

Testing our ability to accurately measure nitrification rates in the oligotrophic ocean: Does adding ammonium affect the nitrifier community composition?

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Microbial Diversity 2011

Abstract

In this mini-project I've tested the effect of adding ammonium on the nitrifying community in coastal seawater. Water was collected from 22 meters from Great Harbor, Woods Hole MA and incubated for 2 days with 3 concentrations of ammonium (0, 0.1 and 5 μM). I found that the nitrifying community significantly changed over the 48 hour time period. Quantitative PCR showed that there was a shift from 20% AOB to AOA community to an 80% AOB by the end of incubation in the highest ammonium addition. There wasn't a significant change in the AOB to AOA ratio when no ammonium or 0.1 μM ammonium was added. Although the AOB to AOA ratio did not change there was still a shift in specific species of organisms, shown by DGGE in all three incubations. Clone libraries from the no ammonium addition also showed that a rare group of organisms outnumbered the original, more diverse, community within 48 hours.

Introduction

The most common method for measuring nitrification rates in the ocean is to incubate with seawater with ^{15}N labeled ammonium for 24-48 hours and then to measure the ^{15}N in the nitrite and nitrate produced. This method relies on the ability to add tracer levels of ammonium to not drastically change the chemical environment of the nitrifying community. In many areas of the ocean this is impossible. Ammonium, the most desired nitrogen compound in the ocean, exists in very low abundance in most of the water column. Many oceanographers continue with these rate measurements by adding the small amounts of ammonium, although not trace levels and inevitably they are stimulating the growth of a new and unnatural nitrifying community.

Different species of nitrifiers, especially between bacterial and archaeal, have a wide range of affinities towards ammonium. Likely, by adding ammonium to the system we are stimulating the growth of certain species over others (Habbena et al 2009). Historically it has been much easier to isolate ammonia oxidizing bacteria (AOB) because they outgrow ammonia oxidizing archaea (AOA) in high levels of ammonium and have a faster growth rate. Although AOA are in higher abundance in oligotrophic seawater because they have a higher affinity for ammonium (Habbena et al, 2009).

In this experiment we will test whether different ammonium additions to oligotrophic seawater will affect the nitrification rate overall as well as the microbial community, specifically the relative abundance of AOA and AOB.

Methods

Experimental Design

For this experiment water was collected from 22 meters using a niskin bottle on a small boat tied to the back of the WHOI dock on July 8, 2011. 5 different 4L polycarbonate bottles were filled with seawater. Table 1 shows the bottle names and conditions.

Table 1.

Bottle Name	Added Labeled Ammonium (μM)
1a	0.1
1b	0.1
2a	5
2b	5
Control	0

Labeled ammonium was added to bottles immediately after sampling, within 1 hour of returning to laboratory. Then subsamples were taken from each bottle initially and at 12, 24 and 48 hours after the ammonium addition. Each subsample consisted of filtering 200mL split onto two different 0.2 μm isopore membrane filters and frozen at -80°C . 50mL of the filtrate was also collected for measuring the isotopes of nitrite and nitrate. Also 15mL of seawater fixed and 3 and 10 mL of water were filtered onto 0.2 μm isopore membrane filters for cell counts. Also before the addition of labeled ammonium samples was taken for measuring the initial nutrient concentrations (ammonium, nitrite, and nitrate) of the seawater. The bottles were incubated in the laboratory, with a temp of 22°C , in the dark for the 48 hours.

DNA and RNA Extraction

Filters were added to 15mL falcon tubes with 6mL of extraction buffer. Extraction buffer was made with 0.6 mL of a stock buffer (250mM EDTA and 100mM Tris HCl), 30 μL of 20% SDS, 60 μL 5M NaCl and PCR grade water. Beads were added and the tubes were bead beaten for 5 minutes and then place in a 60°C water bath. After being heat to 60°C tubes were dropped in liquid nitrogen. They were heated and frozen 3 times. The lysis buffer was now washed with PCI (phenol chloroform in isoamyl alcohol) and centrifuged for 10 minutes at 5000rpm. The PCI was poured off and the lysis buffer was added to a new conical tube. Samples that still looked cloudy after first washing were washed a second time with PCI. Next the lysis buffer was added to an Amplicon ultra-4 centrifugal device to collect the DNA on the filter. The Amplicon tubes were centrifuged at 3000 rpm for 7 minutes. Buffer had to be added multiple times because not all the material could fit into the filter tube. Once the buffer was filtered the filters were washed 3 times with 1X TE centrifuging between each washing. The volume of DNA left in the top of the filter was measured as it was transferred into PCR tubes and put in the freezer.

The DNA was then run on a gel to visualize that extraction was successful. The DNA was also then quantified using Pico Green and a fluorometer. 1 μL of DNA was added with 198.5 μL of 1x TE and 0.5 μL Pico Green.

Quantitative PCR (qPCR)

DNA samples were diluted ten fold prior to PCR reaction. PCR reaction was set up using a master mix which included Picomaxx enzyme, Picomaxx extraction buffer, SYBR green, dNTPs (a concentration of divalent ions), magnesium chloride, DNA and primers. qPCR was done using 3 different primers: 1) amoA in archaea, cluster A (Francis et al, 2005; Beman and Francis, 2008) 2) amoA in bacteria (Rotthauwe et al, 1997) and 3) amoA in archaea, cluster B (Francis et al, 2005; Beman and Francis, 2006). Standards for each PCR product were obtained from Cornelia Wuchter at Woods Hole Oceanographic Institution (WHOI). They were placed in a thermocycler using a temperature program of 95°C for initial melting, 58°C for primer annealing, 72°C for primer extension and 80°C for fluorescence measurement. Also, inhibition tests were carried out with *E. coli* and found that the 1/10 dilution of our DNA extract did not cause any inhibition.

Denaturing Gradient Gel Electrophoresis (DGGE) and Sequencing

DGGE was performed in the Coolen laboratory at WHOI on a Ingeny PhorU system. First the acrylamide gel was made with a gradient from 20 to 70% ureum and deionized formamide solutions. The gel needs 3 to 4 hours to set up before running. The DGGE chamber is filled with 1x TAE buffer at pH 8. The gel is loaded with a 5 μL of loading dye and 5 μL of the PCR product. The PCR is done with a special reverse primer with a GC clamp. The gel is run at 60°C and 200V for at least 5 hours, but up to 12 hours.

At the end of the run, the gel is removed and stained for 20 minutes using SYBRgold (2 μL in 8mL 1x TAE). Then it is destained in water for 30 minutes. The bands are viewed and photographed on a dark reader.

Bands can be isolated and sequenced by cutting carefully and then placing in 75 μL of 1x TE at pH 8. The bands are placed 4°C overnight to elute and then the product is reamplified using PCR using normal primers. This PCR product was then purified using the MinElute system and then quantified on the nano drop spectrophotometer. The DNA was diluted to 20 ng/ μL and then submitted for Sanger sequencing. These sequences are analyzed by BLASTing locally to a functional gene database for amoA downloaded from FunGene.

Clone Library Construction and Phylogenetic Analysis

I attempted to construct 8 different clone libraries, with only 2 being successful. PCR products from archaeal amoA and bacterial amoA functional gene primers were cut and purified using gel purification. The 8 different samples attempted were the initial time point in the control and the 3 48 time points in 1a,

2a and the control, one for each condition. Once the DNA was purified, 4 μL of DNA was added to a tube with 1 μL of TOPO vector and 1 μL of salt solution. These tubes were allowed to sit at room temperature for 15-30 minutes. Then 2 μL of the DNA mixture was added to 70 μL of electrocompetent *E. coli* cells. The cells were then electroporated at 2250V. Immediately after electroporation 250 μL of cells were added to 250 μL of SOC medium in a tube. The tube was placed on the shaker for 1 hour and then 2 and 20 μL aliquots were plated on LB plates with ampicillin and incubated at 37°C for 12-18 hours. Plates were monitored until there were many small colonies. Once there were colonies they were picked and placed in a growth block with 1.2 mL of super broth.

Nutrient Measurements

Nitrite measurements were made using the spectrophotometric described in Strickland and Parsons 1968. 100 μL of a sulfanilamide reagent (1g sulfanilamide in 100 mL 10% HCl) and 100 μL of 1-naphthyl ethylene diamine reagent (0.1g in 100 mL water) were added to a 2 mL sample. Standards were run in parallel at concentrations of 0, 0.25, 0.5, 1 and 5 μM . Standards were made using sodium nitrite stock solution volumetrically.

Ammonia measurements were also made spectrophotometrically using the phenol method. 100 μL of phenol, 100 μL nitroprusside and 200 μL of a sodium citrate and bleach (5:1) reagent were added to a 5 mL samples. Standards of concentrations 0, 0.1, .2, .5, 1, 2, and 5 μM were run in parallel. The detection of this method was only about 0.2 μM since there was a blank of at least 0.1 μM .

Catalyzed Auto Reporter Deposition- Fluorescence In Situ Hybridization (CARD FISH)

Filters with 3mL of fixed cells were first embedded in 0.1% agarose. Then each filter was cut and 2 small pieces were used for FISH with an eubacteria probe (Eub 338, Eub 338 II, and Eub 338 III) and a crenarchaeal probe (Cren 537, Cren 554). For the bacterial probe the filter was permeabilized in lysozyme (100mg lysozyme, 1000 μL 1M Tris HCl, 1000 μL 0.5M EDTA, 8 mL water) at 40°C for 1 hour. For the crenarchaeal probe the filter was permeabilized in proteinase-K (100 μL proteinase-K and 1 mL 1M Tris HCl and 1 mL 0.5M EDTA and 8 mL water). Following permeabilization filters were added to 0.01 M HCl for 20-25 minutes, and then washed twice in water. Then filters were dipped in 95% ethanol and dried. Hybridization was done in 0.7mL eppendorf tubes. 300 μL of hybridization buffer and 15 μL of probe were added to the tubes. They were hybridized at 35°C for 12 to 15 hours in the dark.

Filters were then mounted on a slide using a DAPI mounting solution. For each slide 10 grids of 0.1 mm were counted for DAPI and both probes at 100X on a Zeiss microscope.

Results and Discussion

Harbor water chemical composition

Water collected from 22 meters deep of the WHOI dock had nitrite and ammonium conditions below the detection limit, $<0.05\mu\text{M}$ for nitrite and $<0.2\mu\text{M}$ for ammonium. This is expected for seawater at this depth during the summer since there are many organisms using inorganic nutrients. Nitrate has not been measured yet but will be in the future.

Cell Abundance of Bacteria and Crenarchaea

Initially in the control, 1a and 2a there was $2.63 \pm 0.25 * 10^6$ cells but by the 48 hours the average was only $1.77 \pm 0.11 * 10^6$ cells in the three different conditions. The total cell decrease was not dependent on the ammonium addition (Fig 1a). The number of bacterial and crenarchaeal cells also decreased from 0 to 48 hours in all treatments (Fig 1 b and c). The total bacterial percentage ranged from 38 to 56% in the initial and 48 hour time points for all 3 conditions. The largest drop in bacterial percentage occurred in bottle 2a, the $5\mu\text{M}$ ammonium addition) between the 0 and 48 hours, from 56 to 39% bacteria. The crenarchaeal percentage was only 1 to 2% of cells, which were more difficult to count because they were rare on the slides. The decrease in cells is most likely due to grazers trapped in the bottles since there was no pre-filtering.

Relative abundances of AOB and AOA

Quantitative PCR was performed with primers for bacterial and archaeal amoA. The copy number was then normalized to ng of DNA that was extracted. From this number the percentage of AOB and AOA was calculated and plotted for the three conditions at 4 different time points (except in the initial condition of bottle 1a since DNA extraction was not successful). Figure 2 a-c shows the relative abundance of AOB and AOA for the incubations. For the no ammonium addition and $0.1\mu\text{M}$ ammonium addition there was no systematic change in percent abundance over the 48 hour incubation. The average AOB percent was 25% and AOA 75%. In the $5\mu\text{M}$ ammonium addition (2a) there was a systematic increase in AOB percentage at 12, 24 and 48 hours. At the end of the incubation AOB now dominated the system at 70%.

These results are similar to what I had expected. It has been known that AOB flourish in areas with higher ammonium concentrations like soils, coastal waters and estuaries (Ward et al, 1984; Stark et al, 1996; Santoro et al, 2008) so it makes sense that in the highest ammonium concentrations there was a dominance of AOB. I was surprised that by 12 hours they were able to detect the ammonium and grow fast enough to outnumber AOA. I had also expected the starting community would have a larger percentage of AOA because now multiple times they have been shown to be the dominant ammonia oxidizer in the ocean (Francis et al, 2005; Wuchter et al, 2006; Mosier and Francis, 2008). It was interesting that in the $0.1\mu\text{M}$ ammonium addition there was little change in the community over 48 hours. This could be for a couple of reasons. First that amount of ammonium can only stimulate a small amount of growth which is likely not detectable in that time period. Also, since my

method for measuring ammonium had a detection limit greater than 0.1 μM I can't be sure that the ammonium in the ocean was already higher than 0.1 μM .

Community composition change

In this study I was able to use two different methods to assess the community composition change between the beginning and end of the incubations. The first method I used was DGGE, in which I could visualize the change in fragment GC content from running through a denaturing gel. Fig 3 depicts the gel that I loaded the PCR products for both bacterial and archaeal amoA. The bacterial amoA fragments changed for each time point and in the different ammonium additions. Fig 4a shows the bands, which were sequenced and then BLASTed against an amoA functional gene database downloaded from FunGene. Of the 16 bands BLASTed there were only two closest hits either *beta_proteobacterium_enrichment_culture_clone_SF06E-BC11-B02* (Mosier and Francis, 2008) or *bacterium_amoA.26.Sali.kuLtur* (Eilmus et al, unpublished). The lower bands all had the closer hit to the second AOB. The percent identities of these hits ranged from 83 to 97%.

I also sequenced the bands in the archaeal amoA DGGE gel (Fig 4b) and every band BLASTed to the crenarchaeote enrichment culture clone SF06E-BC11-D01 (Mosier and Francis, 2008). These again had ranging percent identities from 87 to 98%. The bands in the same position on the gel mostly had a similar percent identity to the crenarchaeote enrichment culture suggesting they are a similar organism. Overall the bands in the archaea amoA gel were all clustered closer together. It is hard to know whether this is due to less diversity in AOA's or that AOA's in generally have fewer differences in GC content. Also, AOA amoA is a shorter sequence, which would also contribute to closer clustering on the DGGE gel.

Bottle Effects on the community composition

Since DGGE has only a coarse resolution in community change I planned to make clone libraries for AOA and AOB the initial and final time points for each ammonium addition. In the end only 2 clone libraries worked, for the initial and final time point in the control for AOB. Interestingly, even when no ammonium was added there was a change in the AOB community just due to putting seawater in a 4L polycarbonate bottle.

The first analysis I did on the clone libraries was to BLAST the sequences against the same local functional gene database from FunGene mentioned previously. These results are shown in Figure 5. Since the database is not detailed enough I found that in the initial time point every sequence had a closest hit to *beta_proteobacterium_enrichment_culture_clone_SF06E-BC11-B02* (Mosier and Francis, 2008). Then the 48 hour clone library only now has a closest hit for to *beta_proteobacterium_enrichment_culture_clone_SF06E-BC11-B02* for 40% and the other 60% of sequences best match *bacterium_amoA.26.Sali.kultur* (Eilmus et al, unpublished) This shows that there is a new group of organisms that thrive in the bottle conditions over the initial dominant community.

Since the database was limited and could not capture the diversity in my clone library I decided to also align all my sequences against each other to see how the diversity changed between the two libraries. I aligned the sequences using MUSCLE and then made a tree using Fast Tree. Also, prior to aligning I removed the vector from the sequences and made sure they were all inserted in the same direction and if not I flipped the arrangement. After constructing the tree I used ITOL (Interactive Tree of Life) to visualize the tree assigning a different color to each clone library (Fig. 6). The tree shows that there was no overlap in clustering between the two libraries. This indicates that the 2 main clusters that were in the bottle at 48 hours were rare members of the nitrifying community originally. There also appears to be more diversity in the initial clone library, which diverged only into 2 main clusters after 48 hours.

Conclusions

- The total number of cells, including bacteria and crenarchaea, decreased in the bottles over a 48 hour incubation in all bottles.
- Quantitative PCR of the amoA gene in AOB and AOA, showed there was a shift to a more dominant AOB community in 5 μ M ammonium addition. When no ammonium or only 0.1 μ M ammonium was added there was no systematic change in the ratio of AOA to AOB.
- DGGE showed that there was a shift in community composition in all bottles. Sequencing of the DGGE products showed that there were multiple species although the small database limited the analysis, since all bands closest hit was either one of two different ammonia-oxidizing bacterial sequences.
- Clone libraries from the initial and 48 hour time point in the control showed that even without adding ammonium there were distinct changes in the AOB community. The main AOB group found in the 48 hour time point did not occur initially, meaning it was originally a rare member of the nitrifying community.
- For future work on this project I plan to measure the nitrate and nitrite isotopic ratios to see if the nitrification rate was different depending on the amount of ammonium added.

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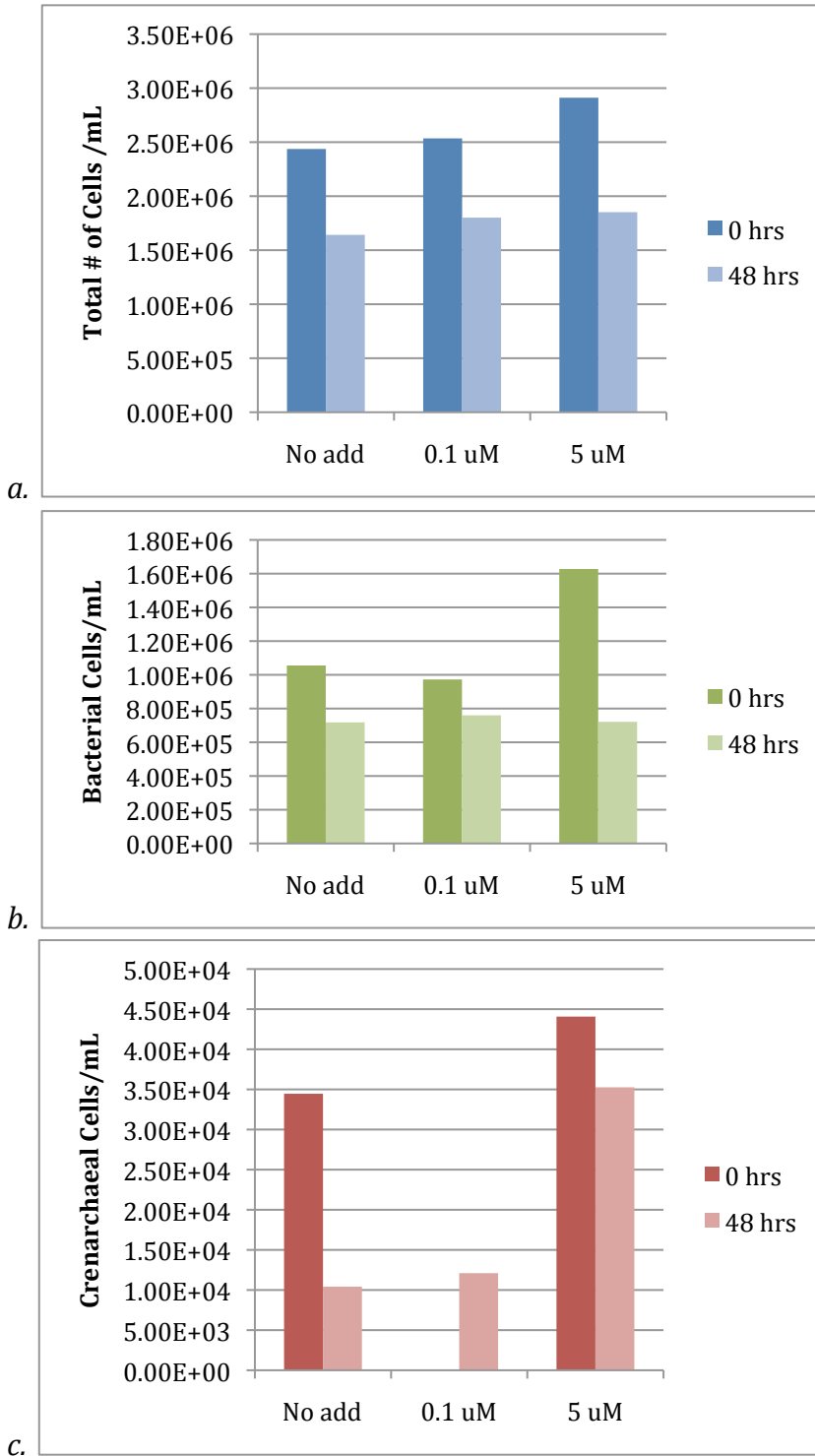


Fig 1. Cell counts using DAPI (a) and CARD FISH bacterial probe (b) and crenarchaeal probe (c) for the control, 1a and 2a bottles for the 0 and 48 hours time points. The crenarchaeal probe did not work for the 1a bottle at 0 hours.

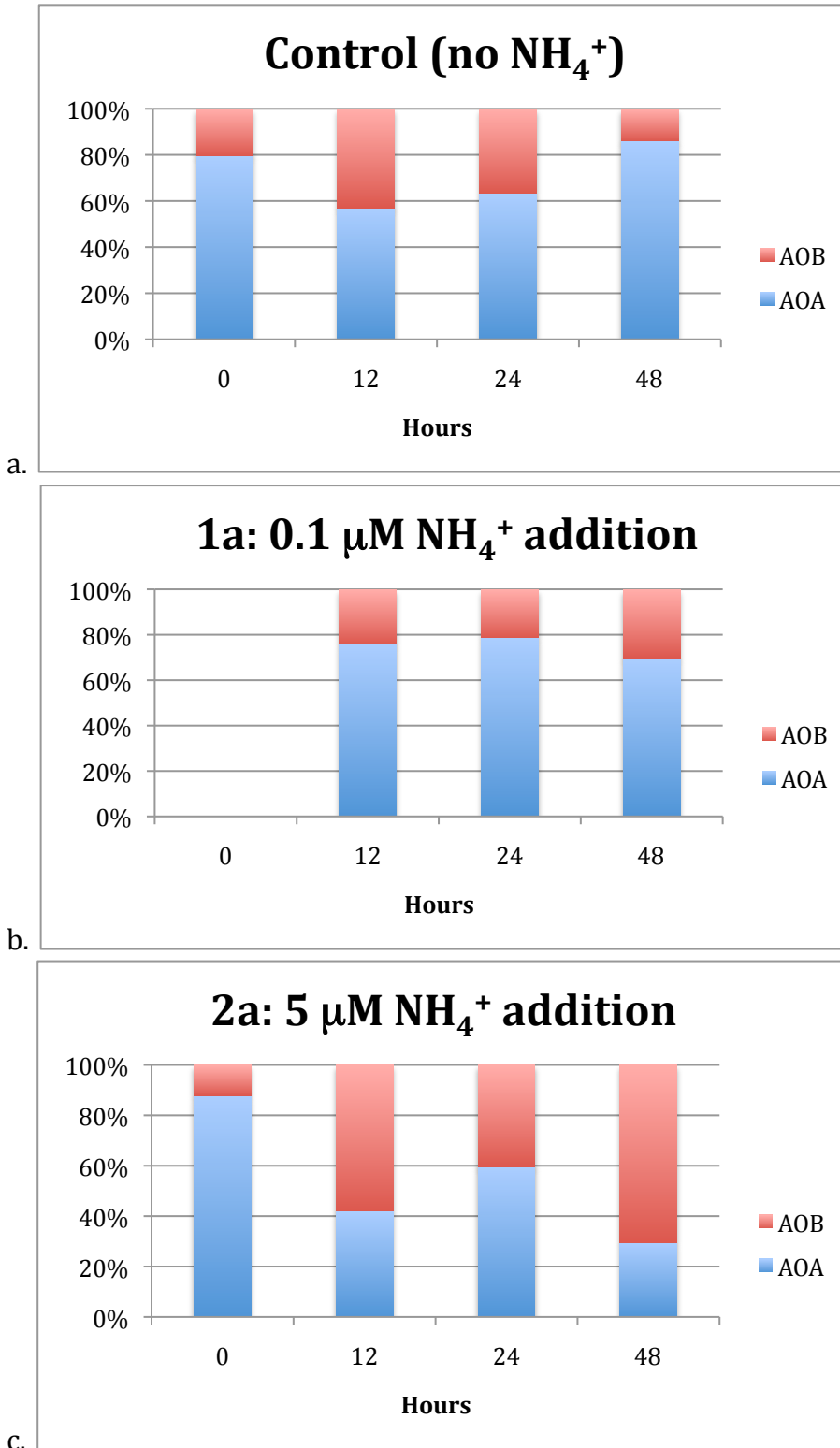


Fig 2. Percentage of AOA and AOB at 0, 12, 24 and 48 hours for the control, 1a and 2a. Percentage calculated from copies of amoA per ng DNA measured using qPCR.

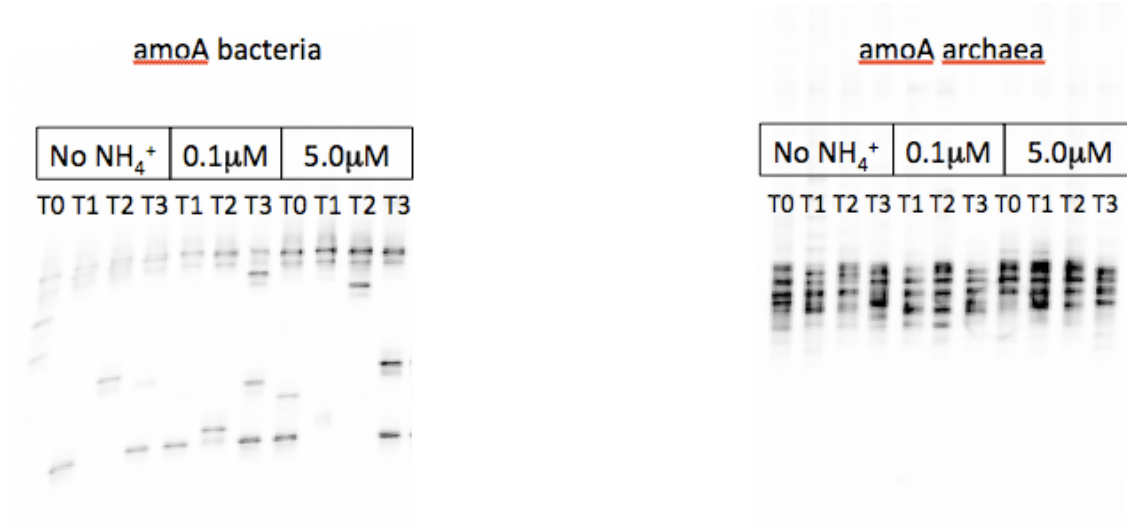


Fig 3. DGGE gel loaded with PCR products amplified using the bacterial (left) and archaeal (right) amoA primers.

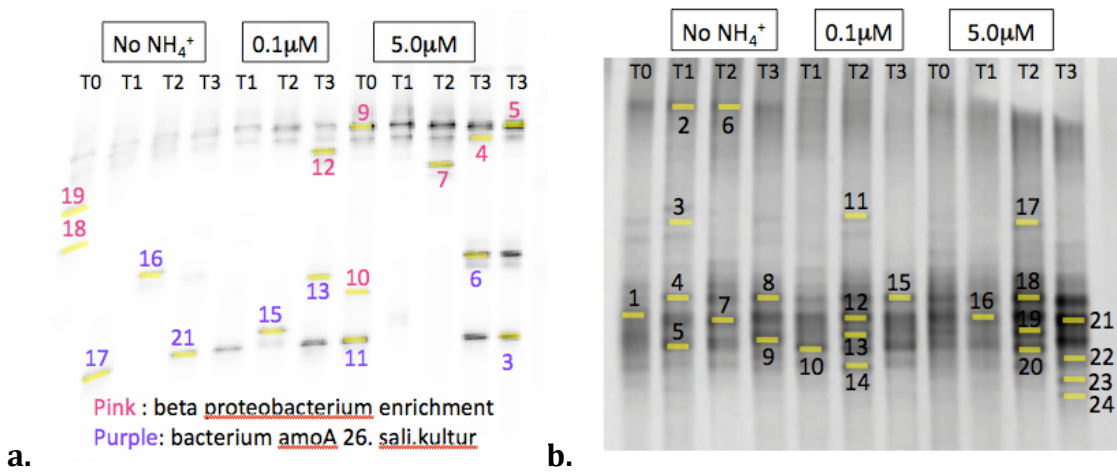
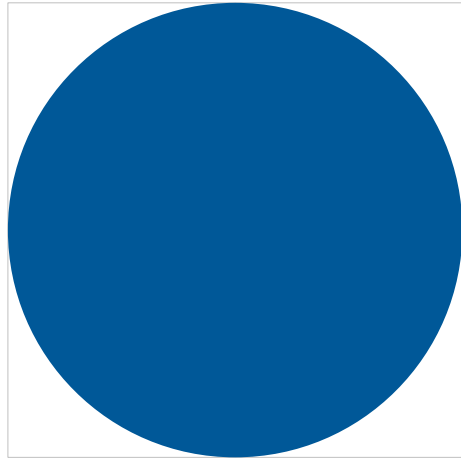
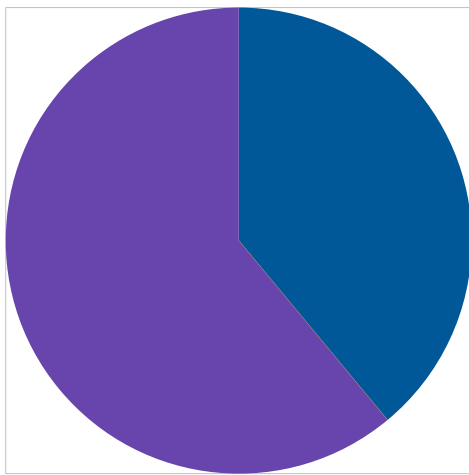


Fig 4 a-b. Depiction of bands that were cut and sequenced from a DGGE gel for bacterial (a) and archaeal (b) amoA. The bacterial amoA gel also shows the BLAST hit for each band.



■ Beta proteobacterium
enrichment SF06E-BC11-B02

a.



■ Bacterium amoA.26.Sali.kultur

b.

Fig 5 a-b. Closest BLAST hits to an amoA functional gene database for 2 clone libraries in the control at 0 hours (a) and 48 hours (b).

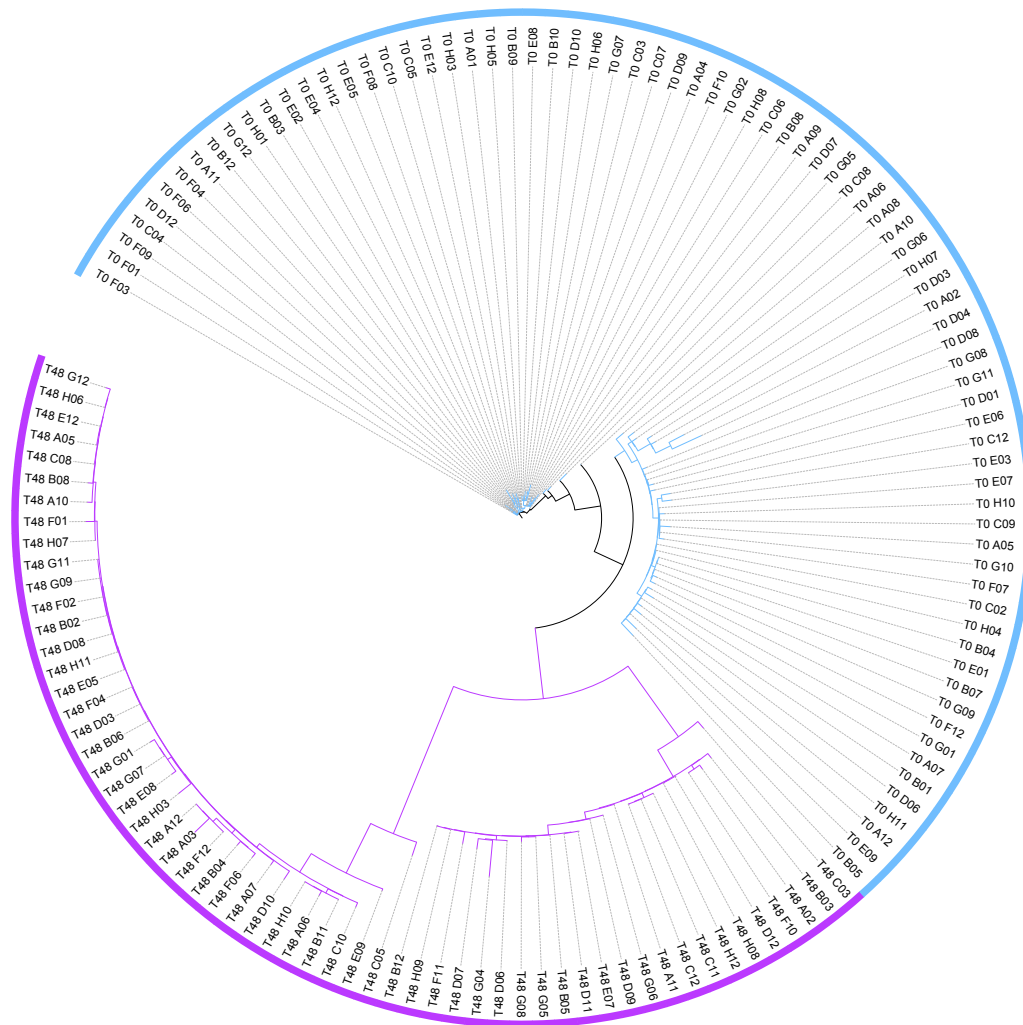


Fig 6. Tree constructed using Fast Tree and then visualized using ITOL for the 2 clone libraries for the control at 0 hours (blue) and 48 hours (purple).