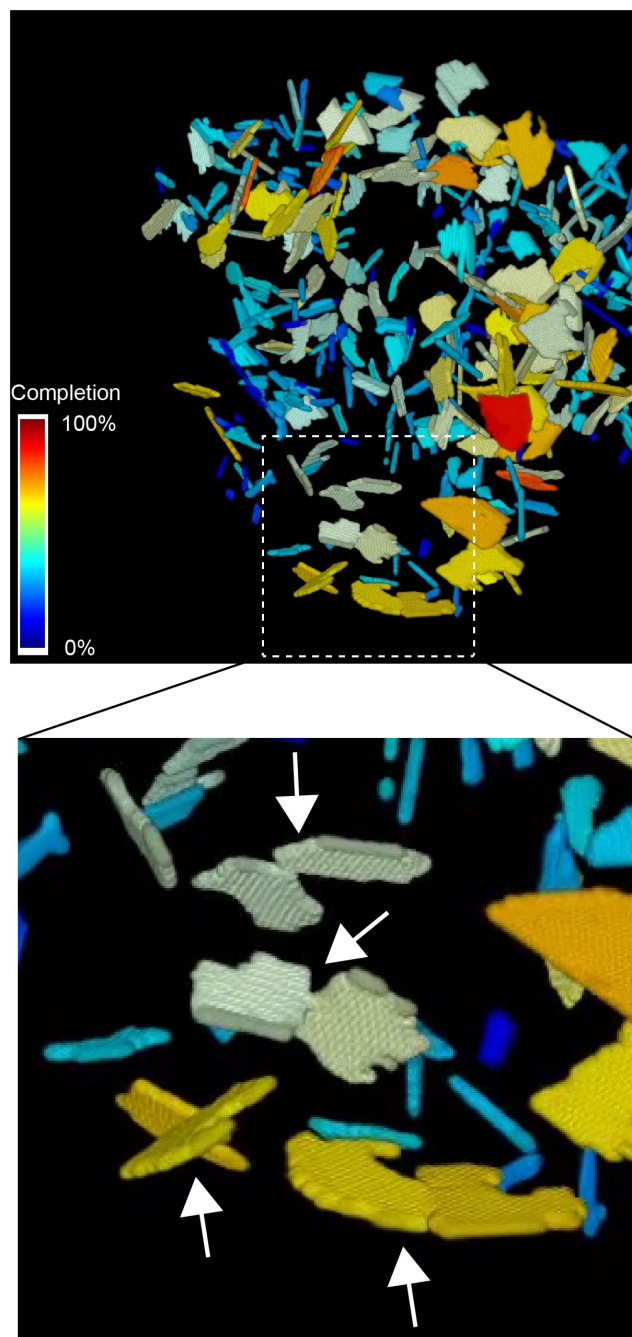


Figure 5.7: *In situ* locations of the ANME dividing walls together with completion information in aggregate E_01. The top picture showed the all the ANME dividing walls in their native positions. The color represented the completion percentages, where a warm color corresponded to a high completeness. The bottom image showed a closeup region marked by the white box in the whole aggregate. The white arrows pointed to two spatial patterns of the dividing wall in two adjacent cells.

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*Appendix A***TRANSITION STATE SETS A MAINTENANCE ENERGY
BARRIER AGAINST DIAZOTROPHIC PRODUCTIVITY**

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A.1 Abstract

The abundance of bioavailable nitrogen is a central regulator of ecosystem productivity. Free-living nitrogen-fixing microbes are known to play critical roles in contributing bioavailable nitrogen to various ecosystems. However, we still have limited understanding on their ecology, leaving major questions unanswered such as why many ecosystems are chronically nitrogen limited, and what consequences do human nitrogen input impose on an ecosystem. Here, we quantitatively explore the impact of external bioavailable nitrogen input on the biomass productivity, and consequently, the system nitrogen accumulation of a free-living, anaerobic nitrogen fixing bacterium *Desulfovibrio vulgaris*. We found that the metabolic decision that prioritizes nitrogen scavenging over nitrogen fixation, a common strategy used by free-living nitrogen-fixing microbes, causes a prolonged period where cells show little growth but apparently consumed nonnegligible amount of energetic substrate. The level of energy flux during the transition state is three orders of magnitude higher than the maintenance energy previously described for maintenance states caused by carbon starvation. This led to a lack of accumulative effect between the bioavailable nitrogen-based and nitrogen fixation-based productivity of the system. At intermediate concentration range, higher nitrogen input can even have negative impact for biomass productivity. Deleting a gene encoding for ammonium transporter, which is essential for ammonium scavenge, drastically shortened the transition time and recovered an accumulative effect between ammonium-based and nitrogen-fixing growth. Our study thus reveals an innate energy barrier for diazotrophic nitrogen buildup.

A.2 Introduction

The abundance of bioavailable nitrogen is a central regulator of ecosystem productivity. The past two decades of exploration has made critical progress in updating our view regarding the nitrogen cycle, particularly in the microbial use of various oxidized and reduced form of nitrogen as energy sources, ultimately converting them into inert N_2 gas. In comparison to these increasing forms of N efflux, the biological influx of N remains catalyzed by a set of nitrogenase enzymes which are evolutionarily related (Kuypers et al., 2018). Recent metagenomic analyses in diverse environments also expanded the known taxonomic diversity of nitrogenase-encoding microbes (Dong et al., 2022; Masuda et al., 2024). However, predicting the contribution of biological nitrogen fixation to ecosystem productivity remains a challenging task.

Energy availability is a central constraint for nitrogen fixation. Although the overall reaction of dinitrogen (N_2) reduction into ammonium is exergonic, breaking the triple bond of the dinitrogen gas is challenging, requiring the hydrolysis of at least 16 ATP per N_2 molecule reduced by dedicated enzymatic mechanisms (Hoffman et al., 2014). Whereas plant hosts supply energy sources to their nitrogen fixing symbionts, free-living nitrogen fixing microbes need to be concerned of competition for energetic resources. In principle, a nitrogen-limited but energy-replete environment should lead to a competitive advantage for nitrogen fixing microbes, and until they produce sufficient bioavailable N to support the growth of the remaining, non-fixing population. However, many ecosystems, especially those without human intervention, remain energy-replete yet nitrogen-limited. On the other hand, the effect of external human input of nitrogen source on various ecosystems remains unwell characterized, particularly regarding its actual effectiveness on productivity and the impact on the resilience of the system against various perturbations. Furthermore, while nitrogen-fixing microbes are known to be taxonomically diverse and adopt a range of metabolic lifestyles, few are characterized quantitatively. For example, Deltaproteobacteria are found to be among the dominant nitrogen-fixing microbes in soil and marine sediments (Dong et al., 2022; Masuda et al., 2024), but are so far little studied.

The total nitrogen availability of a system is comprised of external input and intrinsic N_2 fixation. Since N_2 fixing growth leads to lower yield, it is generally assumed that external input of bioavailable nitrogen should increase the total microbial productivity of the system. That is, in an energy-finite system, with increasing

amount of external input of bioavailable nitrogen such as ammonium and nitrate, microbes are expected to primarily use these sources to yield biomass, and then use the remaining energy to fix nitrogen and yield additional biomass (and the bioavailable N of the system). In this study, we show that the external N and fixed N are not additive for the monoculture of a free-living N_2 -fixing bacteria, and external N input can even have negative impact on population growth yield. We found that high maintenance energy, namely the energy consumption during the non-growth state, creates an innate barrier for ecosystem nitrogen buildup.

A.3 Results

Lack of additive effect between input and fixed N

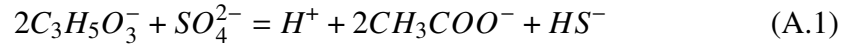
In this study, we quantitatively examine a Deltaproteobacterial strain *Desulfovibrio vulgaris* strain Hildenborough, which was initially isolated from a sewage digester and was the first sulfate-reducing bacterium to be genomically sequenced (Heidelberg et al., 2004). Under the new GTDB taxonomy, this species is now renamed *Nitrodesulfovibrio vulgaris*, classified under *Desulfobacteriota* (Parks et al., 2018, 2020). *D. vulgaris* respire sulfate and reduces it to sulfide, while incompletely oxidizing organic acids such as lactate and pyruvate into acetate (Keller & Wall, 2011). The strain Hildenborough encode a single Molybdenum-type nitrogenase operon containing *nifHDK* (Fig. A.1a). Here, we confirmed the growth of *D. vulgaris* wildtype strain in the absence of a bioavailable nitrogen source (Fig. A.1b,c). Deletion of the nitrogenase subunit gene *nifH* disabled the strain's ability to growth without NH_4Cl (Fig. A.1d), confirming that the bioavailable N-independent growth was attributed to the enzymes encoded by this identified nitrogenase operon. Growing at 37 °C using lactate and sulfate as electron donor and acceptor, respectively, *D. vulgaris* exhibited a doubling time of 8 hours without bioavailable N, compared to a 2.5-hour doubling time when 2 mM of NH_4Cl is supplied (Fig. A.1e). The growth yield of the former is around 50% of the latter. Such a significant decrease in growth rate and yield indicates a strong cost of nitrogen fixation.

To explore the effect of external bioavailable N input on the total biomass and nitrogen build up via nitrogen fixation, we inoculated of an exponentially growing population of *D. vulgaris* (1mM NH_4Cl , N replete) into fresh media with different concentrations of ammonium (Fig. A.2a). Comparing the maximum biomass yield (indicated by OD600) of these wildtype cultures with the $\Delta nifH$ mutant growth under the same conditions revealed a specific regime where nitrogen fixation have additional contributions compared (Fig. A.2b). While ammonium is limited until

it reached 600 μM , this regime is much smaller, spanning only from 0 to 200 μM . Furthermore, with increasing ammonium input, the apparent contribution of the nitrogen-fixing growth decreased linearly and steeply, resulting in a decreasing yield (Fig. A.2b, inset). This unexpected pattern was also observed when we varied the lactate input, with the transition point right shifted with increasing lactate input (Fig. A.2c). Such a biomass productivity of the *D. vulgaris* monoculture in relation to the initial ammonium input is different from a naïve expectation under finite energy supply: after bioavailable N-based growth ended, N_2 -fixation would utilize the remaining energy source to further growth; since the growth yield of ammonium is significantly higher than N_2 (e.g. 100% higher as measured in *D. vulgaris*), an increase in bioavailable N input should lead to an increase in total biomass yield (Fig. A.2d). By contrast, our data exhibit a two-phase pattern: there was no observable added value for ammonium in the lower range of ammonium concentration ($\leq 200\mu\text{M}$), while there was no observable added value of N_2 fixation in the higher range (Fig. A.2e).

Transition state is energy-consuming

Next, we examine the cause of the apparent inability for *D. vulgaris* to accumulate biomass via both bio-available N-based and N_2 fixation-based growth. The main overall energetic reaction adopted by *D. vulgaris* is:



To exclude differences caused by energy usage, we measured the substrate concentrations and found that the lactate input was completely consumed by the end of the cultivation experiments with all levels of ammonium input (Fig. A.3a), accompanied by acetate excretion (Fig. A.3b). The ratio between the total lactate consumption and acetate production was found to be constant, around 0.8, for different culture conditions with different amount of ammonium input (Fig. A.3c). This indicates that the total amount of free energy being produced via lactate oxidation is similar across conditions. Hence, the apparent ‘missing’ biomass yield between expected and experimentally observed amounts is likely to originate from detailed relation between energy consumption and biomass yield that was unconsidered.

One apparent feature emerged was a long transition period between the ammonium-based growth and nitrogen-fixing growth. As shown in Fig. A.4a and b, the growth curves for cultures with ammonium input of 125 μM captures three phases: the first N-replete growth, the second non-growth phase upon ammonium runout (as shown

in Fig. A.4c), and a third phase of diazotrophic growth. This growth pattern resembles the classic diauxic shifts for microbial growing in different carbon sources (Perrin et al., 2020). During the transitional period, little growth was observed, but we observed clear drop in lactate and sulfate concentrations accompanied by an increase in acetate and sulfide concentrations (Fig. A.4c). This indicates that during this nongrowth period, cells maintained a non-negligible level of respiratory activity. The mean lactate consumption rate over the 20 hour period of transition phase was found to be 2.57 mM/hr/OD, in comparison to the 14.88 mM/hr/OD during ammonium-based growth. Such a non-growth state caused by nutrient starvation, in this case the lack to nitrogen source, was termed the ‘maintenance state’. Previously, maintenance state was estimated to consume energy at a rate 3 orders of magnitude smaller in comparison to the exponential growth phase (Price & Sowers, 2004). However, here we found that the energy flux at transition state is 17% of the flux at the exponential phase, which was characteristically different compared to the previously defined maintenance upon energy limitation.

The ratio between acetate production and lactate consumption during the transition phase is around 80% (Fig. A.4d), which is within the variations of the overall measurements (Fig. A.3c). Accompanying the lactate oxidation is the sulfate reduction at a rate of 0.99 mM/hr/OD, which matches the 2.05 mM acetate being produced, given the 1:2 stoichiometry in the energetic reaction. The estimate of the sulfide production is slightly lower, which can be a combine effect of small fraction of incomplete reduction of sulfate into elemental sulfur (Wang et al., 2023) and unmeasured H₂S in the headspace of the culture. Overall, these data indicate that *D. vulgaris* employed similar mode of energetic metabolism during the nitrogen-starved transition phase as the growth phase.

The above observation from substrate measurements indicates that the transition state after the ammonium runout and before nitrogen fixation is a growth-halted, yet energy-consuming state that was previously ignored (Fig. A.4e and A.4f). As indicated in Fig. A.4c, with in initial ammonium input of 125 μ M, around 25% of the energy was consumed during this nongrowth phase. Since a higher initial ammonium input will lead to a higher population upon growth arrest at ammonium runout, and the total energy flux is expected to scale with the population size, increasing amount of energy substrate is expected to be consumed during the transition state as initial ammonium input increases (Fig. A.4g). We thus propose that the transition state energy consumption is the missing element that led to the lack of

additive effect between ammonium-based and diazotropic growth yield (Fig. A.4h).

A simplified model captured the nonadditive effect

While the energy fluxes at the exponentially growing phase and nongrowth phases are quantifiable and easily parameterized for mathematical model adaptation, the quantification of energetics throughout the entire growth history, which involves non-exponential phases, are more complicated. We thus set out to explore whether it is possible to predict the growth-yield relation observed experimentally with a simple model based on empirical data that does not necessarily require an explicit, detailed quantification of growth rates and yields throughout the growth history: First, we consider that the total biomass yield and total Energy consumption can be roughly divided into three phases:

$$Y_{tot} = Y_a + Y_t + Y_n \quad (\text{A.2})$$

$$E_{tot} = E_a + E_t + E_n \quad (\text{A.3})$$

Here Y is biomass yield, E is the limiting energy substrate consumed, a indicates ammonium-based growth state, t indicates transitional state, n indicates nitrogen-fixing state.

We thus set out to obtain empirical data by linking these elements. We found that for both ammonium-based and nitrogen-fixing growth, the biomass yield depends linearly on the lactate initially provided (Fig. A.2a, A.2b). This led to a simple quantitative relation:

$$Y_a = x \times E_a \quad (\text{A.4})$$

$$Y_n = x \times E_n \quad (\text{A.5})$$

Next, we confirmed that the population size at ammonium runout for *D. vulgaris* is linearly correlated with the ammonium input (Fig. A.2c), and we assume that the energy consumption during transition state scale with ammonium concentration, unless it was completely consumed prior to the entry into the nitrogen-fixing phase:

$$Y_t = 0 \quad (\text{A.6})$$

$$E_t \propto c[NH_4Cl] \quad (\text{A.7})$$

Based on the above simplifications, we carried out simulations in Matlab (provided as supplementary codes) and used a single transition-energy parameter to relatively well capture the V-shaped growth-yield relation as observed for different lactate and ammonium input (Fig. A.5d).

A fast-tracked N-fixing decision recovered an additive effect

To quantitatively explore the effect of non-growth energy consumption on biomass production, we varied the level of the former and found that, the non-additive phenomenon is one of the many probable outcomes (Fig. A.6a). With less energy consumption during the non-growth state, which can be caused by lower respiration rate or shorter transition time, there can be a range of ammonium concentrations where the ammonium and N₂-fixing growth is additive. With higher energy consumption during the non-growth state, which can be caused by a higher respiration rate and/or a longer transition time, increasing ammonium concentrations causes a reduction in final biomass in the lower concentration regime, which was observed in our study.

Microbes have been known to maintain a basic level energy metabolism to stay viable and respond to environmental changes. However, such a significant amount of energy consumption as seen in this study suggests other factors may be at play. Nitrogen fixation is known to require intracellular changes, including a large amount of nitrogenase proteins, and such a reorganization of the cellular proteome may potentially contribute to the duration and energy level during this transition. Hence, we carried out LC-MS comparing the proteomes of exponential *D. vulgaris* wildtype strain under ammonium-based and N₂-fixing growth. Indeed, we observed that the nitrogenase subunits are among the most significantly increased proteins inside cells. Additionally, we found that a series of proteins involved in scavenging amino acids and ammonium transporters also increased drastically under N₂-fixing conditions, indicating that nitrogen-source scavenge represent a critical strategy adopted by *D. vulgaris* despite its own ability to fix nitrogen.

Among these machineries are the ammonium transporter AmtB, which increased 60-fold in relative protein abundance (Fig. A.6b). In *E. coli*, AmtB is an ATP-dependent high affinity ammonium transporter, which was able to pump extracellular ammonium at nM range into the cell (Kim et al., 2012). Based on the proteomics data, we hypothesized that ammonium transporter may have two potential functions. First, it scavenges the minute amount of ammonium from external environment upon nitrogen starvation, which was observed in *E. coli*; secondly, it could be essential for the retainment of freshly synthesized ammonia, preventing it from leaking to the external environment.

To explore the effect of AmtB on nitrogen fixation, we investigated the growth kinetics of a $\Delta amtB$ strain (Fig. A.6c). We found that $\Delta amtB$ strain maintained

the ability of fix nitrogen, and *AtmB* is thus not essential for nitrogen fixation. Instead, we observed a much shorter transition time from ammonium-based into N_2 -based growth, reducing from 20 hours to around 5 hours. Hence, *AtmB* primarily play a role of ammonium scavenging from extracellular environment. While the proteomic change required for nitrogen fixation necessitates a time delay after ammonium runout, nitrogen-source scavenging strategy strongly delayed such a transition. Remarkably, disrupting this single *atmB* gene caused a qualitative change in the response of biomass production to ammonium input (Fig. A.6d). The ammonium-based growth and N_2 -fixing growth now appear additive. Hence, the above observed non-additive effect is primarily contributed by the cellular maintenance and nutrient scavenging, while secondarily by the energy required for proteome remodeling.

A.4 Conclusion

In this study, we show that in an energy-limited system, bioavailable nitrogen source-based growth and nitrogen-fixing growth do not exhibit an accumulative effect for a simple culture of nitrogen-fixing bacterium. This has implications for the impact of human nitrogen input into various ecosystems such as agricultural soil.

We found that the lack of accumulative effect was caused by high amount of energy consumption at the transition phase between ammonium-based and nitrogen-fixing growth. This transition phase was observed previous in various other free-living nitrogen-fixing bacteria such as *Azotobacter*. Nitrogen-fixing growth imposes great proteomic and energetic investment. Scavenging of low concentrations of bioavailable nitrogen before entry into a nitrogen-fixing growth is an adaptive strategy that is likely common for facultative nitrogen fixers (this requires more evidence). In our study, we found that a single gene deletion of *atmB* drastically reduced the transition time, and consequently transitional energy consumption. Several transcriptomic studies also showed that *atmB* were highly upregulated for nitrogen fixing growth, suggesting that ammonium scavenging may be a common strategy that can lead to long transition.

The high level of maintenance energy flux is critical for the observed effect. Maintenance effect has been mostly discussed at stationary phase, often associated with energy limitation. Here, we show that the level of maintenance upon nitrogen runout is 3 orders of magnitude higher compared to energy limitation. This has strong implications for the population dynamics and productivity in various ecosystems. Maintenance state beyond the context of energy limitation should be taken

into account in broader context.

A.5 Methods

Medium used in this study

The composition of the medium used in this study was: MgCl_2 8 mM, CaCl_2 0.6 mM, KCl 1.0 mM, KH_2PO_4 2.2 mM, sodium L-Lactate (Cat# 71718, Sigma-Aldrich) (6 mM, 8 mM, and 10 mM), Na_2SO_4 (6 mM, 8 mM, or 10 mM), PIPES 30 mM and NaOH 50 mM. 0.1% trace element solution, containing per liter: nitrilotriacetic acid 8 mM, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 2.5 mM, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 1.3 mM, ZnCl_2 1.5 mM, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ 0.01 mM, H_3BO_3 0.32 mM, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ 0.38 mM, SeO_3^{2-} 0.03 mM, $\text{MoCl}_2 \cdot 2\text{H}_2\text{O}$ 0.2 mM, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 5mM. 0.1% Thauer's vitamin stock solution per liter: d-biotin 20 mg, folic acid 20 mg, pyridoxine hydrochloride 100 mg, thiamine HCl 50 mg, riboflavin 50 mg, nicotinic acid 50 mg, pantothenic acid 50 mg, p-aminobenzoic acid 50 mg, thiocetic acid (lipoic acid) 50 mg, choline chloride 2 mg, vitamin B12 10 mg. NH_4Cl stock solution (100 mM) was added in Balch tubes separately at the end of inoculation to different final concentrations based on condition requirement. The newly made medium was N_2 flushed for at least 30 min before use.

Strains and culture condition

Wild-type *Desulfovibrio vulgaris* Hildenborough, the $\Delta nifH$ mutant and the $\Delta atmB$ mutant were kind gifts from July D. Wall (University of Missouri). Cells were stocked in 50% glycerol in -80°C . Before incubation, cell stock was first revived 1:100 in fresh liquid medium (20 mM sulfate, 20 mM lactate and 2 mM ammonium) till mid-exponential phase. Then the revived cells were inoculated 1:5 in the same liquid medium as the one used in the following experiments till exponential phase. After 2 rounds of revision, the cells were inoculated 1:100 in liquid medium amended by different concentrations of ammonium according to the experimental conditions with biological triplicates. All emendations were done in the anaerobic chamber. After inoculation, the stoppered Balch tubes were flushed by pure N_2 for 5 min with the purpose of supplementing N_2 for the culture. All cultures were grown statically at 37°C water bath in dark. OD was monitored over the course of incubations.

Sampling for metabolites measurements

Liquid cultures were harvested along with incubations. In detail, at each time point, 0.5 ml of culture was taken out using N_2 pre-flushed syringes and needles, and

readily filtered through 0.2 μm filters into 2 ml tubes. Notably, to keep the inner pressure of headspace, 0.5 ml of N_2 gas was supplemented while sampling. As for the flow-through, 50 μl was mixed with 450 μm of Zinc acetate solution (0.5 M) for sulfide measurement, and the rest was immediately flush frozen by liquid nitrogen and stored in $-80\text{ }^\circ\text{C}$ for the measurements of sulfate, ammonium, lactate, and acetate.

Metabolites measurements

Hydrogen sulfide produced by sulfate reduction was detected in technical triplicate by the methylene blue method (Cline, 1969). Quantification was carried out in a plate reader (TECAN Sunrise) by monitoring the absorbance at 670 nm.

Sulfate and ammonium were measured via parallel Dionex Integrion HPIC (ThermoFisher, Waltham, MA, USA) ion chromatography systems with anion and cation columns running in parallel housed at the Caltech Environmental Analysis Center. The ion chromatography system was run as described previously (Green-Saxena et al., 2014; Speth et al., 2022) with the following modifications. A Dionex AS-DV autosampler loaded samples diluted 1:50 in 18 M Ω water to serial anion and cation columns and detectors, which are maintained at $30\text{ }^\circ\text{C}$. Anions were resolved by a 2 $\times$ 250 mm Dionex IonPac AS19-4 μm analytical column protected by a 2 $\times$ 50 mm Dionex IonPac AG19-4 μm guard column (ThermoFisher, Waltham, MA, USA). A potassium hydroxide eluent generator cartridge generated a hydroxide gradient that was pumped at 0.25 ml min^{-1} . The gradient was constant at 10 mM for 5 min, increased linearly to 48.5 mM at 27 min, then increased linearly to 50 mM at 40 min. A Dionex AERS 500 suppressor provided suppressed conductivity detection running on recycle mode with an applied current of 31 mA. Cations were resolved by a 2 $\times$ 250 mm Dionex IonPac CS16-4 μm analytical column protected by a 2 $\times$ 50 mm Dionex IonPac CG16-4 μm guard column. A methanesulfonic acid eluent generator cartridge generated a methanesulfonic acid gradient that was pumped at 0.16 ml min^{-1} . The gradient was constant at 10 mM for 5 min, nonlinearly increased to 20 mM at 20 min (Chromeleon curve 7, concave up), and nonlinearly increased to 40 mM at 40 min (Chromeleon curve 1, concave down). A Dionex CERS 2 mm suppressor provided suppressed conductivity detection with an applied voltage of 4.2 V. Chromatographic peaks were integrated by Chromeleon 7.2.9 using the Cobra algorithm, and were correlated to concentration by running known standards and generating 4-point calibration curves.

Lactate and acetate were measured by HPLC refractive index detector (HPLC-RI) (Agilent 1100). Samples were diluted in 8 mN H₂SO₄ by 5 folds to reduce the salinity. 10 μ l of injection was carried out by 8 mN of H₂SO₄ eluent at a flow rate of 0.6 ml/min over 18 min per sample in the Aminex HPX-87H Column (300 $\times$ 7.8 mm, Bio-Rad).

Protein extraction and proteomics

Sample preparation for proteomics analysis followed a modified FASP protocol (Wiśniewski et al., 2009). Briefly, the collected cells were lysed in lysis buffer (2% SDS, 100 mM Tris/HCl (pH 7.6), 100 mM DTT, supplemented with 1 $\times$ Protease inhibitor cocktail and 1 mM PMSF) at 95 °C for 5 min. Clarify lysate by centrifugation at 16,000 $\times$ g for 5 min at 20 °C and determine protein concentration. Proteins were purified, then digested into smaller peptide fragments using LysC and trypsin proteases. Resuspend the lyophilized peptides in 100 μ l of 0.2% Formic Acid and desalt by HPLC with a Microm Bioresources C8 peptide macrotrap (3 $\times$ 8mm) (200 μ g maximum capacity). Peptide fragments were sequenced and quantified via liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS).

Numerical simulations

Numerical simulations were carried out using Matlab R2022a, with the growth yield of ammonium and lactate measured experimentally, and the transition energy as fitting parameter. The code is provided in the supplementary materials. Detailed codes are shown below:

```
% This model is modified from maintenance_model1.m
% Changed Lactate conc, and removed the nonfixer.
clear all;
Etot=8;
yn = 0.317; % OD per mM N (ammonium) from FW161 strain
data
Pini = 0.001; % initial population OD
lamdanh4 = 0.31; % growth rate
lamdan2 = 0.09;
jnh4 = 14; % Lactate flux
jn2 = 9; %?
Rini=1; %0.01
ttrans=15;
```

```

jratio=0:0.05:1;
jmain=jratio*jn2; % parameter of the consumption rate
    during maintenance
Nini=0:0.005:1.2;
AllFinalP=[];
Pnh4max=Pini*(Etot*lamdanh4/jnh4/Pini+1); % max OD in N
    replete
Pn2max=Pini*(Etot*lamdan2/jn2/Pini+1);
for i = 1:numel(jmain)
    FinalP=Pn2max;
    for j = 2:numel(Nini)
        %% Phase 1: NH4 growth
        Pyn=Nini(j)*yn;
        if Pyn>=Pnh4max
            Ptot = Pnh4max;
        else
            Pnh4=Nini(j)*yn; % Population
            tnh4=log(Pnh4/Pini)/lamdanh4; % time
            Enh4 = jnh4 * Pini * 1/lamdanh4 * (exp(
                lamdanh4 * tnh4) -1); % energy/lactate
            %% transition phase: simplify as
            maintenance
            Etrans = Pnh4 * jmain(i) * ttrans;
            %% Phase 2: N2 growth; t2 needs to be
            resolved
            E2 = Etot - Enh4 - Etrans;
            if E2<=0
                Ptot = Pnh4;
            else
                Pn2ini = Pnh4 * Rini;
                Pstarv = Pnh4 * (1-Rini);
                % Emain = Pstarv * jmain * t2;
                % func2= @(t) jn2 * Pn2ini * exp(
                    lamdan2*t);
                % En2=integral (func2, 0, t2);
            end
        end
    end
end

```

```

        % En2= jnh2 * Pn2ini * 1/lamdan2 * (exp
            (lamdan2 * t2) -1);
        % E2 = Emain + En2;
        syms t2;
        eqn = Pstarv * jmain(i) * t2 + jn2 *
            Pn2ini * 1/lamdan2 * (exp(lamdan2 *
                t2) -1) == E2;
        Stn2= double(solve(eqn,t2,'Real',true))
            ;
        Ptot=Pstarv+Pn2ini*exp(lamdan2*Stn2);
    end
end
FinalP=cat(1,FinalP,Ptot);
end
figure(1);
hold on;
plot(Nini,FinalP,'Marker','none','LineStyle','--');
hold on;
AllFinalP=cat(2,AllFinalP,FinalP);
end
% legend('data
    ','0','0.1','0.2','0.3','0.4','0.5','0.6','0.7','0.8'...

%     , '0.9', '1.0');
ylim([0 0.2]);
cd('/Users/fabs/Work/Projects/Nfixer_Maintenance/
    model202306/')
save('modeloutput_20250223_6mM.mat','AllFinalP','jratio
    ','Nini','jmain','-mat');
% for i=1:numel(Rtr)
%     Rtr1=Rtr(i);
%     FinalOD=[];
%     for j=N
%         %% Ammonium stage
%         OD1=j*Nbio;
%         Lac1=OD1/Ynh4;

```

```

%         if Lac1>=Ltot
%             ODmax=Ltot*Ynh4;
%         else
%             Lac1b=Ltot-Lac1; % left over lac1 after
all NH4Cl is used
%             Lac2=OD1*Rtr1;
%             if Lac2>=Lac1b
%                 ODmax=OD1;
%             else
%                 Lac2b=Lac1b-Lac2;
%                 OD2=Lac2b*Yn2;
%                 ODmax=OD1+OD2;
%             end
%         end
%     end
%     FinalOD=cat(2,FinalOD,ODmax);
% end
%     if Rtr1==150
%         plot(N,FinalOD,'Marker','none','LineStyle
%             ','-','LineWidth',2,'Color','k');
%     else
%         plot(N,FinalOD,'Marker','none','LineStyle
%             ','--');
%     end
%     hold on;
%     AllFinalOD=cat(1,AllFinalOD,FinalOD);
% end
legend('Rtr=0','Rtr=10','Rtr=20','Rtr=50','Rtr=60','Rtr
=80','Rtr=100','Rtr=150','Rtr=200'...
%     , 'Rtr=250','Rtr=300','Rtr=400','Rtr=500');

```

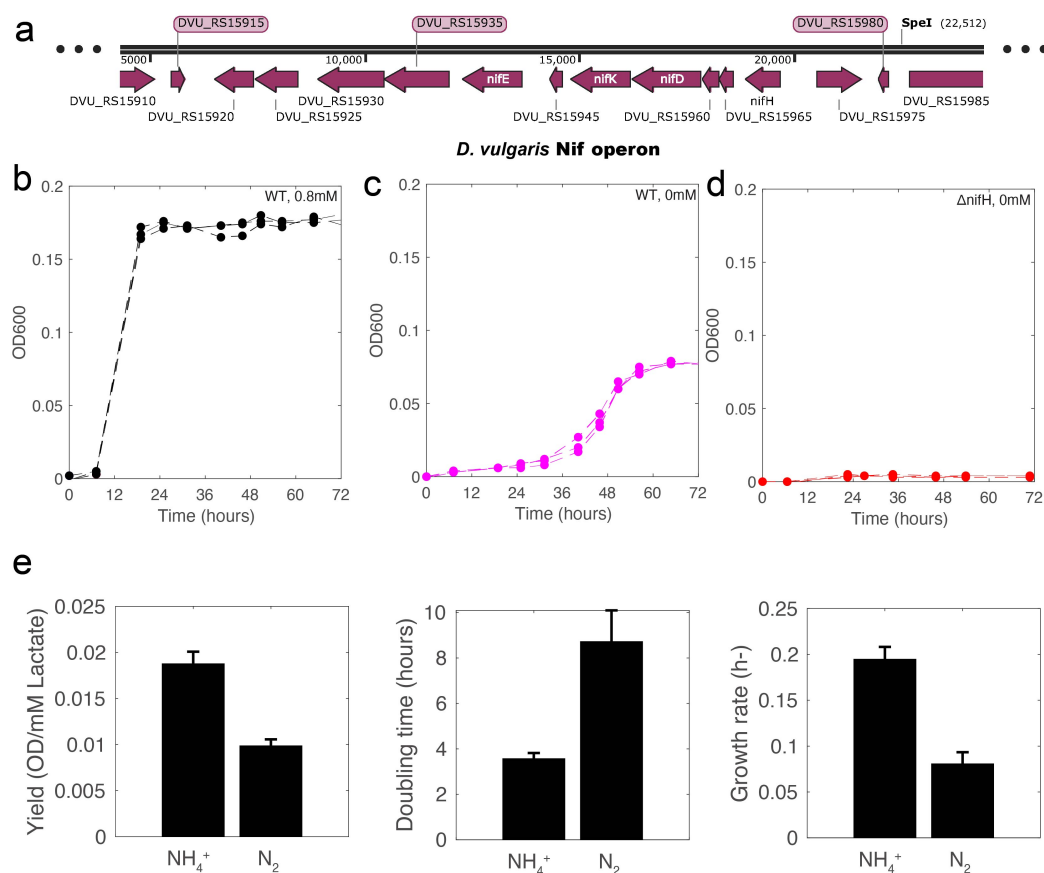


Figure A.1: Characterization of the *D. vulgaris* as a diazotroph. **a**, Nitrogenase-encoding operon of *D. vulgaris*. **b**, growth of *D. vulgaris* wildtype strain in 0.8 mM ammonium. **c** and **d**, inoculation of wildtype and $\Delta nifH$ strains, grown at the same condition, respectively exponentially growing *D. vulgaris* into a medium that does not contain ammonium or other bioavailable N source. **e**, Comparisons between the growth yield, doubling time, and growth rate between ammonium-based and nitrogen-fixing growth of *D. vulgaris* wildtype strain.

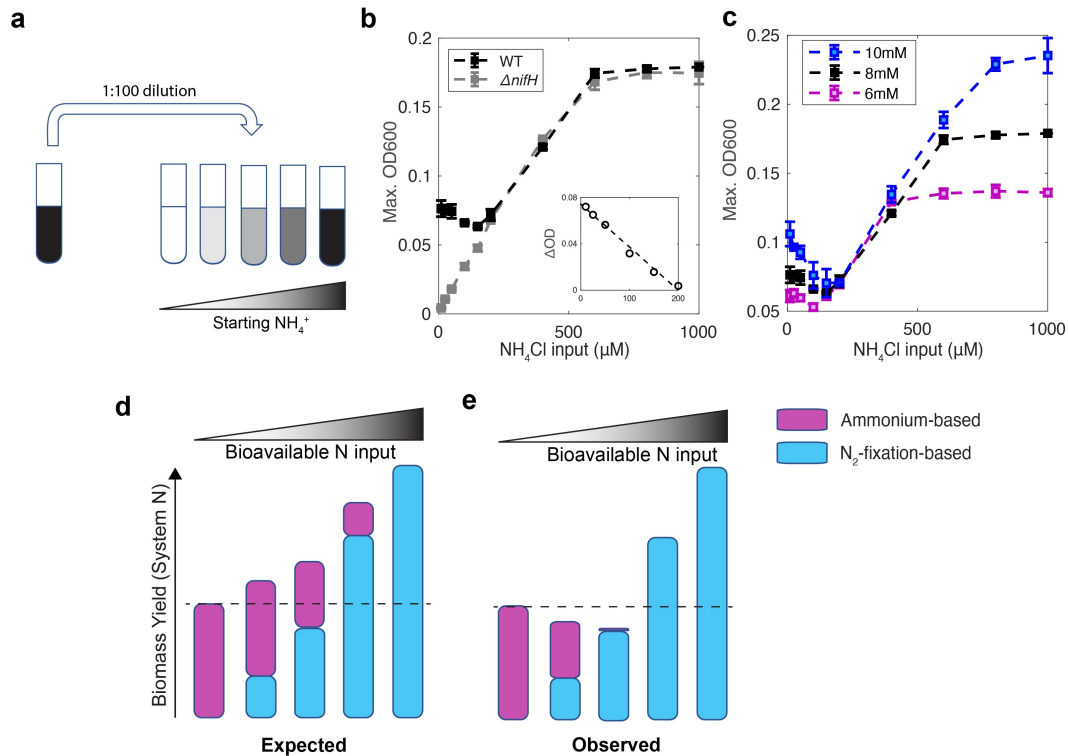


Figure A.2: Apparent non-additive effect of external nitrogen input and internal N_2 fixation in monocultures of *D. vulgaris* strain Hildenborough. **a**, illustration of experimental design, where 1% volume of a N-replete culture at its exponential growing phase is transferred into fresh media with different amount of ammonium input and the same amount of energy sources. **b**, Maximum OD600 reached by the monocultures of WT and $\Delta nifH$ yielded by different NH_4Cl input. **c**, Maximum OD600 reached with different amount of ammonium input with three different initial lactate concentrations. **d** and **e**, Schematics describing the effect of the initial input of bioavailable nitrogen source on the biomass yield (or total system nitrogen buildup) as naively expected (**d**) and experimentally observed (**e**).

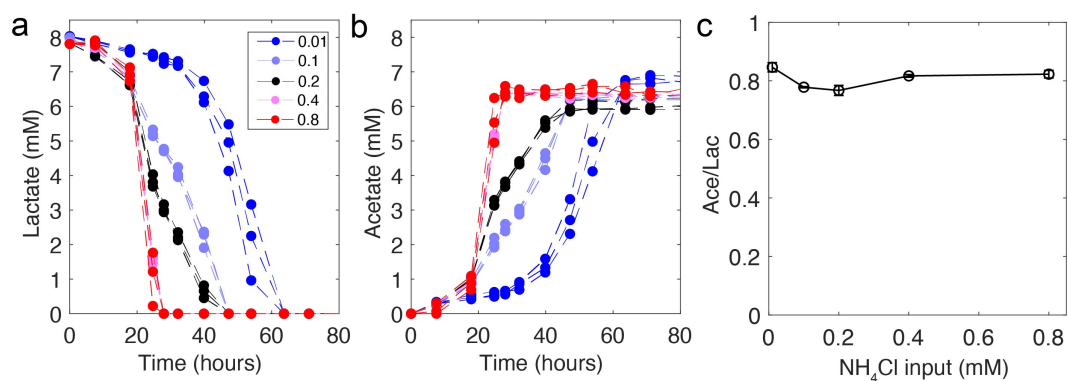


Figure A.3: Energy substrates were completely consumed in cultures with different ammonium input. **a** and **b**, Lactate consumption and acetate production measured over time in cultures of wildtype *D. vulgaris* cultures. Initial ammonium input is indicated in the legend of **a** (in mM). **c**, the ratio between the final amount of acetate produced and the total amount of lactate consumed in different culture conditions.

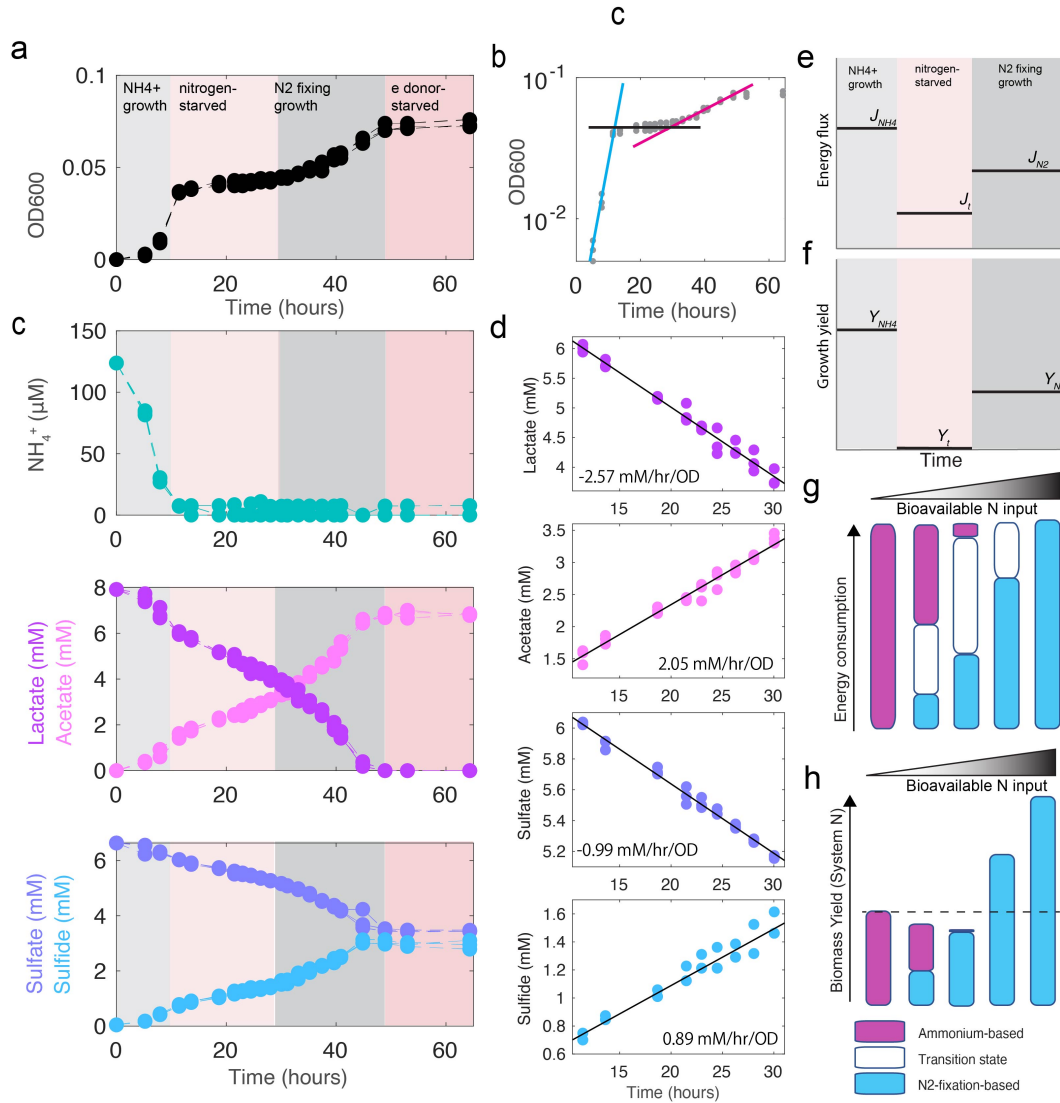


Figure A.4: Transition state consumes energy without growth. **a**, Growth dynamics of *D. vulgaris* culture with 125 μM initial ammonium input. **b**, Growth curve in a with its y axis plotted in the log. Shown are exponential fit for the two growth phases and linear fit for the transition phase. **c**, Substrate change during the growth as shown in a. **d**, transition phase of c, with linear fit shown in black lines.

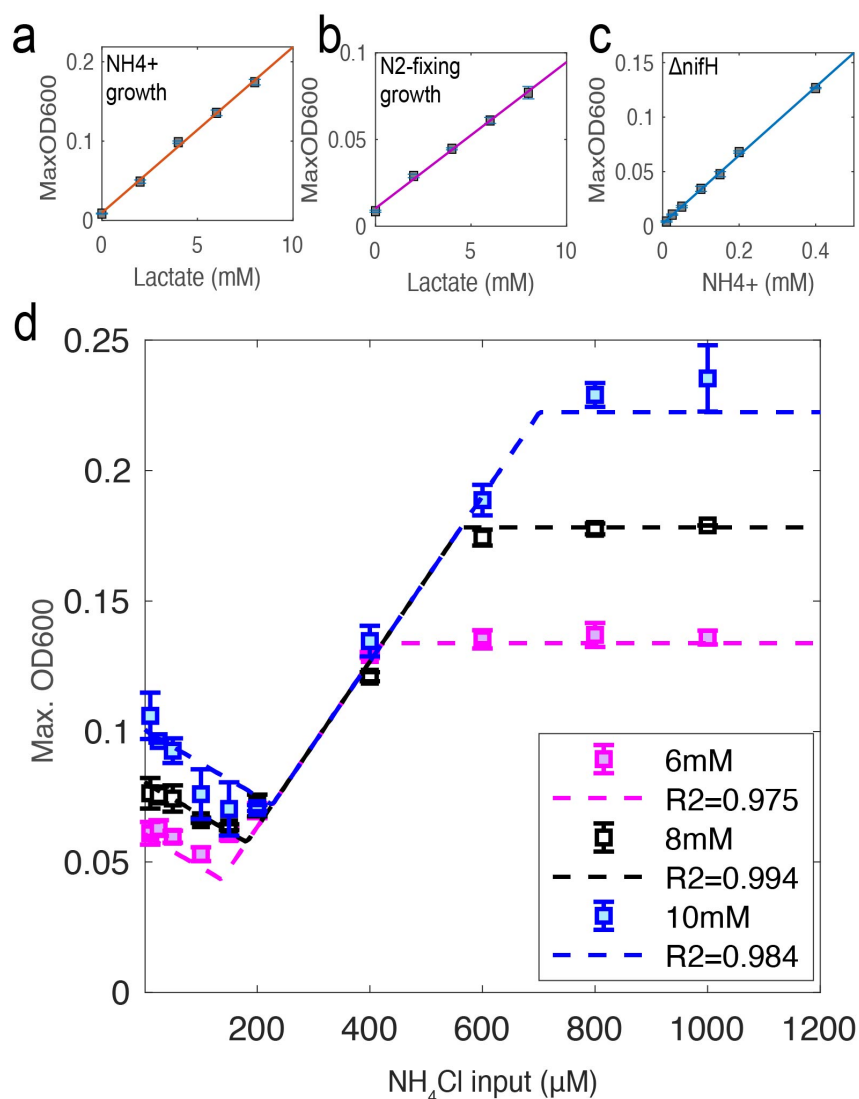


Figure A.5: Empirical data-based simple model captured the experimentally observed non-additive effect. **a-c**, Empirical data showing the linear relation between ammonium concentration, lactate concentration and maximum OD600 value reached by the cultures in the measured range. **b**, Simple phenomenological model captures the experimental data. The data points are shown for mean and standard deviation of maximum OD600 value reached by cultures inoculated with different initial ammonium and lactate concentration. Dashed lines are model-fits with R-square values indicated in the legend. Model prediction indicate that the relation is scalable with different concentrations of energy substrates.

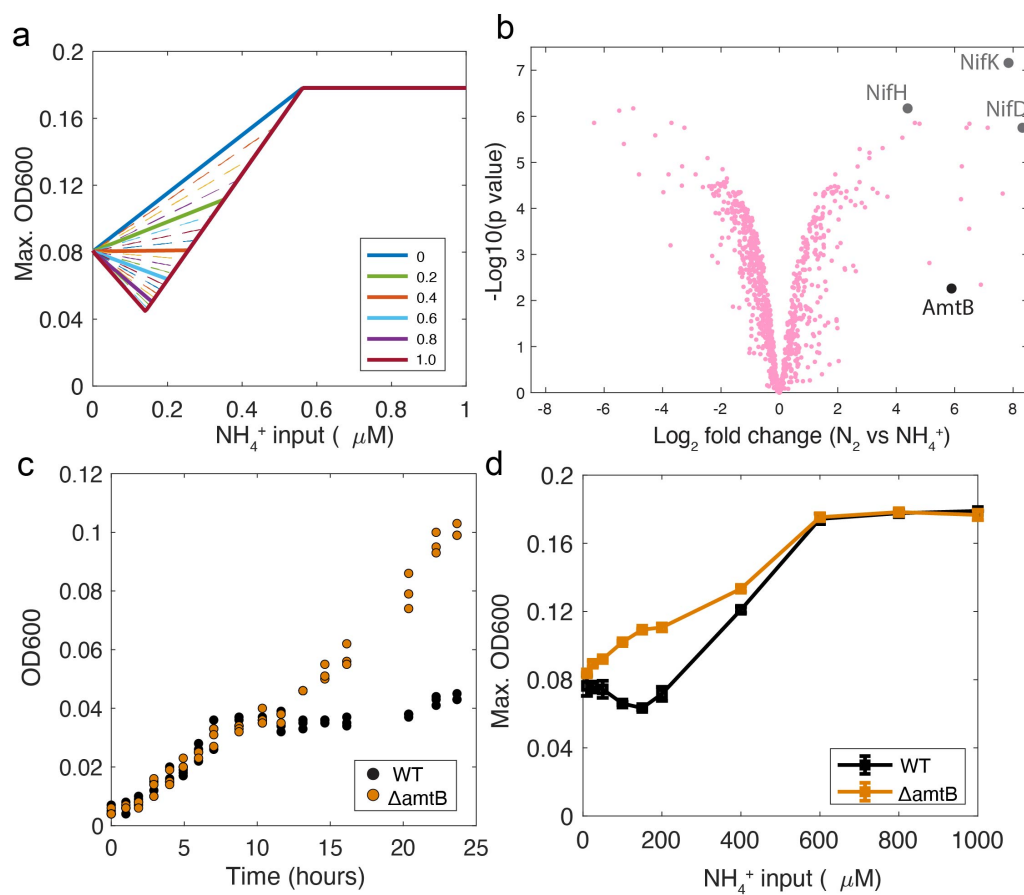


Figure A.6: A fast-tracked N-fixing decision increases growth yield. **a**, Simulations show the relation between biomass productivity and transition energy change. **b**, Proteomic differences between N_2 -fixing growth and ammonium-based growth. **c**, Growth dynamics of *D. vulgaris* WT strain and ΔamtB strain in growth medium with $100 \mu\text{M}$ ammonium. **d**, Maximum OD600 reached by the monocultures of WT and ΔamtB strain yielded by different NH_4Cl input.

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