U.S. Department of Energy: Research Grant FINAL TECHNICAL Report

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Title of Project: Systems Level Dissection of Anaerobic Methane Cycling: Quantitative Measurements of Single Cell Ecophysiology, Genetic Mechanisms, and Microbial Interactions

Grant Number: DE-SC0010574

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Project Results:

1) **Characterizing the syntrophic mechanism of anaerobic oxidation of methane by ANME archaea**

One of the greatest research successes achieved by our multi-disciplinary research team was a breakthrough in understanding the syntrophic mechanism underpinning the metabolic association between methane-oxidizing archaea (ANME) and sulfate-reducing bacteria. Through this work, we developed multiple lines of evidence that direct extracellular electron transfer through a conductive matrix enables conservation of energy in the methane-sulfate redox couple (described below and in McGlynn et al. 2015, Nature; Scheller et al., 2016 Science).

Using laboratory maintained microcosm experiments and flow through bioreactors inoculated with sediments with high rates of anaerobic oxidation of methane (AOM), we completed a suite of stable isotope and chemical manipulation experiments to test hypotheses on the involvement of different terminal electron acceptors and diffusible electron carrying intermediates in the AOM syntrophy. Elements of this work are included in the 2017 Ph.D. thesis of Dr. Hank Yu and have been published in Scheller et al., 2016 and in additional manuscripts currently being prepared for publication. As part of this effort, we tested the hypothesis introduced by Milucka et al. in 2012 stating that ANME archaea are capable of directly oxidizing methane using sulfate with a subsequent passage of zero valent sulfur to S-disproportionating deltaproteobacteria (Yu et al., in preparation). While the energetics of this predicted reaction is favorable, the genetic evidence for the ability to catalyze dissimilatory sulfate-reduction within the ANME archaea is lacking. To further explore this hypothesis, we assembled additional

ANME genomes from metagenomic data and screened the ANME genomes for putative sulfur metabolizing genes. Genes associated with the energy-requiring assimilatory PAPS pathway for sulfate incorporation into biomolecules as well as multiple clades of novel sulfite reductases were identified in the ANME genomes that were distinct from sulfite reductases involved in conventional dissimilatory sulfate-reduction. The expression and biochemical activity and substrate recognition of the ANME sulfite reductases was examined during AOM with different sulfur sources using metatranscriptomics and through heterologous expression experiments (in collaboration with colleagues at Virginia Tech). These data point to a role in sulfur detoxification and assimilatory S metabolism, rather than energy conservation through dissimilatory sulfate-reduction in the ANME-2 archaea. Microcosm experiments using different sulfur intermediates including polysulfide (zero valent sulfur) indicate that high levels of polysulfides are toxic to the AOM consortia, and together this data suggests that the AOM mechanism proposed in 2012 needs to be revisited, or, possibly there are multiple strategies to oxidize methane with sulfate used by different subgroups of ANME.

An alternative hypothesis for the AOM syntrophic mechanism that developed out of our DOE funded research is a methane-fueled syntrophic association through interspecies extracellular electron transfer. Using a multi-pronged 'systems biology' approach that spans multiple spatial scales, we have been testing this mechanism through comparative genomics, transcriptomics, and proteomics, spatial statistical analyses of single cell activities and relationship with distance to partner cells within syntrophic methane-oxidizing consortia, reaction transport modeling, as well as process level AOM activity at the cm scale. Through the integration of information from metagenomic and expression data with single cell activity analysis, fluorescence and EM microscopy, and bulk geochemistry analyses, we have developed new fundamental understanding about these globally-important but enigmatic microbial associations as well as pushed methodological developments that will be of value for exploring a broad range of uncultured microbial interactions in the environment.

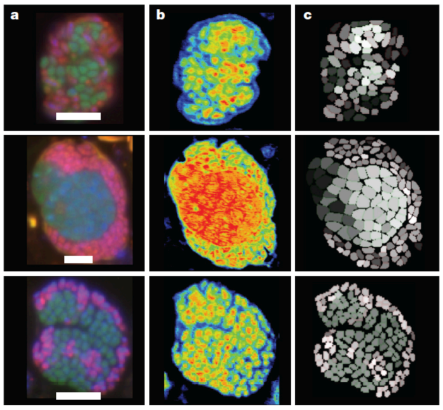

FIGURE 1: Examples of AOM consortia identified by FISH and paired anabolic activity measurement via nanoSIMS. a, FISH-identified consortia showing archaeal cells (green) and Deltaproteobacteria (pink). The top two panels represent consortia of ANME-2c or 2b paired with Deltaproteobacteria. The lower four panels show ANME-2c archaea paired with the seep-specific deltaproteobacterial group, SEEP-SRB1a. Scale bars, 3 mm. b, Corresponding nanoSIMS ion images of biomass show 14N12C2 ion images with warmer colours indicating higher secondary ion counts (maximum 1,500 counts). c, Single cell activities are measured as 15N atom percentages for regions of interest (ROI) representing the FISH-identified archaea and bacteria in each consortium. Lighter shaded cells are more enriched in 15N, which corresponds to higher levels of anabolic activity. From McGlynn et al 2015.
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Specific contributions include the development of a novel analytical framework that integrates geostatistical methods and nanoSIMS acquired isotopic data of spatial patterns of metabolic activity of individual ANME and deltaproteobacterial cells within structured consortia to test different hypotheses regarding the intermediate electron carrier used in the AOM

syntrophic interaction (e.g. chemical (diffusion driven) intermediate or direct interspecies electron transfer); (FIGURE 1).

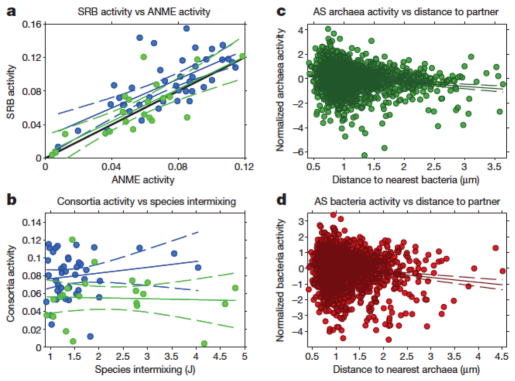

FIGURE 2: Activity relationships between archaea and bacteria in AOM consortia. a, Population-level average of bacterial activity versus archaeal activity for individualAOMconsortia revealing a positive correlation in activity between paired partners. Individual AS aggregates shown in blue. The 1:1 line is shown in black. b, Total consortium activity plotted against the join index (J) of spatial intermixing between archaeal and bacterial partners, where lower J values represent greater mixing (AS in blue: c, d, Activity of single archaeal or bacterial cells, respectively, plotted against distance to the nearest syntrophic partner for the AS data set. In all plots R2 values and solid lines represent linear regressions of the plotted data. Dashed lines illustrate the 95% confidence intervals in slopes and intercepts of the linear regressions. From McGlynn et al., 2015.

Our empirical single cell isotope data and complementary reaction-transport models independently supported a non-diffusion based mechanism underpinning the AOM syntrophy, with a “conductive matrix” model of direct electron exchange providing the best fit to the spatial activity patterns observed from our experimental FISH-nanoSIMS data (FIGURE 2). Comparative genomics of ANME genomes identified previously unreported large multiheme cytochromes (MHC) that are predicted to be a associated with the S-layer - among the largest (MHC’s) described from Archaea to date. TEM staining for heme groups using diaminobenzadine (DAB) within ANME-SRB consortia recovered from methane-rich sediments revealed the occurrence of redox-active proteins within the extracellular space between ANME and deltaproteobacterial cells (McGlynn et al., 2015).

To follow up on the extracellular electron transfer hypotheses, microcosm experiments established under SO4 free conditions and supplied with carbon-13 methane were used to test the potential to decouple the ANME-sulfate-reducing symbiosis by adding an alternative electron acceptor to substitute for the sulfate-reducing bacterial partner. A number of single electron and 2 electron carriers were tested (e.g. AQDS, humic acids, and iron citrate)- many showing a stimulation of methane oxidation above the controls and, notably, some (AQDS) producing AOM rates that were comparable to that achieved with SO4 (FIGURE 3).

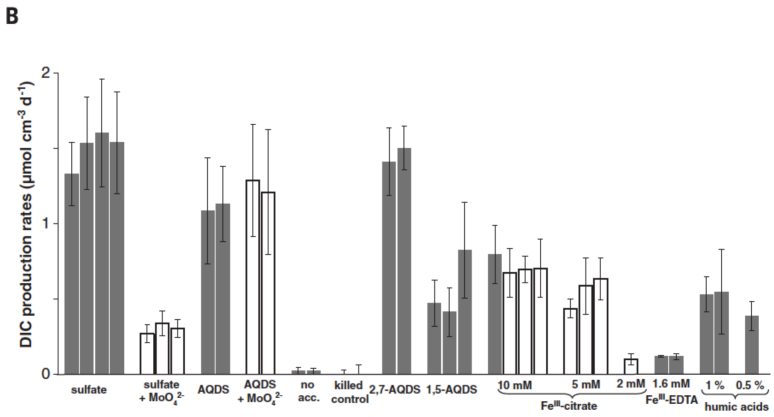

FIGURE 3: 13CH4 based AOM rates showing decoupling of methane-oxidation from sulfate-reduction with the addition of alternative electron acceptors. Shown are the initial calculated rates of AOM with different electron acceptors. error bars represent the 95% confidence interval. White bars depict incubations with sodium molybdate an inhibitor of sulfate-reduction. Data shows similar rates of AOM was achieved using AQDS as with SO4 and that this process is independent of the activity of the sulfate-reducing bacterial partner. From Scheller et al., 2016.

These sulfate-free experiments further support the hypothesis of direct interspecies electron transfer from ANME to their syntrophic bacterial partner, rather than ANME-mediated sulfate reduction. To confirm that CH4 oxidation by ANME archaea coupled to AQDS was decoupled from their bacterial partners, we used stable isotope probing and targeted FISH-nanoSIMS analysis which confirmed that biosynthetic activity for individual AOM consortia recovered from AQDS, humic acids, and iron citrate incubations (n=200 ANME-SRB consortia) was exclusively localized to ANME cells with no observable isotopic enrichment within the co-associated partner deltaproteobacteria (FIGURE 4; Scheller et al., 2016). This novel high-resolution isotopic dataset

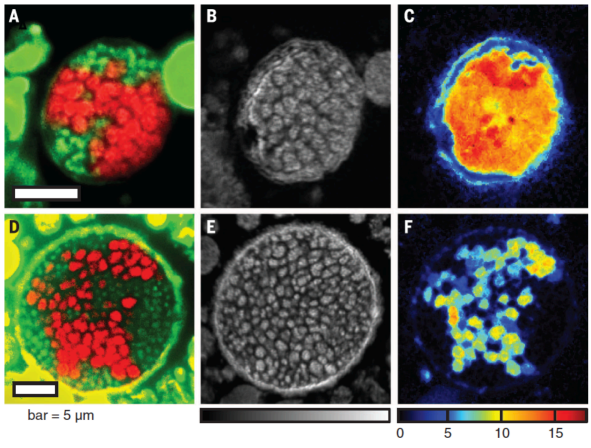

FIGURE 4: Representative FISH-nanoSIMS images from sulfate and AQDS microcosms. The correlation between phylogenetic identity (FISH) and anabolic activity (15N enrichment) for example consortia of ANME-2c archaea and sulfate-reducing bacteria analyzed fromAOM incubations amended with sulfate or AQDS is shown. (A to C) AOM consortium from microcosm with sulfate. (D to F) Consortium from microcosm with AQDS as the sole electron acceptor. In each case, the at%of 15N isotope enrichment was calculated from ratios of secondary ion images of 12C15N– and 12C14N–. (A) and (D) FISH images, with ANME-2c in red and Desulfobacteraceae in green; the FISH signal for the bacterial cells in (D) is weak, probably due to the low abundance of cellular rRNA in SRB in the AQDS treatment without sulfate. (B) and (E) nanoSIMS ion image of 12C14N– for cellular biomass, linear scale (0 to 4500 counts per pixel). (C) and (F) Fractional abundance of 15N (in at %) as a proxy for anabolic activity. Scheller et al 2016.

demonstrates our ability to manipulate the syntrophic association through decoupling of the methane-oxidizing ANME from its electron accepting bacterial partner in the absence of sulfate. The ability to manipulate this uncultured methane-based symbiosis in the lab represents an important advance for AOM research and has served as the springboard for future experiments in the lab, while enhanced genome reconstructions from AOM consortia have assisted in developing new metabolic models for the ANME-SRB syntrophy (FIGURE 5).

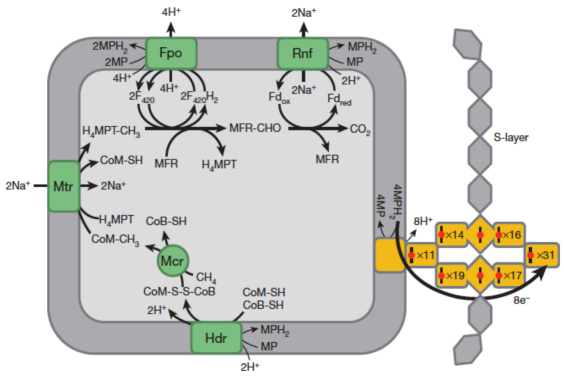

FIGURE 5: A proposal for the energy metabolism of the methanotrophic ANME-2a Archaea.
Reducing equivalents from methane are generated through the methyl branch of the Wood–Ljungdahl pathway and are deposited in the membrane-bound methanophenazine pool (Mp/MpH2) via reactions at the Hdr, Fpo, and Rnf complexes, which oxidize CoM-SH/CoB-SH, F420H2, and Fdred, respectively. All proteins involved in the reverse methanogenesis pathway (green) have been identified in the ANME-2a genome. We have identified proteins (orange) in the ANME-2 genomes that may be responsible for extracellular electron transport. From McGlynn et al 2015

Identifying key microbial groups and metabolic pathways driving anaerobic oxidation of methane in lake sediments coupled to iron and manganese reduction

Microbialy mediated anaerobic oxidation of methane (AOM) is a key process in controlling the flux of methane to the atmosphere from the breakdown of organics in soils and sediments. Nitrate and sulfate-dependent AOM has been well documented and preliminary evidence for several other electron acceptors has been reported – notably heavy metals such as Fe(III) and Mn(IV). An archaeal species, identified as *Candidatus* Methanoperedens sp. BLZ1, enriched on nitrate and methane, was recently hypothesized to also be capable of Fe(III) and Mn(IV) reduction coupled to anaerobic methane oxidation based on short term batch tests (3 days). However, no long-term enrichment or gene expression analysis was conducted to support this hypothesis or to elucidate the metabolic pathways employed by this organism. The aim of our work has been to demonstrate sustained AOM coupled to Fe (III) and Mn(IV) reduction and to identify organisms responsible, and the pathways they employ, through the use of long term enrichment studies coupled with metagenomic and metatranscriptomic analyses.

Two bioreactors, G-Fe and G-FeMn, were established at the University of Queensland using sediment samples from the Gold Creek reservoir. The bioreactors were amended with CH_4 and Fe^{3+} (ferrihydrite) or CH4, Fe3+ (ferrihydrite), and Mn4+ (birnessite) under anoxic, mesophilic conditions. Geochemical analysis of CH4 and Fe2+/
Fe3+ concentrations in both reactors support active anaerobic oxidation of methane and Fe3+ reduction. This activity was stably maintained over the course of 3 years (Ferrihydrite) and one year (birnessite).

Analysis of the microbial community (16S rRNA gene amplicon sequencing) showed an increase in the relative abundance of members associated with Methanoperedens (ANME-2d), increasing from 0 to 54% for G-FeMn and from 0 to 12% for G-FeMn during the 500-day incubation. In the G-FeMn reactor, the genus Methylomirabilis, a previously

described NO3-dependent bacterial anaerobic methane oxidizer, was also observed at moderate abundance (3-14%), but was below 1% in the iron (G-Fe) bioreactor. Metagenomic samples from both the G-Fe and G-FeMn reactors were also analyzed (Leu et al., in preparation). Using GraftM, a bioinformatic program developed by the Tyson Lab (Boyd et al., in prep), 16S rRNA genes were extracted from the metagenomes for community profiling. Similar to the 16S rRNA amplicon profiling, members of the ANME-2d dominated the G-FeMn metagenome (~29%), while this group was detected at lower abundance (3%) in G-Fe metagenome.
The Methylomirabilis OTUs were 6% of the recovered 16S genes in the G-FeMn reactor and undetectable for G-Fe. All other microbial OTUs remained below 10%.

Binning of genomes from the metagenome assembly from the methanotrophic Fe and Mn bioreactors was accomplished using MetaBat (Kang et al., 2014), a program that utilizes empirical probabilistic distances of genome abundance and tetranucleotide frequency to separate contigs into different putative genomes. Based on single copy marker genes, 22 and 27 bins were successfully recovered with >50% completion and <10% contamination rate for bioreactors G-FeMn and G-Fe, respectively. This included 2 ANME-2d bins (>98% completion and <3% contamination), one from each reactor. These putative metal-oxidizing methanotrophic archaea share many metabolic characteristics with the NO3-reducing *Methanoperedens nitroreducans* genomes (Haroon et al., 2013). Both genomes contained a complete set of genes for the 'reverse' methanogenesis pathway except for certain formylmethanofuran dehydrogenase subunits (fmd) that appear to be missing. Both bins contained incomplete sets of genes necessary to mediate respiratory and periplasmic nitrate reduction.

Gene annotations of the G-FeMn ANME bin revealed 18 deca-heme cytochromes that may be involved in extracellular transport of electrons to iron and manganese oxides (as well as used in direct electron transfer between syntrophic partners). Intriguingly, a decaheme c-type cytochrome belonging to the OmcA/MtrC family was unique to this ANME genome. In Shewanella oneidensis MR-1, a known iron reducing bacteria, the OmcA and MtrC genes encode terminal reductases located at the bacterial cell surfaces (Shi et al., 2012). Due to the insolubility of iron and manganese oxides in neutral pH, these extracellular terminal reductases have the ability to bind and reduce metal oxides directly (Shi et al., 2012).

In the G-FeMn ANME bin, the MtrC/OmcA protein and 17 other deca-heme cytochromes were analyzed using PSort, a protein sub-localization prediction tool. The 18 deca-heme cytochromes were predicted to be localized at multiple locations in the cell or as unknown (Table 1). The OmcA/MtrC protein and three other deca-heme cytochromes were predicted to be extracellular, supporting their potential role as

terminal reductases. The localization of the other deca-heme cytochromes at the cell wall and cytoplasm supports a potential extended electron transport pathway, shuttling electrons from within the cell to the terminal reductases, and consequently towards the metal oxides located outside the cell.

Metatranscriptomic analysis revealed upregulation of these genes, supporting their ability to independently mediate AOM using these metals as the terminal electron acceptor. The enrichment of novel *Methanoperedeneceae* in this study highlights the metabolic versatility of this clade and their likely importance in carbon and metal cycling in the environment. Ongoing collaborative work is focused on further characterizing the differences in the genomic potential and impact on biogeochemical cycling by ANME archaea.

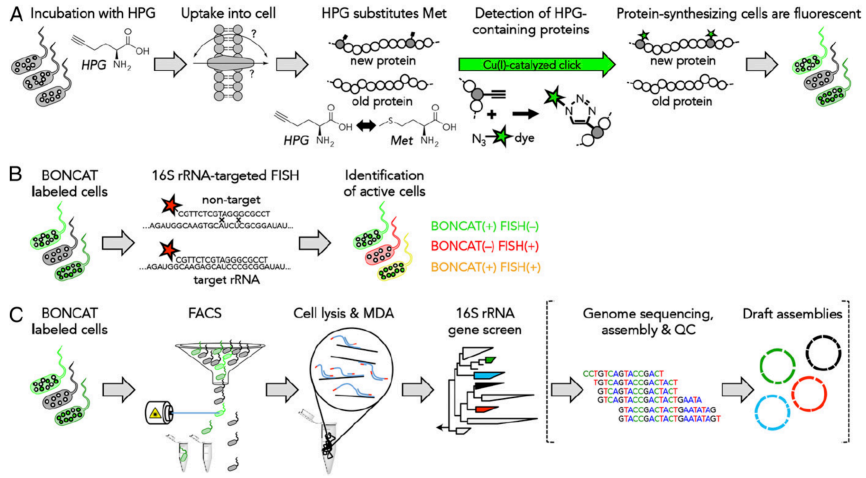

FIGURE 6: Schematic for the visualization, identification, and sorting of translationally active cells using BONCAT-FACS. The bioorthogonal amino acid HPG is added to an environmental sample, which is then incubated under in situ conditions. Individual sorted aggregates are then lysed, genomic DNA amplified by MDA and sequenced.

2) **BONCAT identification of active environmental microorganisms and proteogenomic studies of AOM**

Our proteogenomic studies of AOM assemblages have been ongoing using a combination of conventional metagenomics and environmental transcriptomics as well as new, targeted approaches based on isotopic and fluorescence tracers of cellular activity. The application of 15N stable isotope probing of proteins with collaborators at ORNL yielded information about pathways involved in AOM and protein turnover (Marlow et al., 2016). The long doubling times of the ANME/SRB consortia, combined with challenges from low protein recovery in sediment, incomplete coverage of the sequence database impacted the overall information gained from the results from this work. Despite these challenges, coordinated efforts with the ORNL team led to major improvements in proteins identified from AOM sediment communities- increasing the number of proteins from 400-500 on average in our initial experiments to >1500 proteins in our current work. We are working towards a more comprehensive sequence databases covering the major ANME lineages, we believe we will be able to improve on this further with the inclusion of new genomic information acquired from single ANME-deltaproteobacteria mini-metagenomes, developed in collaboration with the Joint Genome Institute.

A significant challenge of metagenomic studies in soils and sediments is the difficulty in assembling large contigs and composite genomes due to high strain level heterogeneity. This has proven particularly problematic for the AOM sediment system, with multiple subspecies of both ANME archaea and deltaproteobacteria often co-occurring in the same environment. We used a newly developed method called BONCAT (Bioorthognal noncanonical amino acid tagging; Hatzenpichler et al., 2014) which fluorescently labels newly synthesized proteins in cells, in combination with FACS sorting to better characterize the underlying genomic features of different ANME strains and their specific bacterial partners (FIGURE 6). In collaboration with Dr. Tanja Woyke at the JGI, we conducted a pilot project where we flow sorted and sequenced transcriptionally-active AOM consortia from sediment incubations after BONCAT labeling. Initial analysis of Illumina iTAG 16S rRNA diversity from 46 sorted and MDA amplified consortia recovered representatives of the majority of major ANME clades and subgroups described and, with each consortia composed of 1 major ANME archaea and 1 bacterial OTU. Notably, this unique single aggregate analysis revealed the co-occurrence of syntrophic partnerships a specific ANME lineage (OTU) and multiple sulfate-reducing bacteria and vice versa (FIGURE 7; Hatzenpichler et al., 2016). Genomic sequencing of unique consortia is underway and these simplified mini-metagenome datasets are now being incorporated in our environmental proteomics pipeline in effort to capture a greater proportion of the expressed proteins.

This aggregate-specific view of syntrophic partnerships using BONCAT-FACS was also independently studied using ANME and SRB-targeted Magneto-FISH (Pernthaler et al., 2008). Here, a nested set of CARD-FISH probes ranging from Domain-targeted archaeal probes to more subgroup specific lineages (ANME-2c or Seep-SRB1a) were used to assess the specific associations occurring within each group. From this analysis, specific associations between sulfate-reducing bacteria and sulfide-oxidizing bacterial lineages were uncovered in addition to a range of diverse ANME archaea-sulfate-reducing bacterial associations. This work was recently published (Trembath-

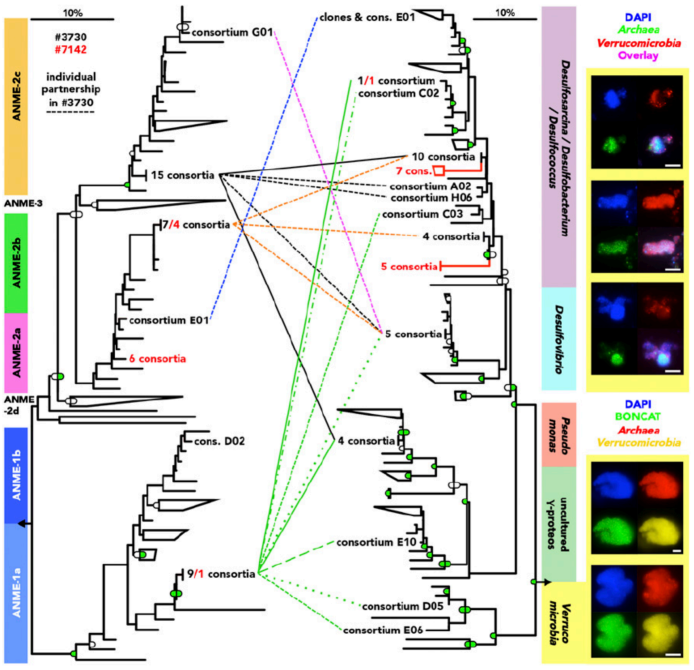

FIGURE 7: BONCAT-FACS results from AOM aggregate sequencing. Identification of microbial partners from 16S rRNA analysis within AOM consortia after activity-based sorting. Taxonomic affiliation of the archaeal (left tree) and bacterial (right tree) partners within 45 individually sorted, translationally active consortia from AOM sediments. This unique dataset shows that for a single ANME or SRB OTU, syntrophic associations with multiple partners are possible. The solid and dashed lines in the figure indicate individual archaeal−bacterial partnerships detected from individual consortia sequencing. Scale bars equal 10% estimated sequence divergence. From Hatzenpichler et al., 2016.

Reichert et al, 2016. Figure 8 shows a compilation of a bootstrapped network analysis of the correlations between specific OTU's recovered from the Magneto-FISH study.

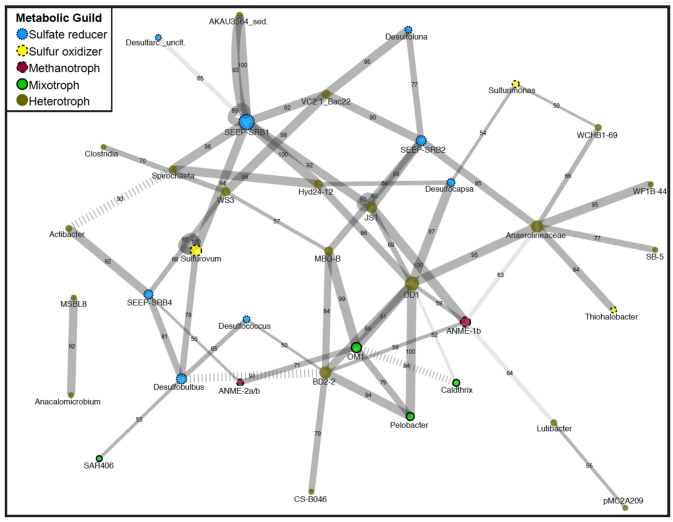

FIGURE 8: Network diagram of Magneto-FISH and bulk sediment samples. Co-occurrence analysis of the top 135 unique OTUs displayed in network form. Nodes represent the taxonomy of the OTUs in the network and edges are the connections between OTUs. Node size is scaled by number of connecting OTUs and colored by simplified, putative metabolic guild (sulfate reducer: blue, small dash; sulfur oxidizer: yellow, medium dash; archaeal methanotroph: magneta, large dash; mixotroph: green, no dash; heterotroph: brown, no outline). Edge thickness is scaled by number of occurrences of this association (from 50 to 100 times) and number of occurrences also included along edge. Negative associations are denoted by hashed lines. From Trembath-Reichert et al., 2016.

3) Genomic identification, EM and chemical characterization of ultrastructural features associated with different ANME-2 lineages involved in AOM

TEM analysis of the ANME archaea and deltaproteobacteria that syntrophically couple methane oxidation to sulfate reduction revealed surprisingly complex ultrastructural features within these uncultured microorganisms. In environmental systems, it is often difficult to ascribe phylogenetic identity to microorganisms in TEM preparations due to incompatibilities in the preparation methods. To overcome this limitation, we have developed a method (FISH-EM) that uses fluorescence staining of microorganisms (FISH) prior to embedding in resin for subsequent thin section preparation and EM ultrastructure and chemical analysis (FIGURE 9: McGlynn et al., in preparation).

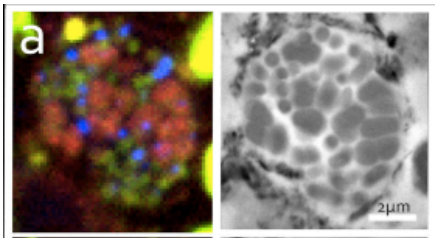

FIGURE 9: Demonstration of FISH-EM with uncultured sediment consortia. Here ANME-2c recovered from methane oxidizing sediment was hybridized by a double (5' and 3') cy3 labeled ANME-2c_760 probe (in red) and Desulfobacteraceae (DSS) bacteria was hybridized by double cy5 labeled DSS_658 probe (in green). DAPI is shown in blue. The corresponding TEM image is shown on the right. The embedded section is ~0.5 micron thick.

Using this approach, we have identified unique ultrastructural features that appear to be unique to the different ANME subgroups, with members of the ANME-2b containing large polyphosphate inclusions while archaea associated with ANME-2c appear to lack these inclusions. Morphological heterogeneity was also recorded in the deltaproteobacterial partners, many harboring carbon rich storage granules and unusual inner membrane invaginations associated with redox active/heme containing proteins

identified through DAB (3,3'-diaminobenzidine-tetrahydrochloride-dihydrate) staining. These features are reminescent to the cristae of mitochondria or inner membrane invaginations harboring particulate methane monooxygenase in aerobic methanotrophs (McGlynn et al., 2016). A low abundance subpopulation of the Desulfobacteraceae in association with ANME-2 was also observed to contain both iron sulfide (gregite) magnetosome-like chains and iron oxide inclusions. Elemental composition was independently confirmed using TEM-EDS and EELS (FIGURE 10: ; McGlynn et al., in preparation).

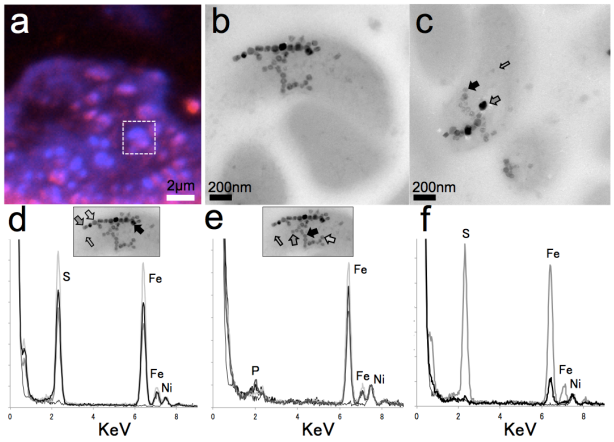

FIG 10: EDS of magnetosome-like intracellular minerals within sulfate-reducing bacteria in association with ANME-2 identified by the ANME-2-538 probe. (A) FISH image with ANME-2-538 hybridized cells in red and bacterial partners in blue (DAPI) B) TEM of the boxed region in A. (C) Another example of mineral phases in partner bacterial cells. D-F) EDS spectra taken from particles in each of the two magnetosome-like chains showing Fe:S composition of the darker material phase in (B) and the absence of S in the lighter phase. Arrows on the TEM images mark the location of EDS acquisition (McGlynn et al., in preparation).

Annotation of the reconstructed genomes from ANME-2d also suggests potential for production of intracellular inclusions and links to acetate production under nitrate-coupled methane oxidation. The metabolic capabilities of microorganisms within AOM bioreactors were assessed after KEGG annotation and compared with transcriptomic data. For example, in an ANME-2d dominated reactor, nitrate limiting conditions stimulated acetate production. Expression data was mapped onto the assembly, revealing high expression in KEGG modules associated with acetate production, acetyl-CoA synthesis and PHA biosynthesis.

4) **AOM consortia response to nitrogen limitation**. Analysis of the impact of nutrient (N) limitation on anaerobic methane oxidation was explored in environmental samples naturally enriched in ANME-SRB consortia. Stable isotope probing experiments amended with 15N2 confirmed dependence of nitrogen fixation on methane. Surprisingly, not all sediment horizons harboring methane-oxidizing aggregates showed active N2 fixation (Figure 11). Instead, this process was concentrated within a few discrete sediment horizons supporting the highest AOM biomass and rates of methane oxidation. FISH-nanoSIMS analysis of 15N2 assimilation by AOM consortia and single cells of SRB confirmed N2 fixation activity with CH4 addition and revealed most fixation activity appears to be carried out by ANME-2 archaea in association with SRB.

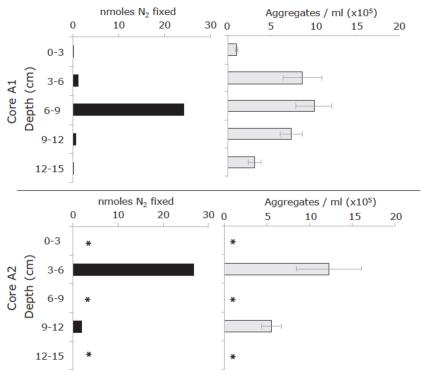

FIGURE 11: Variations in environmental nitrogen fixation coupled to anaerobic oxidation of methane. Total N2 fixed during a 39 week bottle incubation with 15N2 plotted to show the sediment depth horizon (left) and ANME-SRB aggregate abundance from the same incubations recorded at the beginning of the experiment (right; quantified per ml of sediment slurry in the incubation). Error bars indicate one standard deviation in each direction of aggregate counts on 3–5 replicate filters. Asterisks indicate depths for which no sediment 15N-amendment experiments were conducted. From Dekas et al., 2013.

5) **Modeling potential intermediates for the syntrophic association between ANME and SRB.** Research spearheaded by C. Meile at UGA worked on the development of new reactive transport models for methane oxidation by ANME-SRB consortia to guide the computational analysis of the syntrophic interaction between archaeal and bacterial partners. This effort led to the development of a dynamic, three-dimensional, multi-species model that was then used to compare simulated spatial patterns of activities against the empirical single cell activities measured from FISH-nanoSIMS data of individual AOM consortia. The syntrophic mechanism of AOM in microbial aggregates was simulated using the 3 major mechanisms hypothesized for the ANME-SRB interaction, including soluble intermediates like hydrogen, formate, and acetate (MIET), the Milucka et al. (2012) disulfide mechanism, and direct interspecies electron transfer (DIET). The resulting simulation of methane oxidation rates and activity distribution patterns was then compared to observational data, and used to identify likely interactions in the light of this process-based quantitative analysis. A draft of this work is being prepared for submission (He et al., in preparation).

In this manuscript, a number of distributions of archaea and bacteria within microbial aggregates were studied, including archaea at the aggregate core surrounded by bacteria, a well-mixed scenario, as well as intermediate structures with smaller clustering of the two types of microorganisms. Rate expressions accounted for cell densities, substrate availability and reaction energetics, causing the process to shut down when the microbe gains less energy than what is required for maintenance. For mechanisms involving the exchange of chemicals, the model was formulated as diffusion-reaction equations. In DIET, electron transport was implemented depending on concentration gradients in redox molecules and electric field, following the work of Strycharz-Glaven et al. (2011).

The model simulations covered a wide range of parameter values, most importantly cell-specific rate constants. As a consequence, the simulated rates of AOM varied widely for each of the three mechanisms considered. Results from our model simulation suggest that DIET and HS2- models can reach cell-specific rates

determined from laboratory measurements, while MIET models using acetate typically yielded slower rates.

Furthermore, model formulations in which the archaea produce an intermediate chemical species that is used by the bacteria commonly lead to high rates of activity near interfaces between archaea and bacteria. This is because diffusional transport limits the removal of metabolites, leading to thermodynamic shutdown at high cell-specific rates away from a microbial partner that consumes that chemical. The extent of this effect depends on the size of the aggregates (FIGURE 12). This intra-aggregate activity pattern differs from measured single cell anabolic rates (from 15N nitrogen incorporation). The observational data presented in McGlynn et al. (2015) suggest that N update is evenly distributed within an aggregate, and that the average cell-specific N uptake does not vary significantly with aggregate size.

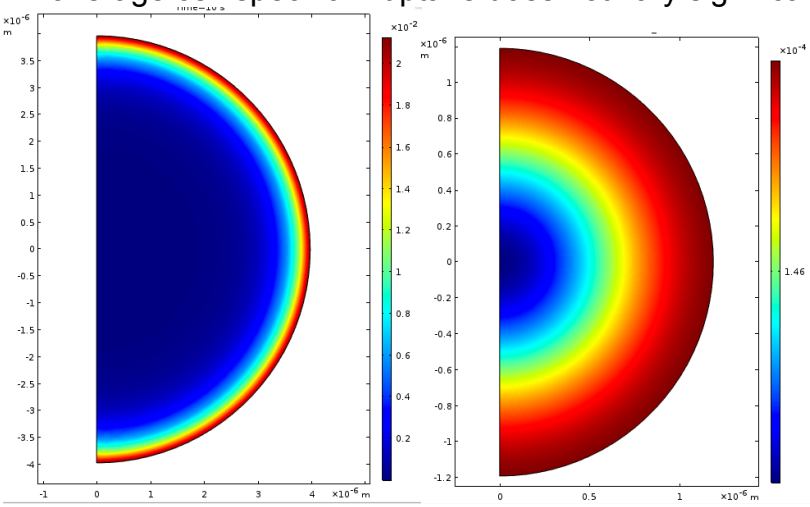

FIGURE 12: Example of rates of AOM in an arrangement where the archaea are located in the center of the aggregate. Shown here are the rates of methane oxidation in the area where archaea are present. Low rates are indicated by the blue color, while high rates are shown in red. The left aggregate has a larger radius than the one on the right, resulting in high rates of methane oxidation restricted to the interface between archaea and bacteria only. He et al in preparation.

To further explore the feasibility of DIET, HS2- and MIET pathways, we selected models that give AOM rates in a range from 0.01 to 100 fmol cell-1 d-1, roughly consistent with field observations. We compared the outcome of these simulations with nanoSIMS measurements from McGlynn et al. (2015), where cellular activity is calculated for regions of interest (ROIs) corresponding to single cells extracted from co-registered fluorescence in situ hybridization and nanoscale secondary ion mass spectrometry (FISH–nanoSIMS) combined with 15N stable isotope probing.

MIET and HS2- pathways showed results that differed from observations (activity rate and pattern). Rates of AOM via mediated interspecies electron transfer (MIET) are limited by the build-up of the intermediate species, resulting in thermodynamic shutdown of the reaction. The HS2- pathway can yield larger rates, yet also leads to distribution of activity at high rates that diverges from observations. However, DIET model results suggest that this mechanism is consistent with measured sediment AOM rates and intra-aggregate activity patterns, while MIET and HS2- pathways tend to yield either lower AOM rates, or show dependence on aggregate size or

spatial distribution of archaea and bacterial cells FIGURE 13; He et al., in preparation).

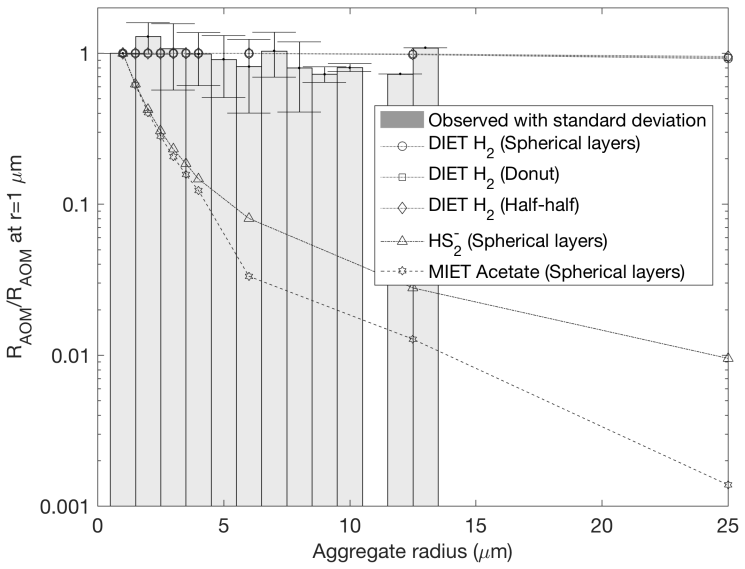

FIGURE 13: Average cell-specific AOM rates, normalized to the value for the smallest aggregates, vs. aggregate radius, for a spatial arrangement in which ANME are surrounded by bacteria (spherical layers). Geometry settings with 2 pockets of archaea (donut) and archaea and bacteria half spheres (half-half) are also shown for DIET models. The simulations shown were picked for rates that either lie within (HS_2^- and DIET) or are close to (MIET) the observed range. MIET and HS_2^- models show a considerably more pronounced decrease of AOM rates with aggregate radius than that of DIET models. Data observed in lab incubations shows no significant relationship between average archaeal activity and aggregate radius. From He et al., (in preparation).

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