Final Report for Award #DE-SC0003940

Title of Project: Integrative molecular and microanalytical studies of syntrophic partnerships linking C, S, and N cycles in anoxic environments

Institution: California Institute of Technology

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Principal Investigator: Victoria Orphan

Project Results:

Although small in scale, microorganisms have a profound and fundamental role in the cycling of carbon and energy on our planet. Integral to the success of these diverse and abundant forms of life are complex microbial interactions and syntrophic associations, occurring on the scale of micrometers. We are only now beginning to fully appreciate the diversity and pervasiveness of syntrophy in nature, with the majority of these intimate metabolic interactions occurring between microbial taxa that have yet to be domesticated in the laboratory and thus require the use of new culture-independent molecular and geochemical approaches.

Syntrophic interactions are central to the biogeochemical cycling of carbon in anoxic environments, including the regulation of methane emissions from sedimentary ecosystems. Molecular and isotopic investigations of methane-rich anoxic sediments worldwide have identified unique lineages of uncultured methanotrophic archaea (ANME's) in association with distinct lineages of sulfate-reducing bacteria, who together mediate sulfate-coupled methane oxidation (AOM). Despite their global importance, these cooperative microbial consortia have been difficult to study in the lab and our knowledge of their function and capabilities has been limited. As part of this early career award, we have been focused on developing multi-disciplinary methodologies that incorporate molecular, microscopy and mass spectrometry techniques to advance our understanding of the genetic, phenotypic, and metabolic differences underlying these as yet uncultured methanotrophs and their syntrophic bacterial partners. Specific examples include the further development of a technique known as Magneto-FISH that was initially published and patented by our group in 2008 (Pernthaler et al 2008). In Magneto-FISH, magnetic beads are used to selectively enrich fluorescently-hybridized aggregates of methanotrophic archaeal cells and physically associated bacteria based on their diagnostic 16S rRNA sequence directly from methane-rich sediments. Selective capture of these methane-oxidizing consortia enabled our group to do targeted molecular and metagenomics investigations of the microorganisms and biochemical pathways associated with methane-cycling in the environment (Trembath-Reichert et al. 2013). In our recent publication, Magneto-FISH experiments were designed with combinations of nested FISH probes that differed in taxonomic specificity for methane-oxidizing archaea and associated sulfate-reducing deltaproteobacteria. Captured consortia from each magneto-FISH experiment were then used for high throughput Illumina TAG sequencing and network analysis, leading to the identification of potentially new associations with methanotrophic archaea and between different groups of bacteria in association with sulfate-reducing microorganisms (**Figure 1**, Trembath-Reichert et al., 2016). Targeted

PCR analysis of metabolic genes amplified from the same Magneto-FISH captures additionally enabled correlations to be drawn between 16S rRNA based identity and functional gene diversity of methanotrophs and sulfur-cycling microorganisms in situ.

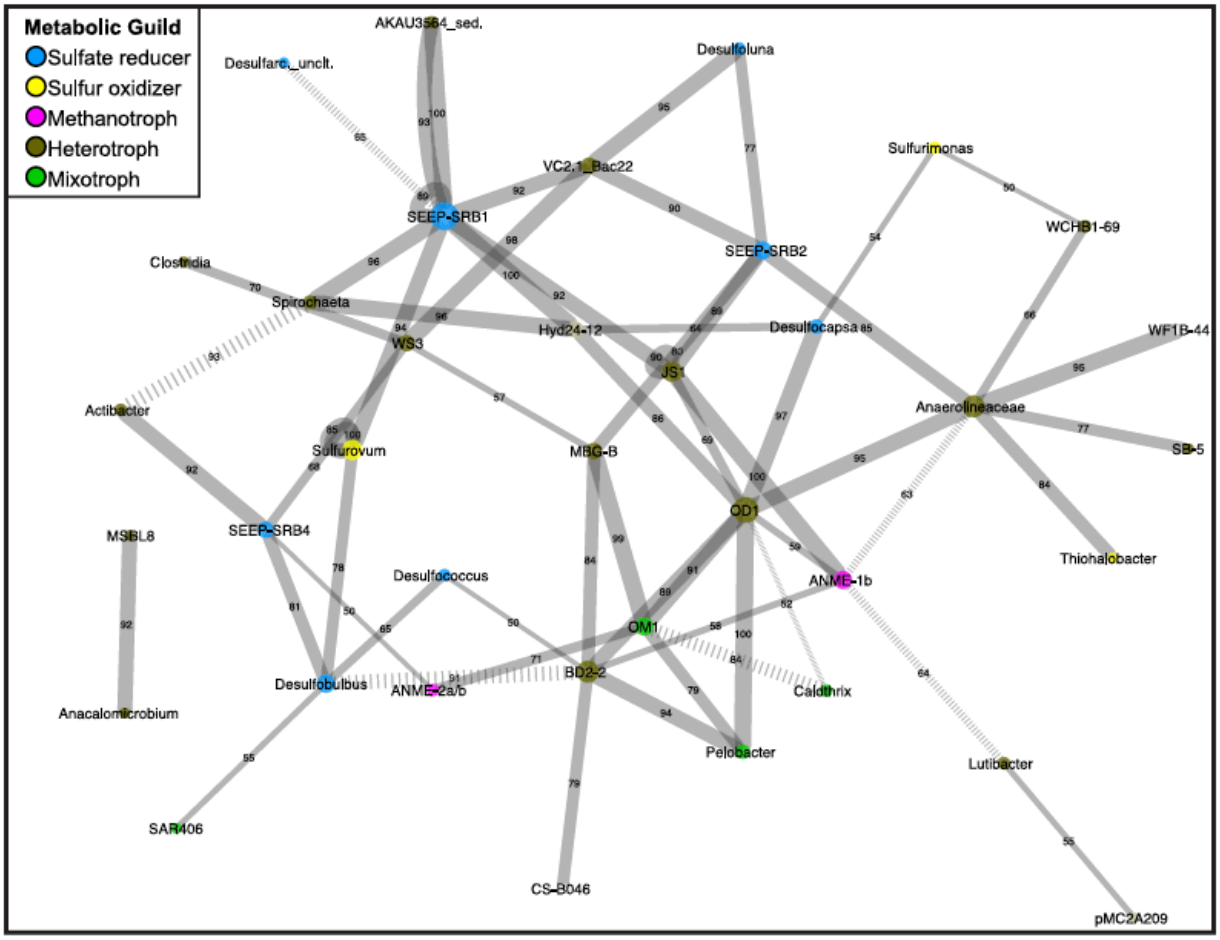


Figure 1: Network diagram of Magneto-FISH and bulk sediment samples. Co-occurrence analysis of the top 135 unique OTUs displayed in network form. Nodes represent the taxonomy of the OTUs in the network and edges are the connections between OTUs. Node size is scaled by number of connecting OTUs and colored by simplified metabolic guild (blue_sulfate reducer, yellow_sulfur oxidizer, pink_archaeal methanotroph, brown_heterotroph, green_mixotroph). Light shading indicates putative assignment. 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. The combined network is displayed using Cytoscape, with the average correlation coefficient across all runs determining the distance between nodes and the number of occurrences in 100 network iterations determining edge width. (from Trembath-Reichert et al., 2016 PeerJ)

The targeted capture experiments using Magneto-FISH are most effective for conducting gene targeted PCR-based amplifications, however quality DNA recovery for full metagenome sequencing was more challenging after the required formaldehyde fixation and MDA amplification which resulted in poor genome assemblies. As an alternative to Magneto-FISH, we have been recently developing methods that target specific populations in natural samples

based on their translational activity using click chemistry approaches (e.g. BONCAT, Hatzenpichler et al 2014 Environ. Microbiology) combined with fluorescence activated cell sorting (FACS). Using BONCAT-FACS on samples showing active methane-oxidation activity, we have successfully sorted and sequenced single AOM consortia without fixation. This new research direction, conducted in collaboration with Tanja Woyke's group at the the Joint Genome Institute, was built upon some of the cell separation protocols and information acquired as part of the early career award research and leveraged to write a successful proposal for the JGI-EMSL FICUS program and DOE-BER Carbon cycling program. Combined with conventional metagenomics analyses of methane-rich sediment samples, we are now poised to conduct comparative genomic analyses for representative single aggregate genomes from all major ANME clades and their associated bacterial partners alongside a suite of reconstructed archaeal and bacterial genome bins recovered from sediment metagenomes. During the course of our metagenomic analysis we recovered two high quality genome bins belonging to novel free living anaerobic members of the Tenericutes (candidate group RF-3 in Greengenes), a bacteria related to mycoplasma. Genome annotation and metabolic predictions from these organisms were used to guide enrichment media design and we were successful in culturing this small fermentative microorganism (named Izimaplasma), who like members of the mycoplasma, lack a cell wall (Skennerton et al., 2016).

The second major component of my early career award was centered around testing hypotheses about the metabolism, interspecies interactions, and environmental adaptation of structurally and phylogenetically diverse AOM consortia using stable isotope labeling experiments coupled with FISH and nanoSIMS ion mapping of syntrophic consortia. High resolution isotopic and elemental analysis using nanometer secondary ion mass spectrometry (nanoSIMS) enables quantitative isotopic and elemental analysis of single microbial cells in environmental samples (e.g. Dekas and Orphan et al 2011). A major goal of our work was to optimize methods that directly couples molecular visualization methods including phylogenetic 'stains' (e.g., rRNA targeted fluorescence in situ hybridization, FISH) and immunocytochemical localization of expressed metabolic proteins (for example methyl coenzyme M reductase; dissimilatory (bi) sulfate reductase; and nitrogenase) with stable isotope analysis of individual microorganisms by FISH-nanoSIMS while also maintaining the critical spatial information within structured AOM consortia. Many of the designed antibodies unfortunately were not successful in our system and this component of our research is still ongoing. However, we were able to make substantial progress in our understanding of nutrient limitation and nitrogen acquisition by methane-oxidizing microbial communities using single cell stable isotope probing techniques (e.g. Dekas et al. 2013 and 2015). Microcosm experiments were designed to test the N2 fixation potential of methane-oxidizing consortia in sediments combined with FISH-nanoSIMS and transcriptomic analysis. These data demonstrated the diazotrophic potential of select sulfate-reducing bacterial groups in addition to the methane oxidizing ANME-2 archaea (**Figure 2**, Dekas et al., 2015). However, in all cases, N2 fixation was only observed to occur in the presence of methane (**Figure 2, 3** Dekas 2013, 2015). In some cases, nitrogen fixation was observed to occur in the presence of 50-150 μM porewater ammonium, suggesting that the regulation of nitrogen fixation by these anaerobic microorganisms may not be as tightly controlled compared to the well-studied aerobic model diazotrophic cyanobacteria and azotobacter.

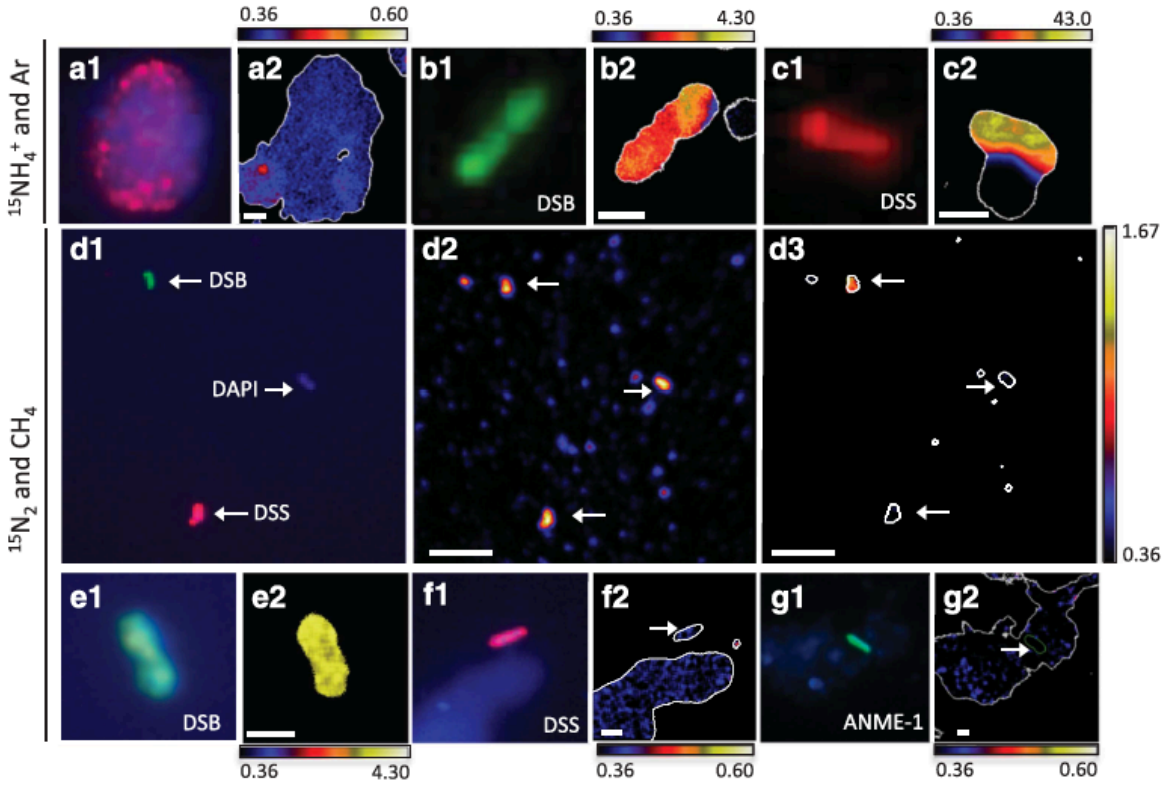
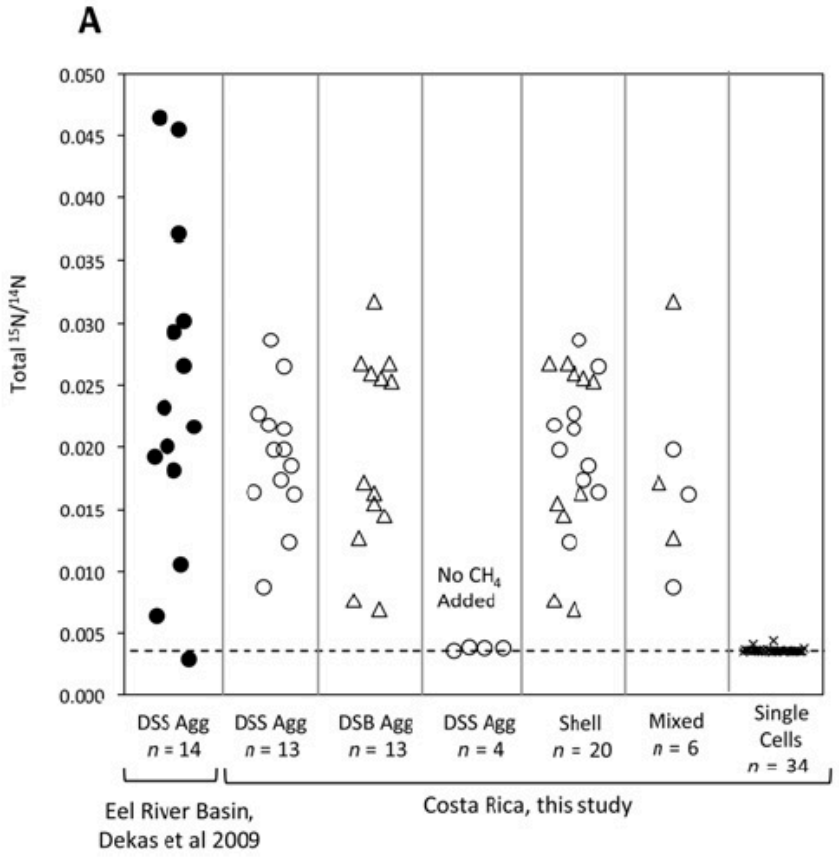


Figure 2: Paired CARD-FISH and NanoSIMS images of sediment archaea and sulfate-reducing bacteria single cells and aggregates incubated with 15NH4+ and Ar (a–c) or 15N2 and CH4. CARD-FISH images show DSB (probe seepDBB_653), DSS (probe DSS_658) and ANME-1 (probe ANME-1_350), counterstained with DAPI (blue) as indicated. (a1) An ANME-2-DSS aggregate with probe DSS_658 in red. a2, b2, c2, d3, e2, f2, g2: NanoSIMS images of the same cells show their isotopic (atom % 15N) composition. (d2) A 12C− ion image of the same cells in (d1 and d3), with 12C− counts ranging from 0 to 900 per pixel.(modified from Dekas et al 2015, ISME J).

Figure 3:: Nitrogen 15N cell enrichment from FISH-nanoSIMS study of ANME-2/SRB consortia and single cells after sediment incubation with 15N dinitrogen. Each data point represents the 15N/14N ratio of the total nanoSIMS CN− secondary ions collected from a single ANME-2/SRB aggregate or single cell. Aggregates of ANME-2 associated with Desulfosarcina/ Desulfococcus (DSS, identified using oligonucleotide probe DSS_658) are depicted as circles, aggregates of ANME-2 associated with members of the Desulfobulbaceae (DSB, identified using oligonucleotide probe seepDBB_653) are depicted as triangles. Single cells with unknown phylogenetic identity were localized by DAPI-staining. Data demonstrates that N2 fixation within methane-rich sediments is conducted by methanotrophic ANME-2 archaea in association with different groups of sulfate-reducing bacteria and occurs in the presence of methane (Modified from Dekas et al 2013, Environ. Microbiol.).



Deciphering the influence of spatial organization on metabolic activity in syntrophic consortia

The majority of syntrophic microorganisms form close physical associations derived from their metabolic interdependence. While there are a number of co-culture model systems in which to study these metabolic interactions, it is much harder to discern syntrophic associations and other forms of metabolic interactions in the environment. As part of this research program, we further optimized the spatial resolution of FISH-nanoSIMS for multi-celled consortia and biofilms using embedding thin sectioning techniques, and, developed high resolution correlated fluorescence and ion images of individual methane-oxidizing and sulfate-reducing bacterial consortia (**Figure**

4, McGlynn et al 2015 Nature). Well curated maps of biosynthetic activity (nanoSIMS isotope assimilation) and cell identity (FISH) were then analyzed with a suite of geostatistical techniques to assess whether predicted patterns of enhanced microbial activity with decreased interspecies spatial distance occurred. The lack of correlation between cell-cell distance and anabolic activity was unanticipated and counter to conventional models of syntrophy based on molecular diffusion. Guided by these findings, we looked to the genomes of these organisms for clues relating to their syntrophic interaction and uncovered genes predicted to encode S-layer associated large multi-heme cytochromes, similar to those used in extracellular electron transfer (McGlynn et al 2015). Working with colleagues at Caltech, we developed alternative reaction-transport toy models to simulate spatial patterns of microbial activity in syntrophic associations based on direct electron transfer or through molecular diffusion of common syntrophic substrates such as hydrogen, formate, and acetate. These models supported our empirical results from the single cell ANME and sulfate-reducing bacteria activity patterns in consortia, resulting in a new hypothesis of AOM syntrophy based on extracellular electron transfer between methane-oxidizing ANME-2 archaea and sulfate-reducing bacteria (McGlynn et al., 2015).

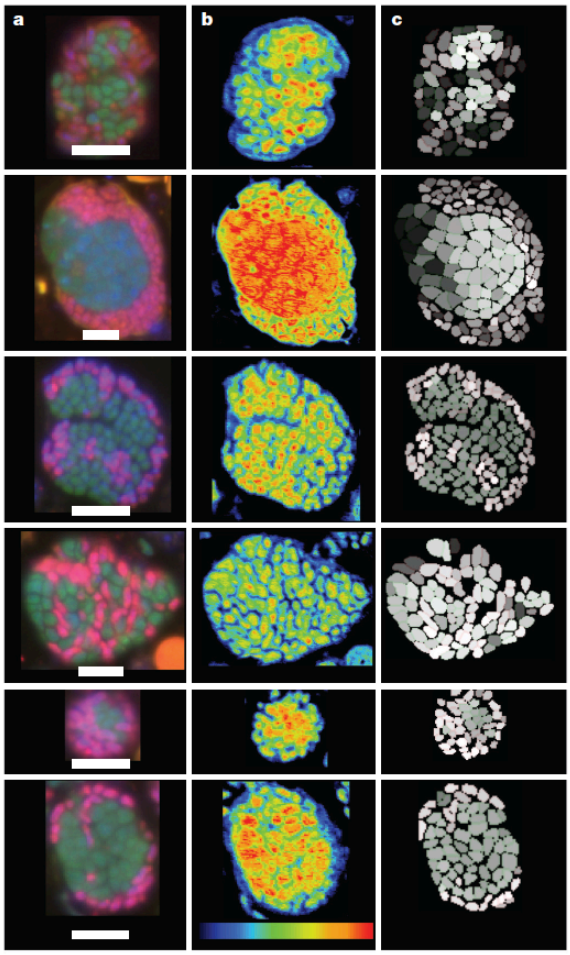


Figure 4: Examples of AOM consortia identified by FISH and paired anabolic activity measurement via nanoSIMS. a, FISH-identified consortia showing archaeal cells (green) and Deltaproteobacteria (pink). The top two panels represent consortia of ANME-2c or 2b paired with Deltaproteobacteria. The lower four panels show ANME-2c archaea paired with the seep-specific deltaproteobacterial group, SEEP-SRB1a. Scale bars, 3 **m**m. 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 with higher levels of anabolic activity and 15NH4 assimilation. Representative aggregates were chosen from the larger data set composed of 62 aggregates. (From McGlynn et al., 2015 Nature)