Shifts in the relative abundance of ammonia-oxidizing bacteria and archaea in the water column of a stratified marine environment, Salt Pond

Sarah Hurley
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Department of Earth and Planetary Sciences, Harvard University
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Abstract:
Salt Pond, a seasonally stratified kettle pond in Falmouth, Massachusetts, provides an ideal analogue for the Black Sea and other stratified marine environments. Nitrification in stratified water columns and oxygen minimum zones (OMZs) connects organic matter remineralization to the anammox and denitrification, the two known mechanisms of nitrogen loss in marine systems. We determined the effect of physiochemical gradients through the chemocline in Salt Pond on ammonia-oxidizing archaea and bacteria using amoA, the functional gene encoding ammonia monooxygenase subunit A. Ammonia oxidizing archaea (AOA) dominated the fresh, oxic surface water with low ammonium concentrations. Ammonia-oxidizing bacteria (AOB) abundance increased through the chemocline into the euxinic, but potentially micoraerophilic deep water. Within the AOA community, sequences were most related to cultured and uncultured putative AOA and grouped into surface, shallow, and deep clusters. The results of this study offer insights into the ecology of AOA and AOB by comparing the environmental conditions in which these groups are numerically dominant.
Introduction

Nitrogen is an essential and often limiting nutrient fundamental to the structure and biochemistry of life. Nitrogen is readily oxidized or reduced and serves as both an electron donor and accepter in energy metabolisms of microorganisms. Microbial metabolisms drive the formation and consumption of different chemical form of nitrogen and therefore drive marine nitrogen biogeochemical cycling.

Nitrification, the stepwise oxidation of ammonium to nitrite and nitrate, is a key process the marine nitrogen cycle responsible for the formation of the large deep-sea nitrate reservoir (Figure 1). Nitrification is a microbially mediated process, performed mostly by chemolithotrophic microbes who glean energy and electrons from this oxidation. Ammonia released from the remineralization of particulate organic nitrogen (PON or PN) is oxidized to substrates for denitrification and anammox. Nitrification therefore connects the recycling of organic nitrogen to the only two presently known nitrogen loss processes.

In productive equatorial and coastal upwelling zones, high fluxes of organic matter and remineralization create oxygen minimum zones (OMZs), enabling denitrification (Hogslund et al., 2008) and anammox (Hamersley et al., 2007). Remineralization releases large amounts of ammonium, driving the high nitrification rates often associated with OMZs. Nitrogen loss from OMZs are estimated to account for 30-50% of total nitrogen loss from the oceans (Gruber & Sarmiento, 1997).

The seasonally meromictic kettle pond, Salt Pond, in Falmouth Massachusetts provides an ideal model system to study nitrogen cycling processes through an oxygen gradient. The water column in Salt Pond closely resembles the chemical and oxygen gradients typically associated with large marine anoxic basins such as the Black Sea (Damste et al., 1993). The water column in these systems consists of an oxic surface layer, sulfidic and anoxic deep waters, and consistent concentrations of chemical species along isopycnals through the transition zone, or chemocline. Studies of nitrification processes, such as ammonia oxidation, in Salt Pond can there be translated to OMZs and larger stratified marine systems.
Ammonia oxidation, the first step in nitrification, is considered rate-limiting and nitrite rarely accumulates in natural environments (Schleper & Nicol, 2010). Traditionally, ammonia oxidation was thought to be catalyzed by Nitrosomonas and Nitrospira in the betaproteobacteria (Head et al., 1993), and Nitrosococcus in the gammaproteobacteria (Purkhold et al., 2000; Ward & O'Mullan, 2002). Metagenomic studies provided the first evidence for the metabolic capacity to oxidize ammonia in marine group 1 Crenarchaeota (Venter 2004, Treusch). The first successful isolation of an autotrophic ammonia-oxidizing marine archaeon, *Nitrosopumilus maritimus* (Könneke et al., 2005), led to the discovery that mesophilic ammonia-oxidizing archaea (AOA) are ubiquitous in the global oceans (Francis et al., 2005b). In the open ocean and suboxic basins, AOA outnumber AOB in the water column (Coolen et al., 2007; Lam et al., 2007).

The first step in ammonia oxidation, the oxidation of ammonia to hydroxylamine, is catalyzed by the membrane bound ammonia monooxygenase (AMO) enzyme (Könneke et al., 2005). AMO is encoded by the amoA, amoB, and amoC genes. Putative crenarchaeal homologues to all three subunits have been identified (Hallam et al., 2006; Treusch et al., 2005). Due to its functional significance and conserved phylogeny, the amoA gene can be used as a molecular marker to study the diversity and abundance of AOA and AOB (Coolen et al., 2007; Lam et al., 2007).

This study attempts to address the following questions on the abundance and diversity of AOA and AOB in the stratified, marine analog Salt Pond:

1. Who is the ammonia oxidizing community?
2. Where does this community occur in the water column?
3. How do physiochemical parameters affect the distribution of ammonia oxidizing archaea vs. ammonia oxidizing bacteria?

The following approaches were taken to address these questions:

1. 454 16S sequencing and community analysis at 9 depths
2. Functional gene (amoA) clone libraries
3. qPCR targeting archaeal and bacterial amoA
4. CARD-FISH and DAPI cell counts
Materials and Methods

Site description:

Salt Pond is a brackish, shallow, seasonally stratified pond located in Falmouth, Massachusetts (Figure 2). It is a glacially formed kettle pond ranging 4.6 to 5.8 meters deep, with a surface area of 0.29 km² (Simmons et al., 2004), and is surrounded by 0.02 km² of upstream salt marsh (http://www.capecodcommission.org/tidalatlas/). The pond exhibits density stratification due to permanent tidal exchange with Vineyard Sound and freshwater inputs from runoff, precipitation, and groundwater. The average daily tidal amplitude is ~0.6 m.

Sampling:

Sampling was conducted from an inflatable rowboat on July 8, 2011. A YSI 600R Water Quality Sampling Sonde (Yellow Springs, Inc., Yellow Springs, Ohio) was used to collect salinity (ppt), temperature (°C), oxygen (% DO, mg/L) and pH data. A profile was taken in two locations ~10 meters apart to determine the water depth and location of the chemocline. The YSI probe was then attached to the intake of a portable peristaltic pump to allow for simultaneous water collection and profiling (Figure 3).

Water samples were collected from 9 depths spanning 0-4.0m. Water samples for molecular work and FISH analysis were collected in acid rinsed 2L polycarbonate bottles. In an attempt to measure sulfide concentrations 1 ml of water was taken from the full 2 L with a tipless syringe and transferred into 25 ml of zinc acetate solution (25 ml Milli-Q water + 1 ml 20% by volume zinc acetate). This procedure did not adequately fix the sulfide or was too large a dilution as no sulfide was detected using the Cline assay (Cline, 1969).

FISH:
Immediately on return to lab, water samples for FISH analysis were fixed using 1% formaldehyde in 50 ml conical tubes. 2 and 5 ml aliquots were filtered onto 0.2 µm membrane filters (Millipore GTTP, 25 mm). CARD-FISH was performed using the following probes:

<table>
<thead>
<tr>
<th>Bacterial</th>
<th>Eub338</th>
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<tbody>
<tr>
<td></td>
<td>Eub338 II</td>
</tr>
<tr>
<td></td>
<td>Eub338 III</td>
</tr>
<tr>
<td>Crenarchaeal</td>
<td>Cren537</td>
</tr>
<tr>
<td></td>
<td>Cren554</td>
</tr>
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<td></td>
<td>Eury806</td>
</tr>
</tbody>
</table>

**Nutrient Analyses:**

50 ml of water was sterile filtered and frozen for nutrient analysis. Ammonia measurements were performed by Chris Algar at the MBL Ecosystem Center according to (Scheiner, 1976). Nitrite measurements were performed spectrophotometrically according to (Strickland J, 1968).

**Community 16s RNA analysis:**

Between 200-300 ml of water from each sampling depth was filtered onto 0.2 µm membrane filters (Millipore GTTP, 47 mm) for DNA extraction. DNA for 454 sequencing was extracted using a PowerSoil DNA Isolation Kit and quantified using a NanoDrop spectrophotometer (Thermo Scientific). SSU rRNA genes were amplified with barcoded primers that also incorporated the Roche 454 Ti adapter sequences. The barcode was on the forward primer. Each barcode was 9 nt long and all the barcodes we used are in the mapping file for the 454 data. The primer targets are 515F and 907R on the *E. Coli* 16S gene.

Forward primer (X denotes barcode, lowercase is the linker between barcode and 16S primer),
Reverse primer (lowercase denotes linker between adapter and 16S primer),

5'-CTATGCGCCTTGCCAGCCCGCTCAGggCCGYCAATTCMTTTRAGTTT-3'

In the "AmplificationPlate" in the mapping file "plate1" was amplified for 32 cycles and "plate4" in the "AmplificationPlate" field was amplified for 36 cycles. The PCR program utilized a touchdown annealing temp for the first 10 cycles from 68-58C. Then there were 12 cycles of three-step PCR (denaturation, annealing, elongation) followed by 10-14 cycles of two-step PCR (annealing and elongation at same temp). A Phusion HF polymerase (2X mastermix) was used to amplify the gene with 8% DMSO and 0.5 uM primers in the final reaction volume. The template was normalized to 15 ng/µL (for DNA above 15 ng/µL) and 2 uL of each template was used for PCR. Post amplification DNA was quantified using the PicoGreen assay and then pooled ~125 ng of each PCR product. This pool was concentrated down to 100 uL using the vacufuge and gel purified using the Montage Kit (Millipore). The gel-purified pool was then shipped to Penn St. for sequencing.

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 NAST aligner tool to align sequences with the core set of 16s alignment templates. Sequences were aligned using the filter_alignment.py protocol in Qiime with lane masking enabled (as described and referenced by Libusha Kelly, course report, 2010).

Clone Libraries and qPCR:

DNA for all other molecular analyses was extracted using a phenol-chloroform, freeze-thaw procedure in the Coolen Lab at WHOI. Total nucleic acid extract was with a ladder in a 1% agarose and visualized with ethidium bromide (Figure 4). DNA extracts were quantified using fluorometry and yielded the following concentrations:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Depth</th>
<th>ng/µL</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>DNA</td>
</tr>
</tbody>
</table>
PCR for archaeal and bacterial amoA clone libraries and qPCR were performed using primers and thermocycling conditions previously described for bacterial amoA (AmoA-1F*/AmoA-2R) specifically targeting the β-AOB (Rotthauwe et al., 1997), and archaeal amoA (Arch-amoAF/Arch-amoAR) (Beman et al., 2008; Francis et al., 2005a). An inhibition test was performed to check for inhibition due to remaining extraction solvents, etc. Significant inhibition was not demonstrated in any of the samples.

Clone libraries using archaeal primers as described above were constructed for depths 0 m, 0.5 m, 1.5 m, and 4.0 m. PCR products were cloned into the TOPO2 TA Cloning Kit (Invitrogen). Sequences in the clone libraries were aligned against each other using Muscle. Sequences in each library were locally BLASTed against the a FunGene amoA database. A tree was constructed using FastTree and visualized in iTOL.

**Results and Discussion:**

**Salt Pond Depth Profile:**

Seasonal density stratification in Salt Pond occurs during the summer months—late June through September—and prevents vertical mixing (Simmons et al., 2004). Sampling on July 8 revealed a distinct chemocline from 1.5 to 3 meters (Figure 5), as reported in previous years (Saenz, 2008). The surface of the pond is oxygenated freshwater with a salinity of 11.37 ppt, dissolved oxygen concentration of 6.4–6.7, and a pH of 8.8. Respiratory oxygen consumption drives down dissolved
oxygen that cannot be replenished by mixing and creates a chemocline. Below the chemocline the salinity increases to 19 ppt, the dissolved oxygen drops to 4.1 mg/L, and the pH decreases to 7. Sulfide concentrations likely increase to below the oxycline to millimolar concentrations (Saenz, 2008).

16S Community Analysis by 454 sequencing:

The major phyla represented by 16S rRNA 454 sequencing are Actinobacteria, Bacteroidetes, Chlorobi, Cyanobacteria, and Proteobacteria. Cyanobacteria are the dominant phyla in water column down to 2.5 meters with the exception of 0.5m where Proteobacteria (Figure 6). While photosynthetically active radiation (PAR) was not measured, this abundance pattern reflects photosynthetic activity in Cyanobacteria and Proteobacteria through the photic zone. At 3 meters there is a slight relative increase in the abundance of Bacteroidetes followed by a complete dominance of Chlorobi (97% of relative phyla counts at 4 meters). The increase in Chlorobi at 3.5-4 meters reveals the spatial niche of low-light adapted green sulfur bacteria in the euxinic bottom waters of Salt Pond. The time constraints of this course did not allow for a very in-depth analysis of this dataset. For example, breaking down the proteobacteria could provide information on the spatial niche and relative representation of purple sulfur bacteria and ammonia oxidizing bacteria.

In order to assess the difference in microbial communities at each depth, weighted and unweighted dendrograms were produced using UniFrac (Figure 7). The UniFrac method measures the phylogenetic distance between sets of taxa in a phylogenetic tree as the fraction of the branch length of the tree that leads to individual descendants from each water column depth (Lozupone & Knight, 2005). Given the gradient of physiochemical conditions in Salt Pond, a logical hypothesis would be that microbial communities at each depth would be the similar to the communities at adjacent water depths. To a coarse degree this appears to be true in the dendrograms. Samples, especially in the more closely chemically related surface and deep water tend to cluster by depth, while samples in the more rapidly changing chemocline are more spread out. In this case the 0.5 m sample may be
reflecting a photosynthetic community different from the otherwise dominant cyanobacteria.

Rarefaction curves for the 16S community analysis do not show saturation at any depth (Figure 8). This is unsurprising as each depth was only sampled once. More notably, the grouping of the rarefaction curves mirrors patterns found in the relative representation of phyla and the UniFrac dendograms, largely that the sample from 0.5 meters was the only sample approaching a plateau.

**qPCR:**

qPCR was performed on the total nucleic acid extracts at each depth to look at the relative abundance of putative ammonia-oxidizing archaea (AOA) and bacteria (AOB) across the physiochemical gradient of the chemocline in Salt Pond. Shifts in the relative abundance of AOA and AOB correlate closely with both dissolved oxygen and ammonium concentration through the water column. In the first ½ meter of the water column AOB dominate ~70% of amoA community. Between ½ and 2 meters, in the oxic to suboxic water just above the chemocline, AOA make up over 95% of the amoA community. Through the chemocline and into the anoxic deep water, the abundance of AOB increases steadily to 66%. In the lower 3 meters of the water column the increase in the abundance of the AOB community covaries with the increase in ammonium concentration.

In the surface water of Salt Pond, the high percentage of AOB may be due to nutrient run off of surface water. In past years, high ammonium concentrations have been observed in the upper meter of Salt Pond, so this may be a temporal variation that was missed on the one sampling expedition. The location of a peak abundance of AOA in the oxic to suboxic nitrification zone just above the chemocline has been similarly observed in the Black Sea (Lam et al., 2007) associated with maximum nitrification activity. In and below the chemocline with the decrease of dissolved oxygen, the remineralization of organic matter and groundwater inputs provide a source of ammonium. As ammonium concentrations increase and dissolved oxygen concentrations decrease, AOB become more prevalent than AOA. This suggests that AOB outcompete AOA at high ammonia concentrations in a potentially
microaerophilic environment created by an influx of ground water from the sediments.

*Functional Gene Clone Libraries:*

Clone libraries targeting the archaeal *amoA* gene were locally BLASTed against the FunGene amoA database. While the results were not particularly illuminating, they were the similar to uncultured and enrichment cultures of ammonia oxidizing archaea and Crenarchaeota (Table 1).

Trees constructed from archaeal amoA clone libraries showed roughly three clustering groups (Figure 10). There is a ‘very shallow’ depth (0-0.5m) cluster, a ‘shallow’ depth cluster, and a very distinct ‘deep’ cluster. This pattern appears in both the tree ignoring branch lengths and the tree incorporating branch lengths. In the tree incorporating branch lengths, the ‘deep’ clustering is more readily apparent as the branch length of the cluster is distinct from both the ‘very shallow’ and ‘shallow’ clusters on either side.

*CARD-FISH and DAPI Counts:*

CARD-FISH counts with crenarchaeal and general bacterial probes revealed a consistent ratio of bacteria to crenarchaea throughout the water column. The crenarchaea generally make up ~1% of the total DAPI counts and the bacteria make up between 70-80% of the counts. 20-30% of the DAPI counts were not fluorescently labeled with either probe. Morphology in the crenarchaeal probe resembled the peanut shape observed in (Könneke *et al.*, 2005).

**Conclusions:**

- Ammonia-oxidizing crenarchaea and bacteria are both present in Salt Pond. AOA are dominant in the oxic surface waters with low ammonia concentrations while AOB increase through the chemocline and into the suboxic, ammonia rich waters
- The putative ammonia-oxidizing archaea are closely related to cultured and uncultured environmental ammonia-oxidizers in the FunGene *amoA* database.
• CARD-FISH revealed a low overall abundance of crenarchaea compared to bacteria throughout the water column.
• The microbial community changes significantly through water column in Salt Pond, highlighting different ecological niches in a stratified marine system.

Future Directions and Remaining Questions:
• Phylogenetic comparison to known ammonia-oxidizing organisms: Given the lack of specificity in the BLAST results from the FunGene amoA database, the next step would be to build a tree with the clone libraries from Salt Pond to compare to known organisms of interest such as *Nitrosopumilus maritimus* and Crenarchaeota found in the Black Sea (Coolen *et al.*, 2007).
• Nitrification rates: Rate measurements for ammonia oxidation and nitrite oxidation would allow us to look at where in the water column, nitrification is occurring relative to the numerical abundance of either AOA or AOB.
• Temporal variation: Salt Pond is a seasonally stratified pond. The chemocline develops around late June, so sampling on July 8, 2011 is representative of an early summer chemocline. The ammonia-oxidizing community may shift with the evolution of the chemocline throughout the summer, and likely redistributes with the vertical mixing in winter.
• Anammox: Below the chemocline Salt Pond becomes euxinic and could potentially support an active anammox community. This could be accomplished through similar methods used in this report or ladderane lipids unique to anammox bacteria.
• Further 16S analysis: The 454 sequencing provided a depth of data, which could not be fully explored during the limited time in this course. Further probing of community structure at each depth would likely further our understanding of spatial niches in the stratified marine environment of Salt Pond.

Acknowledgements:
Thank you to course directors Dan Buckley and Steve Zinder for providing this truly unique and fulfilling opportunity. Thank you to the course TAs (especially Chuck and Lizzy) for your help and patience. Thank you to Cornelia Wuchter and Marco Coolen for primers, probes, lab space, and scientific guidance. Thank you to the waterbears for rocking the hole.

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References:
Cline, J.D., (1969) SPECTROPHOTOMETRIC DETERMINATION OF HYDROGEN SULFIDE IN NATURAL WATERS. *Limnology and Oceanography*, 14(3), 454-&.


Figures:

The Marine N Cycle

Figure 1. The marine nitrogen cycle highlighting the role of nitrification in connecting the remineralization of particulate organic nitrogen and nitrogen loss from the ocean. Modified from (Francis et al., 2007).
Figure 2. A) Location of Salt Pond in Falmouth, Massachusetts on Cape Cod. B) Location of depth profile taken in Salt Pond.

Figure 3. Water samples collect from ½ meters depths in Salt Pond
**Figure 4.** Total nucleic acid extract from each sampling depth.
Figure 5. Water column chemistry and dissolved oxygen in the Salt Pond water column.
Figure 6. 16S 454 sequence data normalized to sequence reads.
Figure 7. A) Weighted dendrogram created through UNIFRAC analysis B) unweighted dendrogram.
Figure 8. 16S 454 Sequencing rarefaction curves at each sampling depth.
Figure 9. Nitrogen species in the water column (left) and quantification of archaeal and bacterial *amoA* copy number.

<table>
<thead>
<tr>
<th>BLAST hits</th>
<th>% identity</th>
<th># of hits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncultured ammonia oxidizing archaeon</td>
<td>94.44</td>
<td>12</td>
</tr>
<tr>
<td>Crenarchaeote enrichment culture clone San Francisco Bay</td>
<td>90.71</td>
<td>3</td>
</tr>
<tr>
<td>Uncultured crenarchaeote</td>
<td>73.08</td>
<td>174</td>
</tr>
<tr>
<td>Uncultured archaeon</td>
<td>69.91</td>
<td>4</td>
</tr>
<tr>
<td>Crenarchaeote enrichment culture clone</td>
<td>97.14-78.35</td>
<td>193</td>
</tr>
</tbody>
</table>

Table 1. Top BLAST hits when from the FunGene *amoA* database.
Archaeal *amoA* Clone Library Tree

**Figure 10.** Trees of archaeal *amoA* functional gene clone libraries created in FastTree and visualized in iTOL.
**Figure 11.** CARD-FISH counts with bacterial and crenarchaeal probes.
Figure 12. Fluorescence image of cells displaying peanut-like morphology of marine Crenarchaeota.