Sulfur-Cycling in Methane-Rich Ecosystems: Uncovering Microbial Processes and Novel Niches

Thesis by
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Conclusions

The work presented in my thesis encompasses two unique ecosystems, terrestrial mud volcanoes and marine methane seeps. The uniting factor leading to their investigation is that both ecosystems are rich in methane, a potent greenhouse gas. Understanding the *in situ* microbial communities and processes can help us to understand what keeps these critical ecosystems in balance. I therefore chose to focus on the sulfur-cycling microorganisms, a prominent and diverse assemblage of microorganisms, in an attempt to uncover novel niches and processes, which would likely be crucial to the functioning of these ecosystems.

Sulfur cycling in terrestrial mud volcanoes has been largely unexplored and here we provided a comparative view of the microbial communities and processes involved in sulfur cycling in multiple terrestrial mud volcanoes of Azerbaijan. Further, mud volcanism in Azerbaijan is one of the controlling factors for the vast oil and gas fields in this area; thus elucidating microbial processes in this region is important as these processes can play an important role in the degradation of hydrocarbon inside the reservoirs. Functional gene phylotypes affiliated with sulfur-oxidizing bacteria (SOB) revealed putative chemosynthetic metabolisms including sequences clustering with obligate aerobes and facultative anaerobes collectively capable of respiring a variety of sulfur species to the with oxygen or nitrate. In addition to finding strong evidence for viable thermophilic sulfate-reducing bacteria (SRB) in this non-thermic mud, we also found that SRB may potentially (based on related cultured phylotypes) be divided into niches based on their carbon oxidation pathways, with incomplete carbon oxidizers potentially preferring gryphon features and the shallow waters of salse lake features. Together these data represent a diverse array of previously unexplored microbial metabolisms in these mud volcanoes. We also found FISH and rate measurement evidence for the anaerobic oxidation of methane coupled to sulfate reduction, a process which we explored further in the more tractable marine methane seeps.

The anaerobic oxidation of methane, serving as a sink for this potent greenhouse gas, is a crucial biogeochemical process and yet little is known about the dynamics that exist between the two partners which effect this process: SRB and anaerobic methane-oxidizing archaea (ANME). In order to study these dynamics we inhibited sulfate reduction and followed the metabolic processes of the microcosm community as well as the effect of aggregate composition and growth on a cellular level using FISH coupled to nanoscale secondary ion mass spectrometry (FISH-NanoSIMS). We revealed that while bacterial cells appear to decay, ANME cells persist in the form of ANME-only aggregates, which are capable of little to no growth when sulfate reduction is inhibited. These data are the first to show what happens to the ANME/SRB consortia growth on a cellular level when sulfate-reduction is inhibited. We also explored factors influencing the diversity of SRB in association with ANME.

Very little is known about factors influencing the distribution and fitness of distinct sulfate-reducing bacteria partnered with anaerobic methane-oxidizing archaea (ANME). Poorly constrained ecological and physico-chemical factors are almost certainly important to this symbiosis as a whole, and present a unique opportunity to uncover additional environmental regulators of sulfate-dependent methane oxidation. We applied a combination of molecular, geochemical and FISH-NanoSIMS analyses of *in situ* seep sediments and methane-amended sediment incubations from diverse locations (Eel River Basin, Hydrate Ridge and Costa Rican Margin seeps) to investigate the distribution and physiology of a newly identified subgroup of the Desulfobulbaceae (seepDBB) found in consortia with ANME-2c archaea, and compared these to the more commonly observed associations between the same ANME partner and the Desulfobacteraceae (DSS). By first investigating the geochemical parameters that co-occur with higher abundances of ANME/seepDBB we uncovered a novel positive correlation between these aggregates and nitrate concentrations. This relationship with nitrate was experimentally confirmed using sediment microcosms, in which the abundance of ANME/seepDBB was greater with the addition of nitrate relative to the unamended control. Additionally, FISH-NanoSIMS revealed significantly higher ^{15}N-nitrate incorporation levels in individual aggregates of ANME/seepDBB relative to ANME/DSS aggregates from the same incubation. These combined results suggest that nitrate is a geochemical effector of ANME/seepDBB aggregate distribution, and may provide a unique niche for these consortia through the utilization of a greater range of nitrogen substrates than the ANME/DSS.

In sum these data uncover novel aspects of the sulfur-cycling microbial communities in two crucial ecosystems rich in natural methane stores. We demonstrated that sulfur oxidation has the potential to proceed both aerobically and anaerobically in terrestrial mud volcanoes. This knowledge helps us understand under what conditions sulfate may be formed to support the active SRB community, some members of which we revealed are likely capable of anaerobic methane oxidation (AOM). We further explored the symbiosis responsible for AOM in marine methane seeps and were able to show just how crucial sulfate reduction is to this symbiosis by inhibiting sulfate reduction and recording a lack of growth in the ANME cells. We then revealed that the unexplained diversity in SRB associated with ANME cells can be at least partially explained by preferential nitrate utilization by one particular partner, the seepDBB. This discovery reveals that nitrate is likely an important factor in marine methane seep ecosystem functioning.

The combination of geochemical and FISH-NanoSIMS work presented here has not only uncovered novel microbial niches, it has also honed these methods for future work in the field. Our combination of fieldwork, *in situ* geochemical measurements, and FISH-NanoSIMS investigations of microcosm incubations allows for a comprehensive view of the microorganisms both in their native environment and in experimental settings. The detailed study of these microorganisms in their native ecosystem allows for a deep understanding of the factors which affect them and their ecosystem and also can generate hypotheses which are testable not only by bulk rate measurements of microbial processes but also at a cellular resolution via manipulated microcosms. Studies such as these allow for a more complete understanding of not only *in situ* communities and processes but also novel factors that may be central to the ecosystem and yet were heretofore unknown.

On a more personal level these studies have led to my development as a scientist and microbial ecologist. I chose to come to graduate school at the California Institute of Technology because I firmly believe that with the right technology and understanding we can begin to restore balance to ecosystems that have been nearly destroyed by global change. With an enlivened mind and a full toolbox I now plan to continue on that journey.

Author Publications (during PhD)

1. Dekas, A.E., Chadwick, G., Connon, S.A., Fike, D., McCay, D., **Green-Saxena, A**., Dalleska, N., and Orphan, V.J. (in prep) Investigating the Biogeochemical Controls on Deep-Sea Diazotrophy and Its Significance to Local and Global Ecosystems

2. **Green-Saxena, A.,** Dekas, A.E., and Orphan, V.J. (in prep) Effects of the sulfate reduction-inhibitor molybdate on anaerobic methane oxidizing community metabolism, ANME/bacterial aggregate composition and cell growth

3. Trembath-Reichert, E., **Green-Saxena, A**., Poretsky, R., Orphan, V.J. (in review) Whole cell magnetic enrichment of environmental microbial consortia using rRNA targeted Magneto-FISH

4. **Green-Saxena, A.,** Dekas, A.E., Dalleska, N., Orphan, V.J. (in review) Nitrate-based niche differentiation by distinct sulfate-reducing bacteria involved in the anaerobic oxidation of methane.

5. Yang, S., Matsen, J.B., Kanopka, M., **Green-Saxena, A.**, Clubb, J., Sadilek, M., Orphan, V.J., Beck, D., and Kalyuzhnaya, M.G. (2013) Global molecular analyses of methane metabolism in methanotrophic Alphaproteobacterium, *Methylosinus trichosporium* OB3b. Part II. metabolomics and 13C-labeling study. *Frontiers in Microbiology* **4**.

6. **Green-Saxena, A.,** Feyzullayev, A., Hubert, C.R.J., Kallmeyer, J., Krueger, M., Sauer, P., Schulz, H.-M. and Orphan, V.J. (2012, Journal Cover) Active sulfur cycling by diverse mesophilic and thermophilic microorganisms in terrestrial mud volcanoes of Azerbaijan. Environmental Microbiology 14(12): 3271-3288

7. Bailey, J.V., Raub, T.D., Meckler, A.N., Harrison, B.K., Raub, T.M.D., **Green, A.M.,** Orphan, V.J. (2010) Pseudofossils in relict methane seep carbonates resemble endemic microbial consortia. Palaeogeography, Palaeoclimatology, Palaeoecology 285 131-142

8. Orphan, V.J., Turk, K.A., **Green, A.M**., House, C.H. (2009) Patterns of 15N assimilation and growth of methanotrophic ANME-2 archaea and sulfate-reducing bacteria within structured syntrophic consortia revealed by FISH-SIMS. Environmental Microbiology 11:1777-1791