Microbial community stability in anoxic sediments under conditions of shifting salinity, oxygen, and sulfate

Libusha Kelly
MIT
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Abstract:

Microbial interactions in anoxic communities play a role in the construction and maintenance of many environments, including marshes, wastewater treatment plants, and the human mouth. To explore the factors that cause community shifts and those that encourage stability in microbial communities, we enriched the anaerobic microbial populations from two anoxic sediments under conditions of high and low salinity. After a preliminary enrichment phase, we further perturbed a subset of enrichments by amendment with sulfate and oxygen. Physiological and genetic analysis indicated that the initial inocula from both sites contained a diverse community of bacteria and archaea, including expected members like sulfate reducing bacteria, methanogens, and acetogens. Our results suggest that oxygen had the most destabilizing effect of all perturbations on the methanogens and acetogens in the enrichments; sulfate amendment did not impact community composition and activity as severely. Salinity appeared to have an affect on community composition and response; the saltwater incubations were consistently more affected under perturbation than the freshwater enrichments. Finally, the sulfate reducing bacterial populations were more stable to perturbation generally than methanogen and actogen populations.

Introduction:

Shifts in anaerobic microbial community structure can affect economically relevant systems, such as the performance of wastewater treatment facilities and human health. Changes in populations of acetogens, methanogens, and sulfate reducing bacteria (SRB) are associated with process performance changes in wastewater treatment plants 3. Populations of similar organisms shift in periodontal disease; patients with disease show evidence of methanogen and SRB community members in their plaque biofilms while healthy controls have only acetogens 1. In marshes, these three groups of organisms co-exist in a dynamic environment that is subject to perturbation.

These observations prompted the following questions: What prompts shifts in these populations? Are shifts in consortia predicable? How rapidly do they occur? Is population structure similar between populations in similar but distinct environments or is there wide variation in community members? Here, we describe a physiological and genetic characterization of microbial consortia in anoxic sediments.
We started by enriching sediment samples from two local sites, the Little Sippewisset Marsh and the School Street Marsh, under high and low salt conditions. We then perturbed some of the enrichments with sulfate addition and oxygen addition and examined community response. Community composition was characterized using clone libraries constructed from samples from each site and with 454 16s sequencing for samples and amended samples from each site that were collected independently from this work. Estimates of acetogen activity were measured using acetate production. Methane production, a proxy for methanogen activity, was measured using gas chromatography. The numbers of SRBs and methanogens in all enriched environmental samples was quantified with qPCR using the marker genes mcrA and dsrA. The effort described here is a small step towards understanding the factors that prompt community shifts and those that encourage stability in microbial communities.

Results:

Clone library and 454 sequencing 16s analysis:
The clone libraries yielded 71 sequences for the SSM sample and 60 sequences for the LSM sample with identifiable taxonomy. A comparison with 454 sequencing results showed some discrepancies in the distribution of the dominant groups represented in both datasets; specifically the Bacteroidetes were represented more in the 454 data than the clone libraries. Cyanobacteria, which were more abundant in LS than SS in the clone libraries, showed the opposite distribution in the 454 data. Euryarchaeota were more represented in the clone libraries for both sites than in the 454 data. Estimates of the most dominant taxa, the Proteobacteria, were similar across both sequencing sets (Figure 2).

The LSM and SSM samples show no obvious clustering of taxa based on a tree constructed from an 80% clustering of all clone library sequences from both sites and the three nearest isolate hits from the GreenGenes database core sequences as of 7/26/10. The inner ring is colored by taxon, archaea are shown in red and bacteria in blue. The outer ring is colored by isolation site, with SSM shown in dark blue, LSM in pink and previously sequenced isolates from the GreenGenes database shown in gray. There are some small site-specific clusters (in the archaeal set, for example) but most of the LSM and SSM sequences are scattered throughout the tree (Figure 3).

Finally, a hierarchical clustering by site and by organism for the 454 16s data revealed that the SSM inoculum and its enrichment with $H_2/CO_2/Na_2SO_4$ were more similar to each other than the LSM inoculum and enrichment. Certain organisms, such as the Chloroflexi and Euryarchaeota, have similar distributions across the four sites and therefore cluster together (Figure 4).

DNA extraction from sediment samples:
Three T0 DNA extractions were performed for LSM and SSM. DNA yields averaged approximately 30 ng/uL. Twenty DNA extractions for the enriched cultures were
obtained on July 15, one day before the spike perturbations. DNA yields ranged from 3.1 ng/μL to 7.9 ng/μL with an average of 5.1 ng/μL per sample. Twenty DNA extractions were performed five days after the spike; these yielded on average 7.4 ng/μL of DNA per sample. The methanogen standard yielded 112.7 ng/μL of DNA and the SRB standard yielded 57.4 ng/μL.

**Acetate production in enrichment cultures:**
Acetate production was greater in freshwater conditions than saltwater. The oxygen amendment (+air) had the most severe effect of the perturbations and was greater in saltwater than in freshwater for both the LSM and the SSM samples (Figure 5).

**Methane production in enrichment cultures:**
All four of the enrichments amended with sulfate spikes recover and continue to produce methane. The enrichments with oxygen amendment do not achieve growth levels similar to either the sulfate spike enrichments or the H₂/CO₂ positive control. Enrichments started with sulfate in them produce less methane in saltwater than in freshwater for both isolation site inocula. The N₂/CO₂ negative controls stay very low (gray), the H₂/CO₂ positive control (blue) is slow to come up in the freshwater amendment but is high (as expected) in the other three sets of enrichments (Figure 6).

**Quantification of methanogen and SRB populations in enrichment cultures:**
In comparing the pre-spike and post-spike counts of methanogens and SRBs, the SRBs appear more stable and less prone to fluctuation at the end point of the experiment per sample no matter what the conditions of the enrichment. Consistent with expectations, the N₂/CO₂ controls did not change much over the course of the experiment, suggesting limited growth in those enrichments. The sulfate spike retards methanogen growth more in saltwater than freshwater, again suggesting that salinity is an important factor in community composition in anoxic sediments, however the oxygen spike had a less apparent impact on the methanogens. The SRB community was very stable to perturbation, neither the oxygen amendment or the sulfate influx affected the numbers of SRBs; it was expected that sulfate amendment would increase the SRB population and that oxygen would retard it (Figure 7).

SRBs and methanogens are proposed to compete in microbial communities; we therefore expected to see anticorrelation of SRBs and methanogens in the samples. In fact, we see some anticorrelation in the pre-spike samples (labeled “before”) but by the end of the experiment the two communities no longer show this pattern (Figure 8).

**Discussion:**
In sum, the results presented here suggest that the SRB community in anoxic sediments is less affected by oxygen, sulfate and salinity than are methanogens or acetogens. Salinity has an impact on the ability of both methanogen and acetogen populations to respond to oxygen and sulfate spikes. This is particularly interesting because the LSM is actually a very saline environment, and this result was
unexpected. Finally oxygen appears to be a stronger stress on methanogen and acetogen populations than sulfate amendment (Figure 9).

Comparisons of the 454 16s data for pre- and post-enrichment clearly demonstrate changes in the overall community structure. As one example, the *Desulfovibrionales* increase more than 1000-fold between the starting inoculum and the enriched culture. Examining these community changes may be useful in considering about how to set up culture conditions to capture rare community members of interest.

Clearly, the results presented here are limited and further work needs to be done to better assess the community structure and response of anoxic sediments in the Little Sippewisset and School Street Marshes to environmental stresses and changes. I hope that this work has at least provided some new ideas and directions for future members of the microbial diversity course.

**Materials and Methods:**

**Sample collection:**

Anoxic sediment samples were collected at Little Sippewisset Marsh (LSM, July 6, 2010, water temperature 30°C, salinity 34%) and School Street Marsh (SSM, July 7, 2010, temperature 34, salinity 4%).

**Community enrichments:**

For both samples, approximately 1g of soil was transferred into each of 20 glass bottles which were prepared according to Figure 1, with each site having ten bottles with different conditions. Briefly, anoxic salt water and fresh water media was prepared according to the lab manual. Samples from SSM and LSM were added to ten total bottles with conditions as follows. Five bottles were salt water-based and five were freshwater-based. Bottles one through three were amended with H₂/CO₂, bottle four was amended with 10mM (freshwater) or 28mM (saltwater) Na₂SO₄, and bottle five was a negative control amended with N₂/CO₂. Bottle one was a control and was maintained with H₂/CO₂ for the duration of the experiment. Bottles two and three were the perturbation condition experiments; bottle two was amended with 10mM (freshwater) or 28mM (saltwater) Na₂SO₄, and bottle three was exposed to air.

**Community 16s RNA analysis (T0):**

DNA was extracted from 0.25g of soil per site using the PowerSoil DNA Isolation Kit (MO BIO). DNA concentration was quantified with the NanoDrop spectrophotometer (Thermo Scientific). Clone libraries were constructed for each site at T0 from unamended sediment samples from LS and SS were prepared using the universal primers U391R and U515F to generate PCR products that were cloned into the TOPO2 TA Cloning Kit (Invitrogen). Independently of this project, 454 16s
data was collected for course use from samples taken at LS and SS as well as for samples enriched with H₂/CO₂/Na₂SO₄.

The RDP classifier as implemented in the Qiime pipeline for community microbial analysis tool was used to assign taxa to all sequences. Trees were built using the GreenGenes online NAST aligner tool to align sequences with the core set of 16s alignment templates. Sequences were filtered using the filter_alignments.py protocol in Qiime with lane masking enabled. Trees were visualized using iTOL. Heatmaps of community composition were generated using the heatmap2 package in the R statistical software environment (R Development Core team 2010: http://www.R-project.org).

Acetate and Methane measurements in enrichment cultures
Acetate production was measured with HPLC, a standard curve for acetate production was calculated with a water standard, 5mM, and 10mM preparations of fatty acids. Methane production was measured using gas chromatography; a standard curve was calculated to link the area under the methane peak to mM concentrations of methane. Calculations were normalized to headspace volume.

Quantification of SRB and methanogen populations in enrichments
Previously published primers for the marker genes dsrA (SRBs) and mcrA (methanogens) were used to quantify SRB and methanogen populations at three time points using the iQ SYBRGreen Supermix kit (Bio-Rad) (Table 1). Primers were diluted to 15uM; 3uL of a 1:10 dilution of template was added to each reaction. Conditions were as follows: 95° for 5 minutes. 35 cycles of (95 for 30s, 60 for 1min, 73 for 1min) and a melt curve from 6-90 +0.3 per cycle. All measurements were done in triplicate. The dsrA standard was an enrichment culture of a sulfate reducing bacteria. The mcrA standard was an enriched methanogen culture. Both cultures were kindly provided by Gargi Kulkami. Standard PCR was run on both cultures according to the protocol in the lab manual with the corresponding primers; amplification bands were seen for both standards.

Copies of each of the target genes per microliter were calculated by absolute quantification with a calibration curve calculated based on the known amounts of DNA in the standard samples for mcrA and dsrA. These quantities are log-transformed in all plots.

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Table 1: Primers for quantification of methanogen (mlas/mcrA) and sulfate reducing bacteria (DSR1-F+/DSR-R) in anaerobic enrichments.
Acknowledgements:
I would like to thank Penny Chisholm for being a great mentor as well as an extraordinary scientist and for letting me take 6+ weeks out of the lab to attend this course. Dan and Steve, thanks for being amazing fearless leaders and for letting us all chase down crazy ideas in our projects. Bekah (the unflappable), Heather, Chu(n)ck, Annie, and Gargi, this course would never have worked without all of you. I am indebted for all of your help and for your ceaseless encouragement. MD Class of 2010, I learned a tremendous amount from all of you and I am honored to have worked alongside you.
**Figure 1: Experimental conditions.** Twenty anaerobic enrichments (ten freshwater, ten saltwater) were prepared from sediment samples collected at Little Sippewisset Marsh and School Street Marsh. Bottles marked “SPIKE” were amended approximately a week after the enrichments were started.
Figure 2: Comparison of community composition at SSM and LSM using clone libraries and 454 sequencing. Bacteroidetes were represented more in the 454 data than the clone libraries. Cyanobacteria, which were more abundant in LS than SS in the clone libraries, showed the opposite distribution in the 454 data. Euryarchaeota were more represented in the clone libraries for both sites than in the 454 data. Estimates of the most dominant taxa, the Proteobacteria, were similar across both sequencing sets.
Figure 3: Phylogenetic tree of SSM and LSM clone library 16s sequences. The LSM and SSM samples show no obvious clustering of taxa based on a tree constructed from an 80% clustering of all clone library sequences from both sites and the three nearest isolate hits from the GreenGenes database core sequences as of 7/26/10. The inner ring is colored by taxon, archaea are shown in red and bacteria in blue. The outer ring is colored by isolation site, with SSM shown in dark blue, LSM in pink and previously sequenced isolates from the GreenGenes database.
shown in gray.

Figure 4: Hierarchical clustering by taxa and sampling sites based on 454 sequence data. The SSM inoculum (School) and its enrichment with H₂/CO₂/Na₂SO₄ (SchoolE) were more similar to each other than the LS inoculum (LittleSip) and enrichment (LittleSipE). Certain organisms, such as the Chloroflexi and Euryarchaeota, have similar distributions across the four sites and therefore cluster together. Figure generated with the heatmap2 script from the gplots package in R, data are scaled by row.
Figure 5: Acetate production in enrichments. Acetate production was greater in freshwater conditions than saltwater. The oxygen amendment (+air) had the most severe effect of the perturbations and was greater in saltwater than in freshwater for both the LSM and the SSM samples.
Figure 6: Gas chromatography measurements of methane production in enrichments. All four of the enrichments with the sulfate spikes (green bars) recover. The enrichments with oxygen amendment (orange) do not achieve growth levels similar to either the sulfate spike enrichments or the H2/CO2 positive control (blue). The enrichments that were started with sulfate (red) appear to produce less methane in saltwater than in freshwater. The N2/CO2 negative controls stay low (gray), the H2/CO2 positive control (blue) is slow to come up in the freshwater amendment.
Figure 7: Quantitative analysis of methanogens and SRB in enrichments. The SRBs appear more stable and less prone to fluctuation at the end point of the experiment per sample no matter what the conditions of the enrichment. Consistent with expectations, the N2/CO2 controls did not change much over the course of the experiment, suggesting limited growth in those enrichments. The sulfate spike retards methanogen growth more in saltwater than freshwater, again suggesting that salinity is an important factor in community composition in anoxic sediments, however the oxygen spike had a less apparent impact on the methanogens.
Figure 8: Comparative analysis of methanogen and SRB in enrichments. SRBs and methanogens are proposed to compete under some environmental conditions. We see some anticorrelation in the pre-spike samples (labeled “before”) but by the end of the experiment the two communities no longer show this pattern.
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**Figure 9:** Relative affects of perturbation on anoxic sediment communities.
References:


