FINAL REPORT
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Syntrophic interactions and mechanisms underpinning anaerobic methane oxidation: targeted metaproteogenomics, single-cell protein detection and quantitative isotope imaging of microbial consortia

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Executive Summary:
Syntrophy and mutualism play a central role in carbon and nutrient cycling by microorganisms. Yet, our ability to effectively study symbionts in culture has been hindered by the inherent interdependence of syntrophic associations, their dynamic behavior, and their frequent existence at thermodynamic limits. Now solutions to these challenges are emerging in the form of new methodologies. Developing strategies that establish links between the identity of microorganisms and their metabolic potential, as well as techniques that can probe metabolic networks on a scale that captures individual molecule exchange and processing, is at the forefront of microbial ecology. Understanding the interactions between microorganisms on this level, at a resolution previously intractable, will lead to our greater understanding of carbon turnover and microbial community resilience to environmental perturbations. In this project, we studied an enigmatic syntrophic association between uncultured methane-oxidizing archaea and sulfate-reducing bacteria. This environmental archaeal-bacterial partnership represents a globally important sink for methane in anoxic environments. The specific goals of this project were organized into 3 major tasks designed to address questions relating to the ecophysiology of these syntrophic organisms under changing environmental conditions (e.g. different electron acceptors and nutrients), primarily through the development of microanalytical imaging methods which enable the visualization of the spatial distribution of the partners within aggregates, consumption and exchange of isotopically labeled substrates, and expression of targeted proteins identified via metaproteomics. The advanced tool set developed here to collect, correlate, and analyze these high resolution image and isotope-based datasets from methane-oxidizing consortia has the potential to be widely applicable for studying and modeling patterns of activity and interactions across a broad range of spatially structured microbial partnerships, including other syntrophic associations, microbial mats, biofilms, and plant-microbe or animal-microbe symbioses in nature.

4. Accomplishments, goals and objectives of the project.
The anaerobic oxidation of methane is often thought of as a sulfate-dependent process despite the fact that other electron acceptors are more energetically favorable. Early work by our team (PI's Orphan and House) demonstrated that AOM can be coupled to the reduction of manganese (birnessite) and iron (ferrihydrite) in sulfate-free seep sediment incubations (Beal et al., 2009 Science). In this first investigation we could not differentiate whether the observed metal reduction was directly linked to methane oxidation or if the presence of metal oxides facilitates the chemical oxidation of sulfur species, producing transient sulfate that is subsequently used in sulfate-dependent AOM. **One major aim of this DOE funded project was to further test these two alternative scenarios for anaerobic methane oxidation using combination of metal amended microcosm sediment experiments, stable isotope analyses, community profiling and metaproteomics.**
Our recent publication in PNAS now has shed new light on metal-coupled methanotrophy in methane rich sediments. Using a combination of geochemical and stable isotope-based analyses, we conducted time course analyses of laboratory incubations of methane-oxidizing sediments spiked with iron oxide (hematite) designed to enable the differentiation between iron-stimulated sulfate-coupled methane oxidation and direct

methane oxidation using hematite as the electron acceptor (Sivan et al., 2014 PNAS). In tandem with these isotopic and geochemistry focused experiments, we also proposed to apply environmental proteo-genomics and ^{15}N labeled protein Stable Isotope Probing (protein-SIP) methods to further characterize the active microorganisms and metabolic pathways activated under these different electron acceptor regimes. Our initial pilot experiments using the custom 'Polyscan' program developed at ORNL to differentiate 15N and 14N labeled proteins in methane-oxidizing sediment microcosms assisted with constraining the degree of 15N enrichment required for reliable detection, suggesting that longer incubation times (months) are often required for the level of enrichment required for the slow growing anaerobic methanotrophic consortia. Preliminary experiments and methods optimization in collaboration with co-investigator Robert Hettich's group at ORNL for this component of the project was completed, however data analysis and synthesis of results of this component of the project are ongoing. An initial targeted study focusing on the analysis of potential metal cofactors associated with respiratory metalloenzymes in the methanotrophic archaea and sulfate-reducing bacteria was published last year (Glass et al., 2013, Environ. Microbiol). The preliminary results of our protein-SIP experiments have been presented at national and international conferences by student Jeffrey Marlow (see presentation list) and a manuscript on this work is being prepared for submission in spring, 2015.

As part of the objectives of this grant, we also studied the role of nutrient limitation (specifically nitrogen availability) in anaerobic methane-oxidizing communities as this has the potential to impact the methane oxidizing capacity and growth of the methanotrophic community. Our earlier discovery of N2 fixation by methane-oxidizing ANME/SRB consortia (Dekas et al., 2009 Science) indicated that nutrient limitation may exert previously unknown controls on the productivity of methane-based ecosystems, and ultimately carbon turnover. Follow-up investigations in the lab and with sediment cores collected from the field revealed a surprising disconnect between the presence of reduced sources of porewater nitrogen (ammonium or nitrate) and methane-dependent nitrogen fixation, with active N-fixation occurring even in the presence of high concentrations of ammonium (Dekas, Chadwick, in preparation). Diazotrophic growth by the methane-oxidizing ANME-2 archaea impacted rates of growth (3 fold slower), but significantly did not appear to change the rate of sulfate-coupled methane-oxidation over the course of several months. Sediment depth profiles revealed a dependence on methane for nitrogen fixation and a relationship between the peak in methane-oxidizing cell aggregate abundance and diazotrophy (Dekas et al., 2014). Similar microcosm experiments were also conducted to examine the role of nitrate either as an alternative electron acceptor or as a source of nitrogen for growth by methane-oxidizing consortia. Results from this work was recently published (Green-Saxena et al., 2014 ISME J), revealing that the availability of porewater nitrate is involved in niche partitioning between different sulfate-reducing bacterial partners associated with the methanotrophic ANME-2c subgroup, influencing their distribution in the environment.

Advancement of single cell stable isotope analysis

Another major emphasis of our funded research was focused on methods development to improve techniques for conducting quantitative single cell stable

isotope measurements using paired FISH-nanoSIMS experiments. Over the course of this grant, we successfully developed a protocol for the separation of microbial consortia from sediment, followed by embedding and thin sectioning using a resin that is compatible with fluorescence in situ hybridization, immunostaining protocols, and nanoSIMS analysis. Using this optimized protocol we have now analyzed >100 paired ANME/ SRB consortia from sediment incubations after enrichment with 15N-labeled substrates and have developed an image analysis pipeline that enables direct cell-cell correlation between FISH and nanoSIMS datasets, identification of centroids for cell specific positioning, network generation describing weighted relationships between cells in an aggregate, followed by various geostatistical methods for discerning patterns in anabolic activity and spatial organization of ANME and SRB in consortia with differing geometries (e.g. well mixed, layered, clustered). Rather than using the initially proposed HAAR training database, we opted to focus on developing scripts based on more widely available programs including Matlab and R. Initial results from this work indicate a positive correlation in activity levels between ANME and SRB partners within individual consortia, indicative of a synergistic interaction between co-associated populations. Detailed investigation of cell specific activities within local microbial 'neighborhoods' (e.g. cells in immediate proximity to a cell of interest) revealed patterns that were inconsistent with a conventional diffusion-dependent scenarios, pointing to alternate mechanisms of substrate exchange. The proposed modeling of these cell specific activity patterns is ongoing. As part of the original grant, we proposed to use quantum dots for immunofluorescence detection of expressed proteins in the methane-oxidizing consortia. Our experiments using custom peptide targets for methyl coenzyme M reductase (mcrA) and nitrogenase (nifD) from our metagenomic datasets were initially used in combination with conventional fluorescence dyes, and, in the case of the assay for mcrA, were found to specifically stain the methane-oxidizing archaea. Given the success of the fluorescence approach combined with a series of failed attempts using quantum dots, we opted to not pursue the quantum dots further and instead focused on the immunofluorescence assays that showed the most promise.

In collaboration with Mark Ellisman's group at NCMIR we have also made a series of methodological advancements in TEM analysis of AOM consortia ultrastructure, revealing the presence of polyphosphate and carbon rich inclusion bodies within consortia and other as yet poorly understood ultrastructural features. The chemical composition of many of these features has been analyzed through combinations of compatible analytical microscopy techniques including electron probe, electron energy loss spectroscopy (EELS), and XRD/ STEM. Additionally, we have optimized a method for 3D reconstruction of AOM aggregates using the Gatan 3View instrument using alternative contrasting agents and methods for increasing conductance in the resin block. Critical information on these uncultured methanotrophic consortia regarding ANME/SRB partner ratios, cell specific biovolumes, inclusion body distribution and abundance, and variation between aggregate types is being processed from these datasets and will serve as a valuable reference for researchers and modelers focusing on AOM (McGlynn et al., in preparation).

5) Summary of project activities

This collaborative project can be subdivided into 3 main research focus areas, all designed to work synergistically towards the goal of increasing our understanding of microbial consortia and metabolic cooperation underpinning biologically mediated anaerobic oxidation of methane with different terminal electron acceptors and under different environmental nutrient conditions. Specifically, these included 1) stable isotope and geochemical analyses of laboratory maintained microcosm experiments with methane-oxidizing sediment communities 2) Development and application of analytical microscopy methods for uncultured sediment-hosted microbial consortia including fluorescence, electron, and ion-based techniques for single cell/ aggregate-level analysis of activity related to variations in syntrophic partners and under different environmental conditions 3) Development of environmental proteo-genomics and protein-stable isotope probing methods for sediment-hosted methane-oxidizing consortia. Our goals/ hypotheses, research accomplishments and findings, and some of the methodological challenges encountered for each of these 3 focus areas are summarized below.

Research task #1: Geochemical and stable isotope investigations of methane oxidation coupled to alternative electron acceptors.

Sediment incubations from active AOM environments were used in 13C labeled experiments to monitor the anaerobic oxidation of methane. These experiments were set up at both Caltech and in our collaborator's laboratory at Pennsylvania State University in order to test the activity of methane oxidation in the presence of different electron acceptors. In sulfate-methane transition zones, the anaerobic oxidation of methane (AOM) is hypothesized to co-occur with the reduction of sulfate, as sulfate is an abundant electron acceptor found in seawater and is diffusion limited in sediments. Beal *et al.* 2009 calculated that other metal oxides are more energetically favorable than sulfate in terms of rates of energy gain; however, sulfate reduction is still thought to be the dominant process in AOM sediments. To test for the relative activity of AOM in the presence of different electron acceptors, samples were incubated with a ^{13}C- labeled methane in the headspace, and the $\delta^{13}CO_2$, is a representation of the oxidation of methane. Sodium molybdate was added as an inhibitor of sulfate reduction to help determine if manganese- or iron-reduction is directly or indirectly coupled to methane oxidation. These results are consistent with earlier published experiments by our research team (Beal et al., 2009) and point to SO4 reduction as the dominant pathway for methane oxidation in methane seeps, with a secondary mechanism for AOM that is present in the unamended sediment and also is stimulated by the addition of metal oxides. Interestingly, the addition of the sulfate-reduction inhibitor sodium molybdate to SO4 amended incubations resulted in AOM rates that were comparable to the metal-oxide incubations without sulfate, again pointing to a second mechanism of AOM within this system (Figure 1).

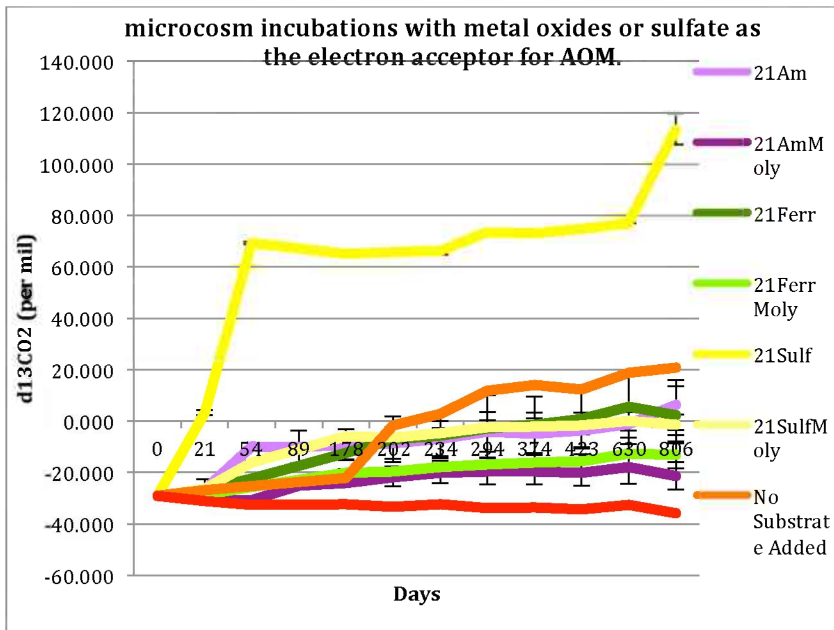


Figure 1: microcosm incubations with 13CH4 designed to test whether methane oxidation occurs in presence of manganese (Am) iron (ferr) oxides, sulfate (sulf), or no addition (no substrate added). Sodium molybdate (Mol) was added to inhibit sulfate-reduction. Consistent with findings of Beal et al 2009, sulfate stimulated the highest rates of AOM, but other treatments revealed modest levels of AOM activity above the the killed control (in red). Brandt et al., (in prep).

In a parallel set of microcosm experiments, environmental sediment samples that still contained in situ concentrations of sulfate were amended with hematite, a less bioavailable form of iron oxide. In this case, the rates of methane oxidation were higher in treatments containing both FeOx and SO4 relative to bottles with SO4 alone. Analysis of 34S and 18O in the residual sulfate over the course of the incubation enabled the differentiation between conventional sulfate-reduction, AOM coupled SO4 reduction and disproportionation. Results from direct comparison of d18O and d34S trends in the iron amended treatments and SO4 incubation (figure 2) were suggestive of methane-coupled sulfate reduction modeling results (Figure 3), rather than sulfate-reduction coupled to organic matter remineralization, supporting the hypothesis that iron oxides can stimulate SO4 coupled methane oxidation, rather than serving as the direct electron acceptor (Sivan et al., 2014 PNAS).

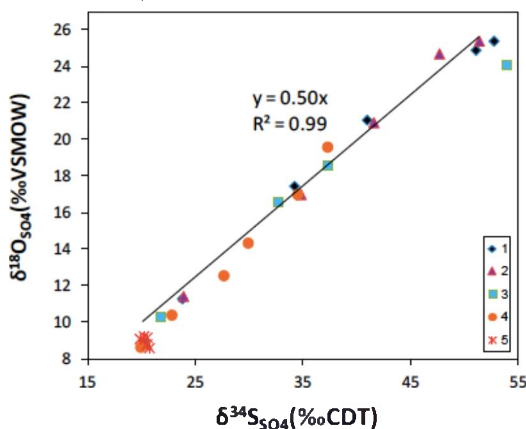


Figure 2 : d34S and d18O values for sulfate recovered in AOM incubations with and without hematite addition. From Sivan et al, 2014.

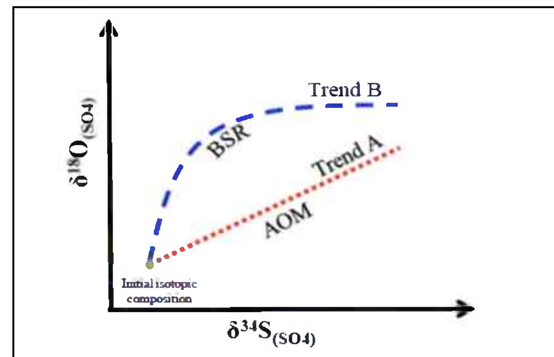


Figure 3: Typical isotope ratio trends of d34S and d18O for sulfate in natural environments modeled after Antler et al. High rates of sulfate reduction with minimal back reaction of sulfur intermediates lead directly to a linear relationship with low positive slope (trend A) whereas lower rates of sulfate reduction with more back reaction of sulfur intermediate lead to the apparent linear phase (higher slope) and asymptotic equilibrium for d18O-SO4 value typical for sulfite exchange with water and oxidation (trend B). From Sivan et al., (2014) PNAS.

Geochemical and stable isotope investigations of nutrient availability on AOM consortia.

Occurrence and response of AOM consortia to nitrogen limitation.

Our early work demonstrated that the uncultured anaerobic methane oxidizing ANME-2 archaea could fix nitrogen in syntrophic consortia with sulfate-reducing bacteria using an unusual nitrogenase that appeared to only be found in methane-charged sediments (Dekas et al., 2009). This was unexpected for two main reasons- the first related to the calculated energy demand for nitrogen fixation juxtaposed against the low energy afforded from sulfate-coupled methane oxidation; the second associated with the potential availability of reduced nitrogen within methane oxidizing sediments, suggesting the requirement to engage in this energetically expensive process to fulfill their nutritional needs was minimal. To advance our understanding of this phenomenon and its potential role in the AOM consortia, we conducted a series of experiments in the lab and field to 1) determine how widespread nitrogen fixation is in geographically distributed methane-oxidizing sediments 2) assess the heterogeneity of N2 fixation relative to geochemical gradients

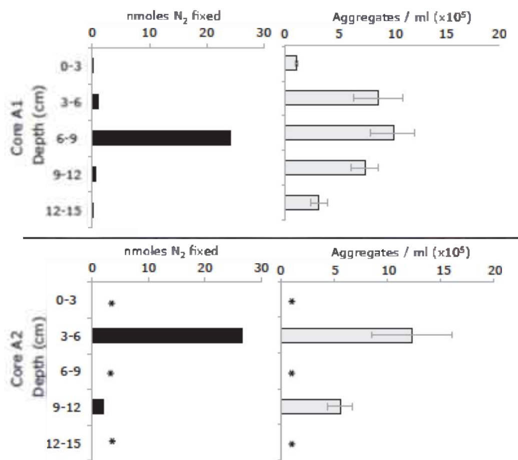


Figure 4 (From Dekas et al 2014) Total N2 fixed during a 39 week bottle incubation plotted to show the original sediment depth horizon (left) and ANME-SRB aggregate abundance from the same incubations recorded at the beginning of the experiment (right; quantified per ml of sediment slurry in the incubation). Results indicate patchy environmental distribution in nitrogen fixation activity associated with methane-oxidizing consortia, frequently limited to sediment depths showing highest abundance of consortia or methane oxidation activity. 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.

and microbial community structure in methane-based ecosystems, 3) empirically test the relationship between ammonium and nitrogen fixation by AOM consortia and 4) increase our understanding of which groups of microorganisms are responsible for bringing new nitrogen into ecosystems with high CH4 flux. Answers to some of these questions are addressed below, with highlights from published manuscripts and work in preparation. The distribution of N2 fixation in CH4 based anaerobic ecosystems was tested using 15N labeled dinitrogen gas or 15N ammonium amendments of sediment microcosms incubated with and without a methane headspace. Unexpectedly, the respiration activity of the methane oxidizing community was similar between incubations under conditions that were either limited or replete with nitrogen, however AOM consortial growth (as assessed by aggregate counts and uptake of 15N into cell biomass by nanoSIMS), was significantly lower in the absence of fixed nitrogen. These initial experiments indicate that the catalytic ability of the consortia to oxidize CH4 is maintained under nitrogen

limitation, however as more of the energetic currency of the cell is devoted to the costly process of nitrogen fixation, doubling times are slowed. Incubation experiments with freshly collected field samples further demonstrated that nitrogen fixation activity by methane oxidizing consortia was heterogeneous at different sediment depths (See figure 4). Despite the abundance of AOM consortia throughout the sediment column at depths ranging between 3 and 12 cm and similar incubation conditions (CH4, SO4 and N) for all sediment horizons, nitrogen fixation was only found to be significant at the 6-9 cm horizon (top panel figure 4).

Field measurements of nitrogen fixation in AOM communities were also observed in sediments containing high μM concentrations of ammonium, suggesting the regulation of the N2 fixation pathway may be less sensitive to reduced nitrogen sources relative to other well studied diazotrophs (e.g. cyanobacteria). Amendments of sediment with increasing concentrations of ammonium (0-2 mM, n=5 different treatments) in tandem

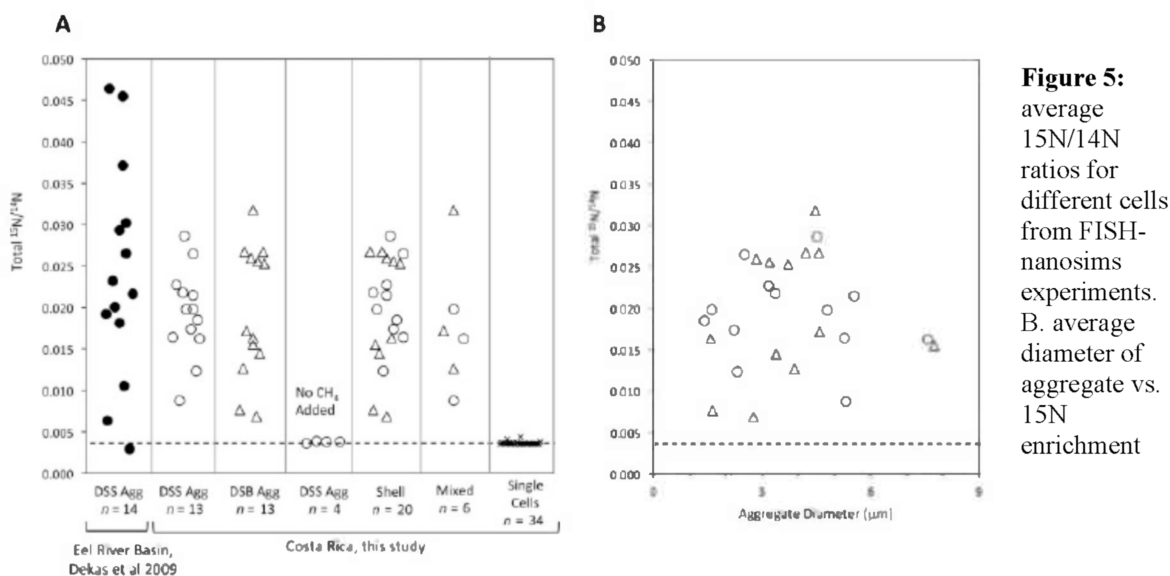


Figure 5: average 15N/14N ratios for different cells from FISH-nanosims experiments. B. average diameter of aggregate vs. 15N enrichment

with 15N2 enabled the tracking of active 15N2 fixation under different nitrogen conditions. These experiments indicated that there was still detectable nitrogen fixation by anaerobic methanotrophs at bulk porewater ammonium concentrations of up to 500 μM (Chadwick, Dekas in preparation). Recent publications have also reported similar phenomena in anoxic sediments dominated by sulfate-reduction as well as in some pure culture hyperthermophilc methanogens and clostridia, suggesting this may be a more general property of anaerobic diazotrophs. Additional experiments are needed to better understand the specific regulation mechanism for N2 fixation within AOM consortia.

To identify specific diazotrophic microorganisms in methane-oxidizing sediments, we analyzed some of our 15N2 microcosms using fluorescence in situ hybridization coupled with nanoscale secondary ion mass spectrometry (FISH-nanoSIMS), following the experimental design laid

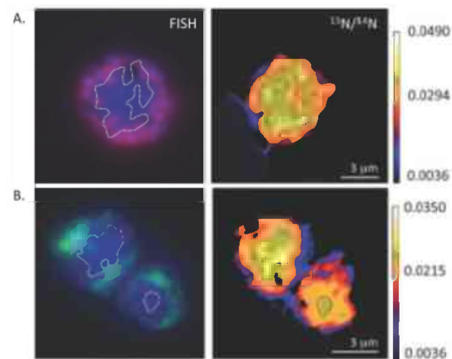


Figure 6. FISH-nanoSIMS image of 15N2 assimilation by DSS/ANME-2 and DSB/ ANME-2 aggregates from Dekas et al., 2014.

out in Dekas et al., 2009. In Dekas et al. 2009, we demonstrated active N2 fixation by ANME-2 archaea when in association with sulfate-reducing bacteria related to the Desulfosarcina/Desulfococcus group, or DSS (Figure 5 panel A left side; Figure 6; Dekas et al., 2014). Here, we used FISH probes targeting 2 different sulfate-reducing bacterial partners of the ANME-2 (the DSS as well as members of the Desulfobulbaceae, DSB) to determine whether nitrogen fixation depended on the specific syntrophic partnership. Aggregate level nitrogen fixation activity was found to be similar between the ANME-2/ DSS and ANME-2/DSB consortia from the same incubation (Figure 5 panel A). We also did not observe any difference in N2 fixation activity in aggregates of different spatial organization (shell vs mixed, panel A) or with size of the aggregate (panel B). 15N enrichment from N2 fixation was reliant on methane addition and, for the most part confined to ANME-2 associated microorganisms rather than single cells in the incubation (Figure 5, panel A). An exception to this were members of the Desulfobulbaceae, who were observed to be enriched in 15N both as single cells as well as in association with ANME-2 (Dekas et al., submitted).

Research task #2

Significant variation in aggregate morphology observed in nature for methane-oxidizing ANME-archaea and sulfate-reducing bacterial consortia. Reaction-transport based models predict close physical associations between microbial partners in well-mixed configurations for efficient electron transfer during syntrophic sulfate-coupled methane oxidation, however naturally occurring ANME-SRB consortia often span a range of sizes, and aggregate architecture, from highly mixed to well separated with patchy distribution or layered. To understand the dynamics between spatial architecture, cellular activity, environmental conditions and consortia membership, we established methane oxidizing sediments under different nutrient regimes in the laboratory and in parallel developed new protocols for separation, embedding and sectioning microbial consortia from sediments in order to maximize the spatial resolution and co-registration between fluorescence in situ hybridization and nanoSIMS ion image datasets.

Incubation experiments with 15N ammonium or 15N nitrate were established to

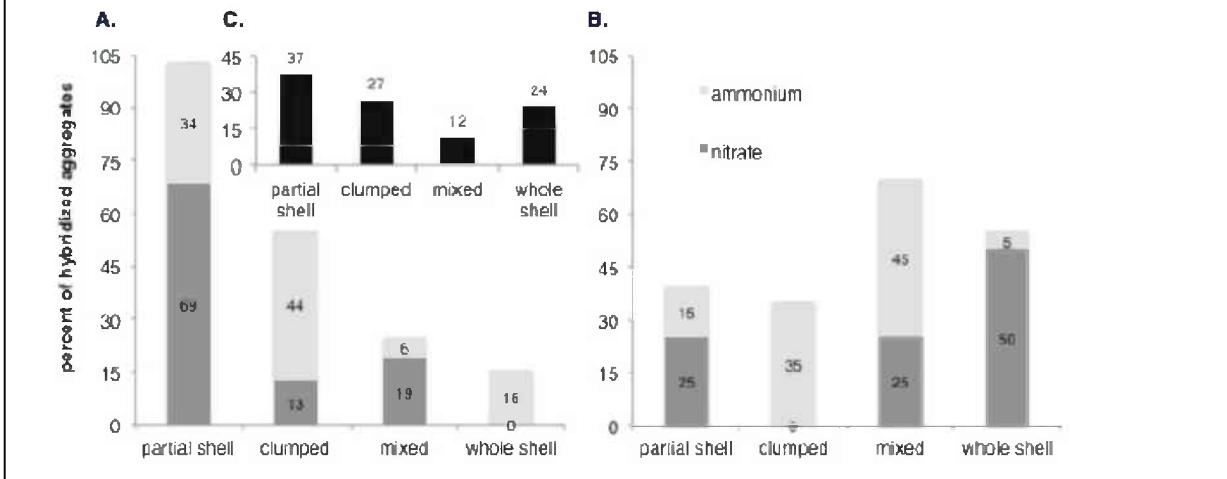


Figure 7 (From Green-Saxena et al., 2014 ISME J) Relative proportions of aggregate morphologies (A. Desulfobulbus/ ANME) and (B. Desulfosarcina/ANME) stained with specific FISH probes from ammonium and nitrate amended incubations. General distribution of aggregate morphology for in situ sediments from Eel River Basin is shown in inset.

examine ecophysiological differences between ANME-2 and different sulfate-reducing bacterial syntrophs (specifically members of the Desulfosarcina/ Desulfococcus, DSS and Desulfobulbaceae, SDB) in regard to their ability to utilize different nitrogen sources and variation in aggregate geometry. FISH experiments revealed that consortia comprised of ANME-2/DSS were frequently found as layered 'shell-type' morphologies, while ANME-2/ DSB aggregates typically had fewer sulfate-reducing bacteria, colonizing as an outer layer on only a portion of the ANME-2 'half shell' morphology, which were more prominent in the nitrate amended incubation (Figure 7; Green-Saxena et al. 2014).

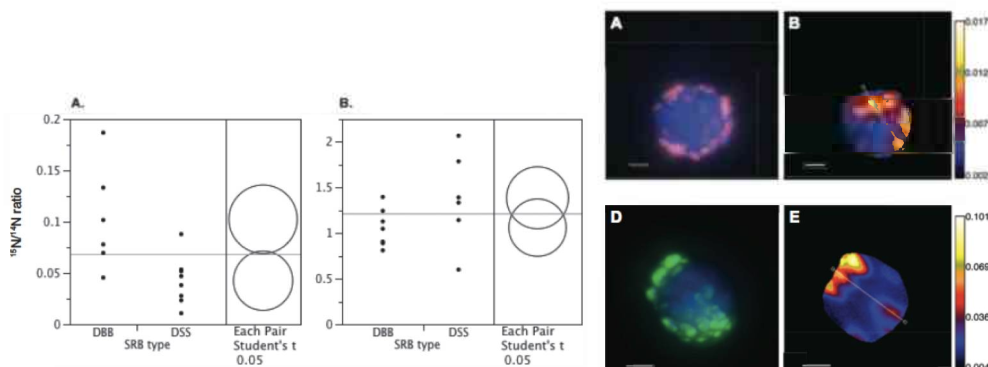


Figure 8: Green-Saxena et al., 2014 ISME J 15N enrichment measured via NanoSIMS in ANME/seepDBB and ANME/DSS aggregates from incubations of Eel River Basin methane seep sediment and amended with either (A) 15N-nitrate or (B) 15N-ammonium.

FISH-nanoSIMS analysis of the ANME-2/DSS and ANME-2/DSB consortia from both the 15N-nitrate and ammonium amended microcosm experiments showed that consortia harboring members of the Desulfobulbaceae (DSB) had higher assimilation rates for nitrate relative to their ANME-2/DSS counterparts from the same incubation (Figure 8 panel A, Green-Saxena 2014) while uptake of ammonium was equivalent in both aggregate types (Figure 8 panel B). The potential for nitrate utilization by the Desulfobulbaceae was identified in the reassembled partial genomes for this group and also is consistent with their distribution in the environment, revealing a correlation between DSB consortia abundance and in situ porewater nitrate (Green-Saxena et al., 2014). This study not only identifies unique physiological properties for different methane oxidizing syntrophic consortia, but also highlights a role for nutrients in addition to concentrations of electron donor and acceptor (methane and sulfate) in structuring these anaerobic methane-oxidizing ecosystems.

Methodological developments for high resolution single cell analysis of uncultured microbial consortia in sediment matrices: co-registration of TEM- FISH-nanoSIMS datasets.

Single cell FISH-SIMS

The multi-species microbial consortia which catalyze the anaerobic oxidation of methane live in a heterogenous sedimentary environment. We have established protocols that allow the isolation of a large number of these microbial consortia, fluorescence-based identification of microbial partners and nanoSIMS analysis of biosynthetic activity

at the level of individual cells in consortia while maintaining the context of their cellular neighborhoods (activity and identity of neighboring cells).

We found that it was possible to isolate a large number of microbial consortia from methane oxidizing sediments by applying density gradient fractionation followed by concentration by filtration. After this concentration step, we embedded the consortia in a plastic resin for generating thin sections that is compatible with FISH microscopy. An example of this is seen in the top right of the figure. There, in an approximately 4mm square area are nearly 700 individual microbial consortia which have been identified by fluorescence in situ hybridization (FISH). With this map of microbial consortia, we were able to analyze metabolic activities of individual cells by following 15N incorporation from 15NH4+ as a general biomarker.

These analyses provided us with three critical pieces of information: i) phylogentic identity of cells, ii) the metabolic activity of these cells, and iii) their spatial relationships to one another (Figure 9). With this knowledge, we have been able to test a number of hypotheses concerning the interactions of these cells as they relate to the anaerobic oxidation of methane. In addition to furnishing major insights into the relationships which exist between these cells, these experimental and analytical techniques will be of value for microbiologist in general, where developing an understanding of cellular relationships within spatially structured communities is of paramount importance to achieving an understanding of the metabolic and physiological consequences associated with microbial neighborhoods.

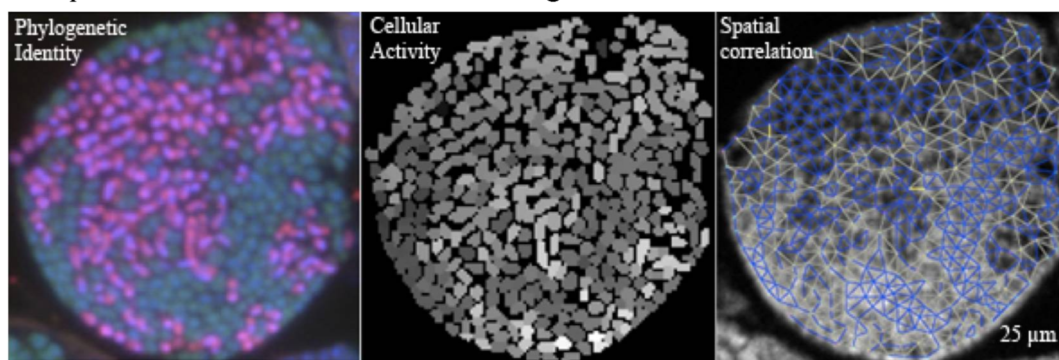


Figure 9: Example of our correlative analysis pipeline which includes paired FISH data (left) nanoSIMS acquired 15N/14N ratios (middle), and a network overlay weighting the spatial relationships between cells in the aggregate (right). An example of a spatially well-mixed anaerobic methane oxidizing syntrophic consortium is shown, with pink cells representing sulfate-reducing bacteria and green cells associated with methanotrophic archaea by FISH. Spatial statistical analyses were subsequently applied to individual consortia to assess patterns of their metabolic activity in relation to proximity to nearest syntrophic partner, spatial structure, and distance to environment, and the species pairings for each aggregate.

FISH-EM: Fluorescence in situ hybridization - electron microscopy

Electron microscopy has vastly improved our awareness of cellular structure. Although the technique has been wildly utilized in environmental microbiology, a significant knowledge gap often exists in correlating observations under the electron beam to the phylogenetic identity of observed cells. We sought to bridge this gap and have developed methodology to allow phylogenetic assignment and electron microscopic analysis of microbial consortia recovered from natural environmental samples. These

sample preparations are also compatible with nondestructive EELS analysis (electron energy loss spectroscopy) enabling chemical identification of specific intracellular granules, and, as a final step, destructive analysis with nanoSIMS for assessing isotopic enrichment.

We found the FISH probes utilized for phylogenetic identification of cells were preserved through the process of plastic embedding and preparation for observation by transition electron microscopy. In our method, FISH is done prior to EM and we have named the technique FISH-EM. Although this technique does not lead to observations comparable to those where heavy metal stains and electron contrasting agents are applied, the cells themselves are maintained in relatively intact form, complete with the presence of internal structure. An example of this is seen in the figure. In this case, the 2 partner microorganisms in the consortia were identified as ANME-2 archaea with a specific Desulfosarcina/Desulfococcus bacterial partner. Interestingly, within the Desulfosarcina SRB partner, two distinct types of magnetosome-like structures were observed, as identified by energy dispersive spectroscopy and electron energy loss spectroscopy. We have observed a number of sub-cellular features which appear to correlate with phylogenetic identity in ANME-SRB consortia, and we are currently cataloging the distribution of these. To our knowledge, this is the first comprehensive electron microscopy study of a phylogeneticallly identified microbial communities derived from the natural environment.

Research task # 3: Metaproteomic analysis of sediment hosted methane-oxidizing microbial consortia.

As part of this project, we formed a collaboration with Dr. Robert Hettich and his team at Oakridge National Laboratories to develop environmental proteomics applications for examining the specific metabolic pathways expressed by sediment microorganisms during net methane oxidation coupled to different electron acceptors. Initial analyses in the first year were focused heavily on 1) testing different protein extraction protocols in clay rich sediments containing methane-oxidizing consortia and 2) optimizing the reference metagenomic database for maximal detection of proteins.

1) Protein extraction tests included sterile sediment and clays spiked with Methanosarcina as well as direct extraction from methane seep sediment. Results from these control tests and environmental sample extractions suggested the method outlined in (Chourey et al. 2010, J. Proteome Res). was optimal for this work and was used in all subsequent sample preps. An exception to this was the iron and manganese oxide amended sediment, where the protein extraction yield and protein detection were low (14-100 proteins). This delayed our progress on this component of the grant and resulted in a further round of extraction protocol testing including a commercial MOBIO protein extraction kit, traditional SDS-TCA, SDS-TCA-FASP, and SDS-cutoff filter cleanup. From these comparative analyses, we determined the MOBIO kit gave substandard results relative to traditional SDS extraction, with the SDS-TCA-FASP preforming the best with ~1500 proteins identified (2 peptides, 1% FDR) in MnO_2 amended sediments. With this optimized protocol in hand, we are planning on completing the metaproteomic analysis of our metal amended sediments by the end of 2014. This follow up work will serve as a complement our recent publication (Sivan et al., 2014, PNAS), providing a mechanism to test whether there is genetic evidence for the expression of genes involved

in dissimilatory sulfate-reduction during anaerobic methane oxidation in metal amended sediments in the absence of detectable sulfate.

2) To develop a relevant genome database for protein identification, we used a combination of our in house assembled metagenomic data from methane seeps (including the starting sediment used for the metal and sulfate-amendment experiments), sequence information from magnetically captured (Magneto-FISH) methanotrophic archaea (ANME-2) and sulfate-reducing bacteria, and genomes from related pure culture archaea and bacteria. Six samples of seep sediment were initially used to test the extraction protocol (see above) and effectiveness of the database for protein identification. These initial analyses detected up to 550 proteins (lowest 115 proteins) using stringent search parameters (threshold of 2 peptides identified at least 1 unique, 1% FDR), identifying proteins associated with the archaeal methane oxidation pathway as well as proteins

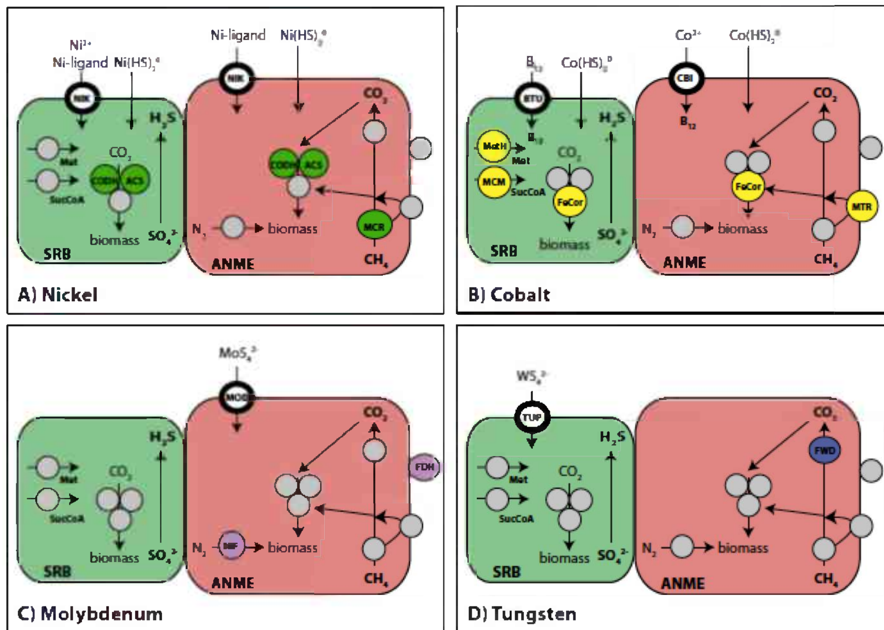


Figure 10 (from Glass et al., 2014)
Schematic of nickel (A, green), cobalt (B, yellow), molybdenum (C, purple) and tungsten (D, blue) metalloenzymes and transporters identified in methane seep metagenomes and metaproteomes with closest identity to *Desulfobacterales* (SRB, green cells) and *Methanosarcinales* (ANME, red cells). All proteins shown in colour have been shown to be expressed in methane seep metaproteomes with the exception of MetH and MCM.

common in dissimilatory sulfate reduction and sulfide oxidation, notably, this also included select proteins predicted to have unusual metal cofactors (e.g. tungsten). A manuscript focusing on the trace metal concentrations in methane seep sediments and the occurrence of key metallo-enzymes (including this putative tungsten containing protein) in methanotrophic archaea and sulfate reducing bacteria based on our environmental and Magneto-FISH metagenomes and proteomes was published in 2013 in Environmental Microbiology (Glass et al., 2014 Environ. Microbiol, see Figure 10).

Refinement of the reference database for proteomic searches has been an ongoing process and, with the lower cost of next generation sequencing platforms, we added 3

additional metagenomes sequenced on the Illumina HiSeq platform that originated from sediment used in our microcosm metal and 15N-SIP amendment experiments. The addition of sequences associated directly with the microcosm experiments used for proteomic analysis increased the number of peptides identified, however not all of the best matches were associated with their corresponding sediment, but rather were spread across the 3 new metagenomes. Establishing the most appropriate reference sequence database for environmental proteomics is an ongoing challenge not only for this project, but all metaproteomic studies of complex environments. There is a constant trade-off between maximizing the relevant gene diversity potentially represented in the sample and creating a database that is too large to efficiently search and increases the likelihood of non-unique matches. Tallying the total number of proteins identified across our methane oxidizing sediment environmental proteomes, recovered 22,313 unique proteins in the combined dataset, 74% of those identified were matches to our metagenomic data, 26% were associated with genomes of related cultured microorganisms.
We also explored the impact of varying other search parameters during protein identification. Traditionally, protein identification requires the detection of two constituent peptides, one of which is unique to the protein in question. To evaluate the relationship between detection confidence and the number of proteins identified, we tested four other approaches. The results of these trials – the number of proteins identified and the overlap of protein identifications between two experimental runs of the same protein extract from a methane-oxidizing sediment incubation– are provided in Table 1. Table 1 indicates that the relaxation of the “two peptide rule” is more significant in increasing the number of detected proteins (leading to a 297% increase in detections on average) than a less rigorous false detection rate (which led to an average 24% increase in detections). Incorporating results from 1% FDR–1 unique peptide searches is statistically valid, and we believe that such detections are relevant and should be included in environmental proteomics results. As part of our final publications on the proteomic data, we will discuss the challenges associated with both protein recovery and metagenomic database generation and offer recommendations for optimization with environmental sediment and soil samples.

Table 1: Search parameters and protein identification results.

Search Parameters			Results	
False Detection Rate	# of Peptides Required	# of Unique Peptides Required	Number of Unique Proteins Identified	% of Identified Protein overlap between Run 1 and Run 2
1%	1	0	12013	54.9
1%	1	1	11166	52.4
1%	2	1	2885	51.3
3%	1	1	14134	49.1
3%	2	1	3476	66.3

Using the most conservative of these protein identification protocols (1% FDR–2 peptides–1 unique peptide), a sulfate-based SIP metaproteomics experiment incubated for

160 days with $^{15}NH_4^+$ revealed dozens of enriched proteins (Fig. 11a). When cross-referenced with a specifically tailored metagenomic database, many sulfur- and methane-metabolism gene products were identified, as well as proteins associated with energy production, protein folding, cell division, and outer membrane processes (Fig. 11b). The SIP proteomics methodology allows us to specify which subset of proteins has been synthesized during the experimental period (see next section for additional information). Connecting such information with geochemical data in the case of metal-containing experiments may point to physiological responses to the presence of oxidized metals and imply functional relationships between AOM and metal reduction. From our proteomic investigations of methane oxidizing sediment communities, 29% of the 22,313 total proteins were identified as hypothetical. Interestingly many of the same hypothetical proteins were observed to be expressed across independent AOM datasets, suggesting potential significance in the methane-oxidizing community. Future investigations will focus on a subset of these 'AOM specific' hypothetical proteins, prioritized by expression in datasets and conservation in specific operons.

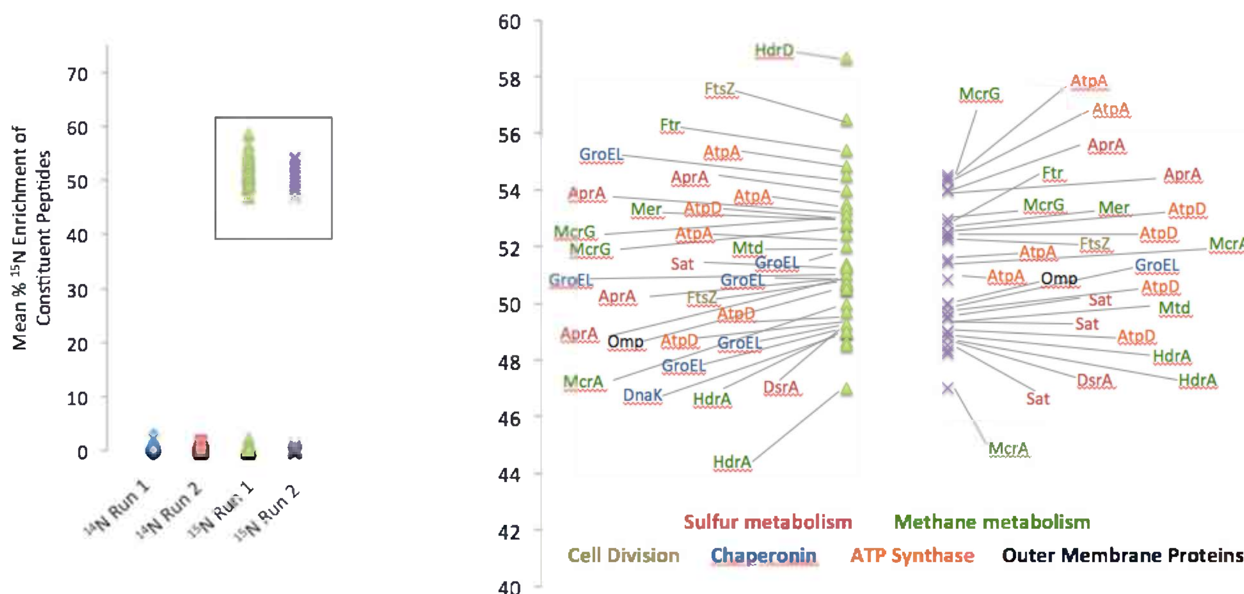
An important aspect of our environmental proteomics work was to test the potential to use 15N labeled substrates for tracking the de-novo synthesis of proteins in these slow growing anaerobic methane-oxidizing consortia. For these experiments, we used methane-oxidizing sediment amended with 15N labeled ammonium, a nitrogen source that was previously shown by PI Orphan (using fluorescence in situ hybridization coupled to secondary ion mass spectrometry (FISH-SIMS) to be actively assimilated by ANME and SRB consortia and represented a robust proxy for cell doubling times. 15N-ammonium labeled sediments and 14N-amended controls from different sediment microcosm experiments were harvested at 2-3 different time points and used for Protein-SIP analysis.

TABLE 2: AOM Sediment Microcosms for 15N protein SIP	label
1: Sulfate as electron acceptor	14NH4
2: Sulfate as electron acceptor	15NH4
3: Fe(OH)3 as electron acceptor	14NH4
4: Fe(OH)3 as electron acceptor	15NH4
5: MnO2 as electron acceptor	14NH4
6: MnO2 as electron acceptor	15NH4
7: No added electron acceptor	14NH4
8: No added electron acceptor	15NH4
9: MnO2 as electron acceptor, with NaMoO4 added as sulfate-reduction inhibitor	15NH4

The earlier time point in the initial set of sediment experiments showed low detectible 15N enrichment in the identified proteins. This sediment was later revealed to have lower rates of methane-oxidation activity compared to other microcosm experiments which helped to explain the low incorporation. A second series of sediment incubations with higher rates of methane oxidation did result in a suite of 15N-enriched proteins relative to the 14N controls, many again associated with enzymes in the methane-oxidation and sulfate-reduction pathways. These experiments, along with a set of 15N amended incubations from the iron and manganese oxide microcosms (9 different treatments, 3 time points (Table 2) are in the process of being extracted using the

modified protein extraction protocol and will be analyzed on the Oribi-Trap Elite instrument in the coming weeks.

Figure 11: Protein enrichment results from a preliminary stable isotope probing (SIP) experiment. In a), the percent of 15N enrichment is shown for both experimental runs of 14N and 15N-incubated samples. b) offers an expanded view of the boxed area from a), with functional annotations. (Marlow et al., in prep.).



6a. Publications to date:

1) Sivan, O, G. Antler, A.V. Turchyn, J.J. Marlow, and V. J Orphan. 2014. Iron oxides stimulate sulfate-driven anaerobic methane oxidation in seeps. Proc. Natl. Acad. Sci USA. www.pnas.org/cgi/doi/10.1073/pnas.1412269111
2) Glass, J.B., Yu, H., Steele, J.A., Dawson, K.S., Sun, S., Chourey, K., Pan, C., Hettich, R.L. & Orphan, V.J., 2014. Geochemical, metagenomic and meta-proteomic insights into trace metal utilization by methane-oxidizing microbial consortia in sulfidic marine sediments. Environ. Microbiology. V16: 1592-1611.
3) Green-Saxena, A., Dekas, A., Dalleska, N. and Orphan, V.J, 2014. Nitrate-based niche differentiation by distinct sulfate-reducing bacteria involved in the anaerobic oxidation of methane. The ISME journal. 8, 150–163
4) Dekas, A.E., Chadwick, G.L., Bowles, M.W., Joye, S.B. and Orphan, V.J., 2014. Spatial distribution of nitrogen fixation in methane seep sediment and the role of the ANME archaea. Environmental Microbiology. 16: 3012-3029.
5) Glass, J. and Orphan, V.J., 2012. Frontiers: Trace Metal Requirements for Microbial Enzymes Involved in the Production and Consumption of Methane and Nitrous Oxide. Frontiers in Microbiological Chemistry, 3.

6) McGlynn, S.E., Boyd, E.S., Peters, J.W. and Orphan, V.J., 2012. Classifying the metal dependence of uncharacterized nitrogenases. Frontiers in microbiology, 3.
7) Dekas, A.E. and Orphan, V.J., 2011. Identification of diazotrophic microorganisms in marine sediment via fluorescence in situ hybridization coupled to nanoscale secondary ion mass spectrometry (FISH-NanoSIMS). *Methods Enzymol*, 486: 281-305.

6a. Conference presentations:

Marlow J, Steele J, Skennerton C, Chourney K, Pan C, Li Z, Hettich R, Orphan V. 2014. Environmental Proetomics of Methane Seeps: An Activity Based Examination of Metabolism and Functional Diversity, International Society for Microbial Ecology, Seoul, South Korea.

Marlow J, Steele J, Skennerton C, Chourney K, Pan C, Li Z, Hettich R, Orphan V. 2014. Environmental Proetomics of Methane Seeps: An Activity Based Examination of Metabolism and Functional Diversity, California Institute of Technology, Geoology Club Seminar Series, Pasadena, California, USA.

Marlow J, Steele J, Skennerton C, Chourney K, Pan C, Li Z, Hettich R, Sivan O, Turchyn A, Antler G, Orphan V. 2014. Clues from Geochemical and Proteomic Investigations of Metal-Linked Anaerobic Methane Oxidation, Ninth International Symposium on Subsurface Microbiology, Pacific Grove, California, USA.

McGlynn S, Chadwick G, Deerink T, Ellisman M, Orphan V. 2014. FISH-EM and CLEM-FISH: Ultra-structure of environmental microbial populations, The Joint Meeting of Japanese Environmental Microbiology, Shizuoka, Japan.

McGlynn S, Chadwick G, Kempes C, Orphan V. 2014. The Spatial Relationship to Metabolic Activity in Syntrophic Communities, Goldschmidt, Sacramento, California, USA.

McGlynn S, Chadwick G, Kempes C, Orphan V. 2014. Spatial Relationships to Metabolic Activity in Syntrophic Communities, The Joint Meeting of Japanese Environmental Microbiology, Shizuoka, Japan.

McGlynn SE, Chadwick G, Kempes C, Deerinck TJ, Chourey K, Pan C, Haroon M, Hettich RL, Ellisman MH, Tyson G, Orphan VJ. 2014. Resolving metabolisms of diverse anaerobic methane oxidizing consortia at the single cell level, 2014 Genomic Science Contractors-Grantees Meeting XII.

McGlynn SE, Chadwick G, Kempes C, Deerinck TJ, Mackay M, Ellisman MH, Orphan VJ. 2014. Structured metabolic relationships within marine microbial aggregates involved in anaerobic methane oxidation, Annual Southern California Geobiology Symposium, Los Angeles, California, USA.

McGlynn SE, Mackey M, Thor A, Chadwick G, Deerinck TJ, Ellisman MH, Orphan V. 2014. Compositional contrasts in sulfur metabolizing bacteria in consortia with ANME archaea, Agouron Sulfur Cycle Symposium, Rancho Palos Verdes, California, USA.

Scheller S, Orphan V. 2014. Microbial Electrolysis Experiments Designed to Decouple Anaerobic Oxidation of Methane from Sulfate Reduction, 11th Annual Southern California Geobiology Symposium, Los Angeles, California, USA.

Scheller S, Yu H, Orphan V. 2014. Probing putative intermediates in the anaerobic oxidation of methane with sulfate via rate measurements and community shift analysis, Gordon Research Conference: C1.

Yu H, Scheller S, Orphan V. 2014. Functional insights into the sulfur-metabolizing genes in methanogenic and methanotrophic archaea, International Society for Microbial Ecology, Seoul, South Korea.

Dekas A, Chadwick G, Connon S, Orphan V. 2013. Investigating the activity and interaction of ANME-SRB aggregates and free-living cells in methane seep sediment via mRNA analysis and FISH-NanoSIMS, Gordon Research Conference: Applied and Environmental Microbiology.

Dekas A, Chadwick G, Fike D, McCay D, Green-Saxena A, Dalleska N, Connon S, Orphan V. 2013. Investigating Benthic N2 fixation at Diverse Deep-Sea Sites, 10th Annual Southern California Geobiology Symposium, Pasadena, USA.

Glass JB, Steele JA, Dawson KS, McGlynn SE, Reinhard CT, Orphan VJ. 2013. Metal micronutrients for anaerobic oxidation of methane, Goldschmidt, Florence, Italy.

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McGlynn S, Boyd E, Peters J, Orphan V. 2013. Classifying the Metal Dependence of Uncharacterized Nitrogenases, Gordon Research Seminar on Bioinorganic Chemistry, Andover, New Hampshire, USA.

McGlynn S, Chadwick G, Orphan V. 2013. Investigating the Metabolic Landscape of Methane Oxidizing Microbial Communities in the Orphan Lab, California Insitute of Technology Micro-Mornings Symposium, Pasadena, California, USA.

McGlynn SE, Chadwick G, Hatzenpichler R, Deerinck TJ, Sun S, Chourey K, Pan C, Hettich RL, Ellisman MH, Orphan VJ. 2013. Resolving cellular metabolisms of diverse anaerobic methane oxidizing consortia at the single cell level, Genomic Science Contractors-Grantees Meeting.

Scheller S, Orphan V. 2013. Microbial Electrolysis Experiments Designed to Decouple Anaerobic Oxidation of Methane from Sulfate Reduction, Gordon Research Conference: Enzymes, Coenzymes and Metabolic Pathways, Waterville Valley, New Hampshire, USA.

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Dekas A, Chadwick G, Fike D, McCay D, Green-Saxena A, Dalleska N, Connon S, Orphan V. 2012. Investigating Benthic N2 fixation at Diverse Deep-Sea Sites Reveals Diazotrophy in the Presence of High Levels of NH4+, International Society for Microbial Ecology, Copenhagen, Denmark.

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cell FISH-NanoSIMS, American Geophysical Union Fall Meeting, San Francisco, USA.

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Glass JB, Adkins JF, Orphan V. 2012. Trace metal bioavailability in methane seep porewaters at Hydrate Ridge: comparisons between clam beds, microbial mats and adjacent seep habitats, Astrobiology Science Conference (AbSciCon), Atlanta, Georgia, USA.

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McGlynn SE, Marlow JJ, Chadwick G, Deerinck TJ, Sun S, Chourey K, Pan C, Hettich RL, Ellisman MH, Orphan VJ. 2012. Correlative Compositional Imaging and Protein Profiling in Marine Anaerobic Methane Oxidizing Microbial Communities, Genomic Science Contractors-Grantees Meeting.

Dekas A, Bowles M, Joye S, Orphan V. 2011. Diazotrophy in the Deep: Combining single cell and bulk geochemical techniques to understand the diversity and significance of benthic nitrogen fixing microorganisms, 8th Annual Southern California Geobiology Symposium, Los Angeles, California, USA.

b. Web site or other Internet sites that reflect the results of this project

N/A

c. Networks or collaborations fostered

Collaboration with Dr. Orit Sivan, Ben Gurion University, Israel

d. Technologies/Techniques

N/A

e. Inventions/Patent Applications

N/A