Trunk River Sulfide Oxidizing Bacteria

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Abstract

A study of the sulfide oxidizing bacteria and the water/sediment chemistry at two sites in the Trunk River. The Trunk River was selected to study sulfide oxidizing bacteria due to the strong sulfide smell that was observed at the site. The Cline assay was used to determine the sulfide concentration and a sulfate assay to determine sulfate concentration in the pore water and enrichment cultures. The profile of the cores showed that the concentration of sulfide increased with depth and sulfate decreased with depth. The salinity and pH of the two sites varied between each other and with the tides. Overall the Mouth site had a lower pH and higher salinity while the Deep had a higher pH and lower salinity. The differences between the two sites suggests that the organisms that live near the Mouth must be more adapted to high salinity and a lower pH while in the Deep the organisms are adapted to a high pH and lower salinity. However, because the two sites are not drastically different it is possible for some organisms to live in both communities which is supported by the clone library and TRFLP results. The colorless sulfur oxidizing bacteria use reduced sulfur compounds as their electron donor with oxygen or nitrate acting as the terminal electron acceptor in respiration. A common pathway for sulfur oxidizing bacteria involves the *sox* gene cluster. From this cluster of genes primers for the *soxB* gene were selected to look at the SOBs. The function gene clone library for *soxB* showed that many of the clades consisted of only clones from the Mouth or Deep however some did mix supporting the idea that the chemistry of the sites overlay enough for some of the community to be the same. This was also supported by the TRFLP data of 16S rRNA from the two sites where most of the peaks were different, however a few peaks were present in both electropherograms showing the slight over lap of the communities. Several isolates were obtained from the enrichment cultures; however, the PCR products for the *soxB* gene did not return good sequencing results. The 16S rRNA returned one promising results of a *Thiovirga*. Overall the results suggest that the communities of the two sites contain some of the same sulfide oxidizing bacteria, but mainly are composed of different organisms.

Background

The Trunk River was selected to study sulfide oxidizing bacteria (SOB) based on the strong sulfide smell that was observed at the site. As the site had two distinct areas, the mouth which was in direct contact with seawater and had less of a sulfide smell while the pond area further upstream was more sheltered from the influx of seawater and smelled strongly of sulfide.

In the environment, sulfur cycles between being fully oxidized as sulfate and fully reduced as sulfide. Many of the intermediate redox reactions are both biologically mediated and spontaneous. Sulfate can be reduced to organic sulfur compounds through assimilatory sulfate reduction or directly to sulfide through dissimilatory sulfate reduction by sulfate reducing bacteria (SRB) (Robertson *et al.*, 2006). In the other half of the cycle, sulfide can be oxidized to sulfur by spontaneous processes or biological oxidation that is either aerobic or anaerobic

(Robertson *et al.*, 2006). Biological oxidation is mediated by sulfur oxidizing bacteria (SOB) that can directly oxidize sulfide to sulfate (Robertson *et al.*, 2006). The microorganisms involved in the oxidation processes are always competing with naturally occurring abiotic reactions to obtain energy.

There are two types of sulfide oxidizing bacteria, photosynthetic and colorless, which have little to no taxonomic relatedness between or within the two types (Robertson *et al.* 2006). This report will focus on the colorless sulfur bacteria which use reduced sulfur compounds as a source of growth energy for respiration (Robertson *et al.* 2006). Most of these organisms require oxygen as the terminal electron acceptor but some can also grow anaerobically using nitrate (Robertson *et al.* 2006). Many of them are facultative chemolithotrophs and can be found growing in environments with a pH between 1-9 and temperatures ranging from 4ºC-95ºC (Robertson *et al.* 2006).

Sulfide oxidation has been observed at low pH where the microorganism can more effectively compete with the rate of abiotic mineral oxidation to obtain energy (Bang et al., 1996). In neutraphilic environments these organisms exist at the oxic/anoxic interface to take advantage of the reduced sulfide in the anoxic layer while still having oxygen available for the oxidation (Jorgensen *et al.*, 1983).

Many sulfur oxidizing bacteria use the functional *sox* (sulfur oxidizing) gene cluster to produce proteins necessary for this process to occur (Friedrich *et al.*, 2001, Meyer *et al.*, 2007). *sox gene cluster*. The *sox* gene cluster consists of seven essential genes, *soxXYZABCD* that code for proteins required for sulfur oxidation *in vivo* (Friedrich *et al.*, 2001). These proteins are localized to the periplasm in different SOB (Friedrich *et al.*, 2001). Homologs of the proteins SoxA, SoxB, SoxY, and SoxZ have been determined from the available sequence data of SOB (Friedrich *et al.*, 2001). Primers are available for *soxB* which acts as an indicator for thiosulfate oxidation ability (Meyer *et al.*, 2007). This gene can be used as an indicator for the presence of other *sox* genes in SOB as they are typically found in a gene cluster (Meyer *et al.*, 2007).

In this project I intend to test the hypothesis that if the environmental conditions are significantly different then the community of SOBs would be different between the two Trunk River areas. Using primers for the *soxB* gene I will be able to look at the differences in the sulfide oxidizing community. Also using Terminal Restriction Length Fragment Polymorphism (TRFLP) with 16S rRNA I will be able to see how the total community differs between the sites. I will also characterize the sites by measuring pH and sulfide and sulfate concentration. Enrichment cultures DNA extracts will also be compared to the original inoculums.

Materials and Methods

Samples were collected from two sites at the Trunk River, one close to the mouth of the river (Mouth) and the other further up the river (Deep). Cores at both sites were collected at low tide and a second set of cores for the Deep location collected at high tide. For the low tide samples the Unisense microelectrodes for oxygen and sulfide were immediately used on site, however the sulfide

microelectrode broke and further measurements were taken back in the lab by sectioning the cores. Pore water was extracted at 1-2cm intervals and the sulfate and sulfide concentrations determined using the sulfate assay and the Cline assay, respectively (Cline 1969). The pH of the samples was determined using pH paper.

Enrichment cultures were set up using 1mL slurry of the sediment and surface water from each site. Enrichments were incubated under aerobic, anaerobic, and hypoxic conditions with either 5mM sodium thiosulfate or 5mM sodium sulfide. For 500ml of medium use 500mL of 1X saltwater or freshwater base then add 5mL of 100X NH_4Cl, 5mL of 100x K phosphate solution, 5mL of 1M MOPS Buffer pH 7.2 , and 2mL of resazurin for the anaerobic cultures . Bromothymol blue at a concentration of 0.02g/mL was added to each culture to act as an indicator. After autoclaving add 1 mL of 1M sodium bicarbonate, 0.5mL of 12-vitamin stock, 0.5mL vitamin B_{12} solution and 0.5mL of HCl dissolved trace element stock. The aerobic and hypoxic cultures contained an agarose plug of sodium sulfide to reduce the rate of abiotic oxidation. The anaerobic cultures had 20mM potassium nitrate added as an electron acceptor. All cultures were incubated at room temperature. Transfers of enrichments were made when turbidity could be observed. Sodium sulfide and sulfate concentrations of the enrichments were also determined by using the sulfate assay and the Cline assay.

Sodium sulfide plates were adapted from the Microbial Diversity lab manual for both freshwater and saltwater base. Saltwater or freshwater base plus 30mM sodium sulfate, 6g/L sodium thiosulfate, for anaerobic cultures also include 2g/L sodium nitrate, 15g/L agar and 0.02g/L bromothymol blue. After autoclaving per liter add 10ml of 1M sodium bicarbonate, 1mL of 1000x vitamin solution and 1mL of 1000x trace elements solution. Enrichment cultures were streaked for isolation when turbidity in the liquid cultures was observed.

DNA was extracted from the initial sample sites using MOBIO Power Soil DNA isolation kit following the standard procedure. This DNA was used for a functional gene clone library of *soxB* for each site. The primers used were soxB693F ATC GG**N** CA**R** GC**N** TT**Y** CC**N** TA soxB1446B CAT GTC **N**CC **N**CC **R**TG **Y**TG (Petri *et al.* 2001). A previously developed PCR procedure consisting of a denaturing step of 2 minutes at 94°C, then 10 cycles with an annealing temperature of 55°C, a 30s elongation at 72°C, 40s annealing and 30s denaturing at 94°C (Petri *et al.*2001). Additional 25 cycles will be performed with the annealing temperature at 47°C (Petri *et al.,* 2001). This was modified so that the first annealing temperature was lowered to 52ºC and the second raise to 52ºC. BSA was added to the environmental samples to get cleaner product.

A pCR4-TOPO cloning vector was used that contained resistance to kanamycin. Twenty three colonies were picked from each library for sequencing. The sequencing data was aligned using Muscle before being loaded into MEGA for tree building and distance matrix calculations.

DNA was also extracted from two anaerobic enrichment cultures using the MOBIO Ultra Clean DNA Isolation Kit. This DNA along with the initial site DNA was used for Terminal Restriction Fragment Length Polymorphism (TRFLP). The

primers were for 16S rRNA with the 8F being flouresenctly label and the R1492 unlabeled. Restriction enzymes were used for each site and enrichment, Sau96I and RSAI.

Results and Discussion

The Trunk River was picked as a sample site due to the strong smell of sulfide that was observed during the class trip. This smell suggested that sulfide oxidizing bacteria would be present to take advantage of the energy source. Samples for this project were taken from two points in the Trunk River located in Falmouth, MA (Fig.1).

Figure 1. Sample sites at the Trunk River in Falmouth, MA (Google Maps).

The cores were taken from the Mouth of the river at low tide and sectioned for sulfide. The concentration was determined to be below the detection limits for the assay. This may also have been due to the sandy sediment that did not contain a lot of pore water making if difficult to obtain enough sample to measure accurately. Cores from the Deep part of the river taken at high tide were sectioned at 1-2cm intervals sulfate and sulfide. The concentration of sulfide increased from 1.7mM at 1-2cm to 16.7mM at 12-13cm then down to 8.6cm at 13-14cm in the darker core (C1) which appeared to have more organics present. The increase in sulfide with depth is consistent with the oxygen levels decreasing with depth thereby reducing the amount of abiotic oxidation that could be occurring. However the last data point has a decrease in sulfide although it is furthest from the surface. This discrepancy could be due to oxygen exposure while transporting and sectioning the core. For the second sandier core (C2) the concentration of sulfide increased from 0.7mM at 0-1cm to 5.3mM at 11-12cm below the sediment layer. The concentration was again

increasing with depth but it did not increase as drastically. This is probably due to the decrease in pore water of the sandy sediment. It may also be due to better drainage and circulation of water through the sediment thereby increasing oxygen levels and decreasing sulfide.

The sulfate concentration for C1 decreases from 6.0mM at 0-2cm to 0.5mM at 8-10cm and then increases to 6.8mM at 13-14cm. The first two points is what would be expected and the final point mirrors the decrease observed in the sulfide profile that may be due to incorrect handling of the core. The sulfate concentration for C2 starts at 8.57mM and then decreases to 5.5mM at 11-12cm (Fig. 2). The concentration of sulfate in C2 does not closely mirror the profile for sulfate. This may be due to the sample size being less than optimal for the assay.

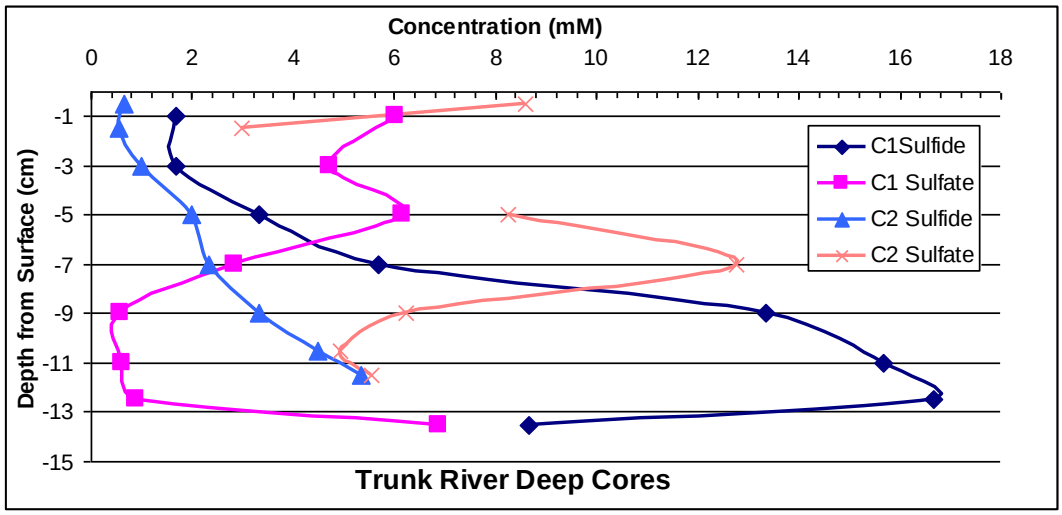

Figure 2. This graph shows the sulfide and sulfate profiles of Core 1 and Core 2 with depth shown as going deeper into the sediment. These cores were taken from the Deep section of Truck River at high tide.

The pH of the sediment was pH 7 for all of the cores obtained, however the pH of the surface water varied with the tide. At low tide the surface water at Mouth had a pH 6 and at the Deep pH of 8. The salinity at low tide at the Mouth was 4ppt and at the Deep 3ppt. At high tide the pH of the surface water at the Mouth was pH 5.8 and at the Deep was pH 6.1. The salinity at high tide was 7ppt at the Mouth and 5ppt at the Deep. (Table 1) The differences between the two sites suggest that the organisms that live near the mouth must be more adapted to high salinity and a lower pH while in the deep the organisms are adapted to a high pH and lower salinity. However because the differences are not very large it is likely that some organisms will be able to live in both conditions.

	Mouth High Tide	Mouth Low Tide	Deep High Tide	Deep Low Tide
Salinity (ppt)	7	4	5	3
pH	5.8	6	6.1	8

Table 1. The pH and salinity of the two sites at high tide and low tide.

After characterizing the sampling site the next step was to enrich for sulfide oxidizing bacteria. Three oxygen levels, two water types, and two sulfide sources were compared to attempt to pulla range of organisms out of the environment.

After one liquid transfer plates were streaked for the anaerobic cultures. After six days eleven very small colonies were picked off the anaerobic culture plates and placed directly into the 16S rRNA PCR reactions. From these reactions only seven had bands when run on an agarose gel. The colonies that worked were labeled A1, A2, A3, A4, A7, A8, and A9. The location of the inoculums and conditions for liquid growth are summarized in Table 2.

	Saltwater/Freshwater	Site	Electron donor
A1	SW	Deep	$Na_2S_2O_3$
A2	SW	Deep	Na_2S
A3/4	FW	Deep	Na_2S
A7	FW	Mouth	Na_2S
A8	FW	Deep	Na_2S

Table 2. The liquid culture conditions from which the isolates were streaked. SW, saltwater, FW, freshwater. A3/4 had two different colony types on the streak plate.

The sulfide and sulfate concentrations was determined from the original liquid culture from these plates were streaked (Fig. 3). The highest concentration of sulfide was seen in the uninoculated medium with only sulfide as would be expected since there are no organisms present to mediate the oxidation process. The concentration of sulfide in the rest of the cultures was below the detection limit of the assay. The concentration of sulfate is low in the sulfide background while the concentration is higher in the active cultures showing that the sodium sulfide and sodium thiosulfate are being oxidized to sulfate. However the cultures that were inoculated from the same site and incubated with 5mM sodium sulfide released much more sulfide than the one with sodium thiosulfate. This suggests that these organisms prefer sodium sulfide over thiosulfate.

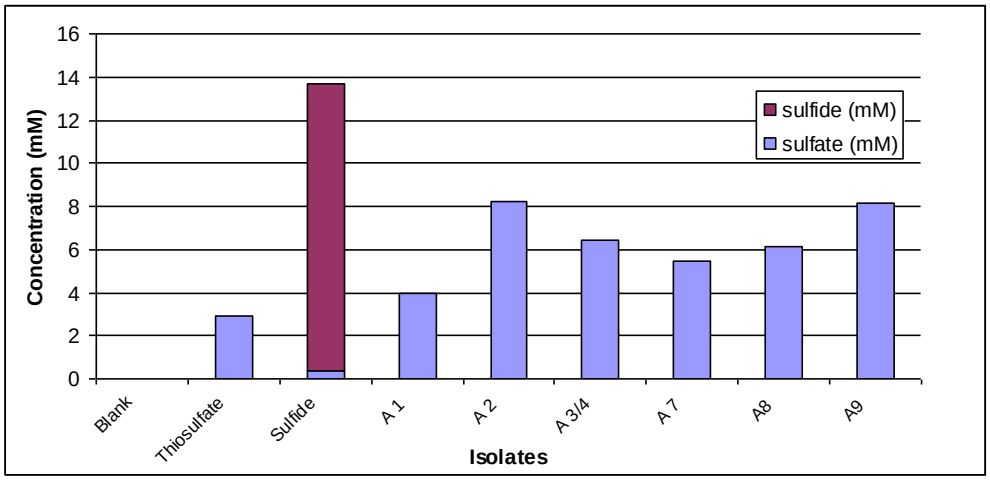

Figure 3. This graph shows the concentrations of sulfate and sulfide in the liquid culture that was used to obtain these isolates.

Eleven aerobic colonies were picked off of plates for single colony 16S rRNA PCR. From these reactions only seven has a clear single band on the agarose gel. These isolates are S1, S2, S3, F1, F2, F4, and F5. The conditions for growth and the sample site are displayed in Table 3.

	Saltwater/Freshwater	Site	Electron donor
S1	SW	Deep	$Na_2S_2O_3$
S2	SW	Mouth	Na_2S
S3	SW	Deep	Na_2S
F1/2	FW	Deep	Na_2S
F4/5	FW	Deep	$Na_2S_2O_3$
M1	SW	Mouth	$Na_2S_2O_3$
M2	SW	Deep	$Na_2S_2O_3$
M3	SW	Deep	Na_2S
M4	FW	Mouth	Na_2S

Table 3. The liquid culture conditions that the different isolates were obtained from. SW/FW indicates saltwater or freshwater respectively. For F1/2 and F4/5 two colonies were observed on each plate and picked for sequencing. The 'S' and 'F' isolates are aerobic. The 'M' cultures were incubated in the hypoxic chamber.

The Cline and sulfate assay were performed to determine the concentrations of sulfide and sulfate in both the aerobic and hypoxic cultures. In all the cultures the concentration of sulfide was below the detection limit. The concentration of sulfate in most of the inoculated cultures was higher than the background levels except in M2. This may be due to M2 being unable to utilize sodium thiosulfate however the same inoculum M3 with sodium sulfides was able to release sulfate. This suggests that not all organisms are able to oxidize all forms of reduced sulfur.

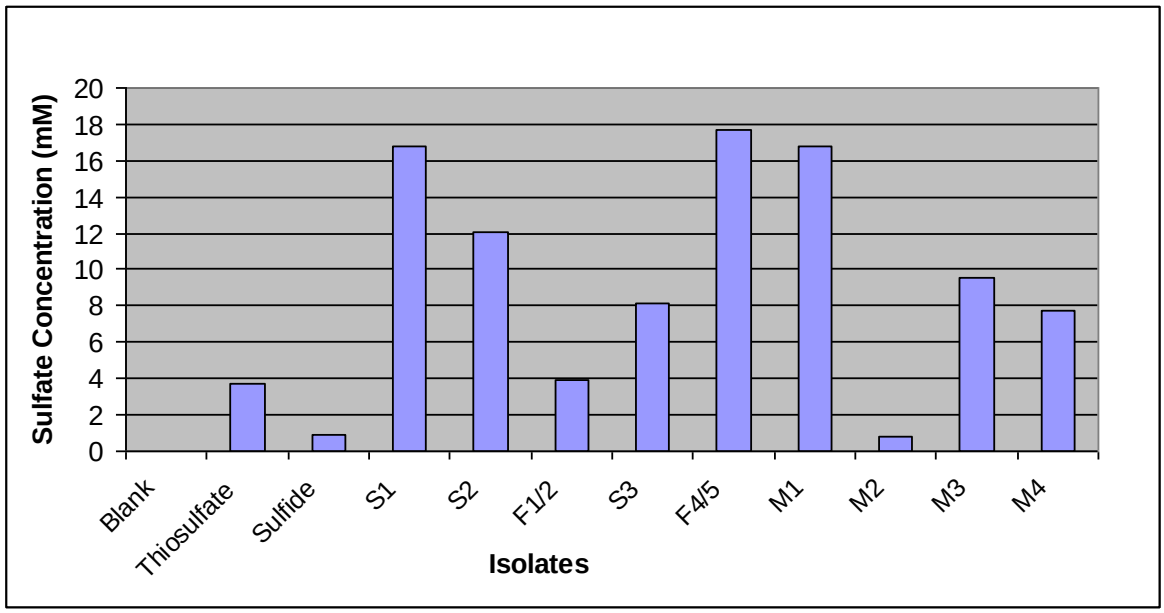

Figure 4. The concentration of sulfate in the aerobic and hypoxic cultures.

All of the isolates with a positive16S rRNA PCR product were sequenced. Of the anaerobic cultures only A1 had a good sequence and was determined to be Bacteria[100%] Proteobacteria[100%] Betaproteobacteria[100%] Burkholderiales[100%] Alcaligenaceae[100%] Castellaniella[100%] using the Ribosomal Database Project (RDP) classifier (Wang *et al.* 2007). For the aerobic culture isolates classifications was obtained for F1, F2, F4, F5, S1, S2, S3. The classification for these are F1 Bacteria[100%] Bacteroidetes[100%] Sphingobacteria[36%] Sphingobacteriales[36%] Flexibacteraceae[34%] Fabibacter[20%] , F2 Bacteria[100%] Proteobacteria[100%] Gammaproteobacteria[100%] Aeromonadales[100%] Aeromonadaceae[100%] Aeromonas[100%], F4 Bacteria[100%] Proteobacteria[100%] Gammaproteobacteria[100%] Pseudomonadales[100%] Pseudomonadaceae[100%] Pseudomonas[100%], F5 Bacteria[100%] Proteobacteria[100%] Gammaproteobacteria[100%] Chromatiales[100%] Halothiobacillaceae[100%] Thiovirga[100%], S1 Bacteria[100%] Proteobacteria[100%] Gammaproteobacteria[100%] Pseudomonadales[100%] Pseudomonadaceae[100%] Pseudomonas[100%], S2 Bacteria[100%] Proteobacteria[100%] Gammaproteobacteria[100%] Aeromonadales[58%] Aeromonadaceae[58%] Oceanisphaera[49%], S3 Bacteria[100%] Proteobacteria[100%] Gammaproteobacteria[100%] Alteromonadales[100%] Shewanellaceae[100%] Shewanella[100%] (Wang *et al.* 2007). These were all put into a tree using RDP's tree builder and also known sequences were incorporated (Fig. 5) (Cole *et al.* 2007 and 2009). Several of these the *Pseudomonas* and *Halothibacillaceae* have family members that are able to use reduced sulfur compounds. The isolates were also used for a functional gene PCR of *soxB*. The anaerobic isolates did not amplify for *soxB* and all of the sequences obtained for the aerobes where bad. This may have been due to the small size of the colonies or that the PCR needs to be further optimized. The isolates F4, F5, and S1 are the most likely to be able to oxidize reduced sulfur due to their relatedness to known sulfide oxidizers. The lack of amplification for *soxB* could also suggest that they use a different pathway for sulfide oxidation.

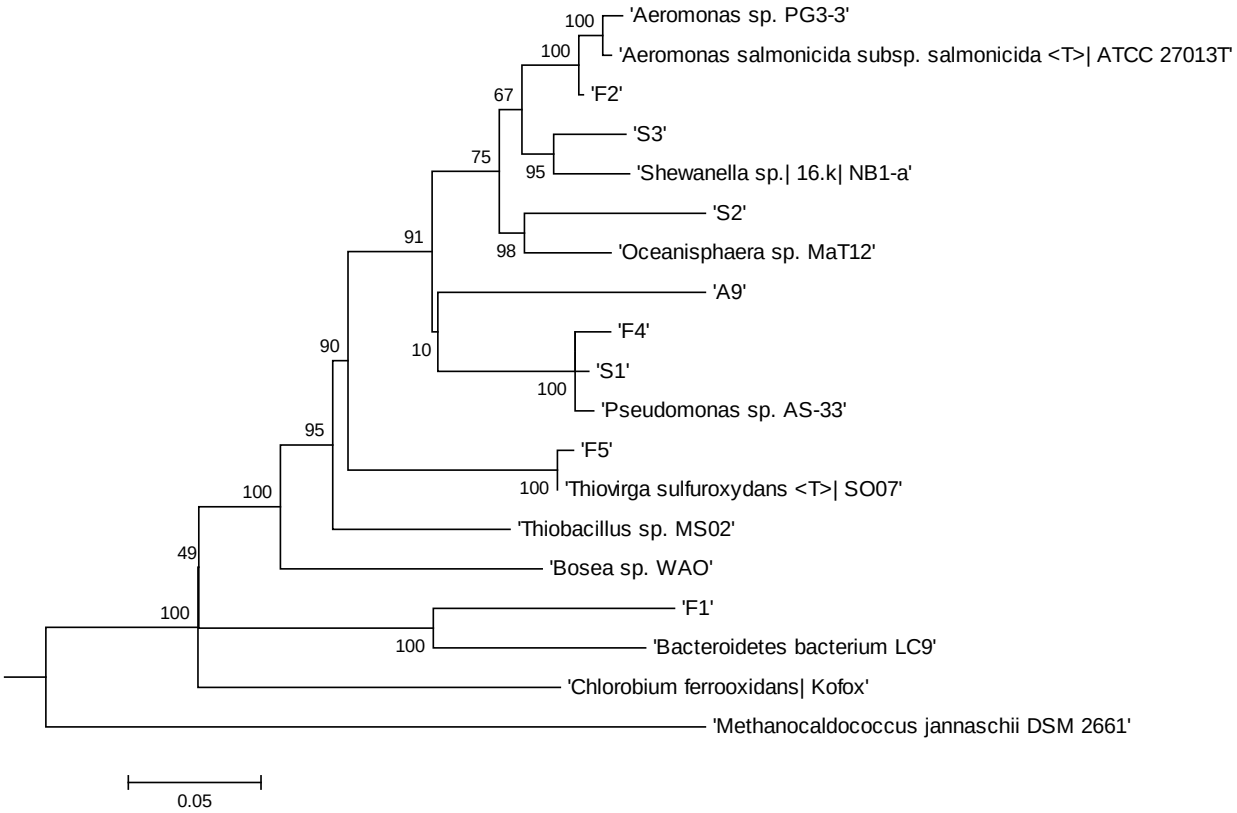

Figure 5. 16S rRNA tree build with RDP's tree builder of the isolates and known sequences.

Two clone libraries were constructed from DNA extracted from each sample site. Twenty three clones were picked for each site and sequenced. After removal of the bad sequence reads only 39 total clones remained. The *soxB* clone tree shows that some of the clones do cluster according to where they were sampled; however there is also some mixing of the clones (Fig. 6) (Tamura *et al.* 2007). A second tree includes the various blast hits that matched the clades (Fig. 7) (Tamura *et al.* 2007).

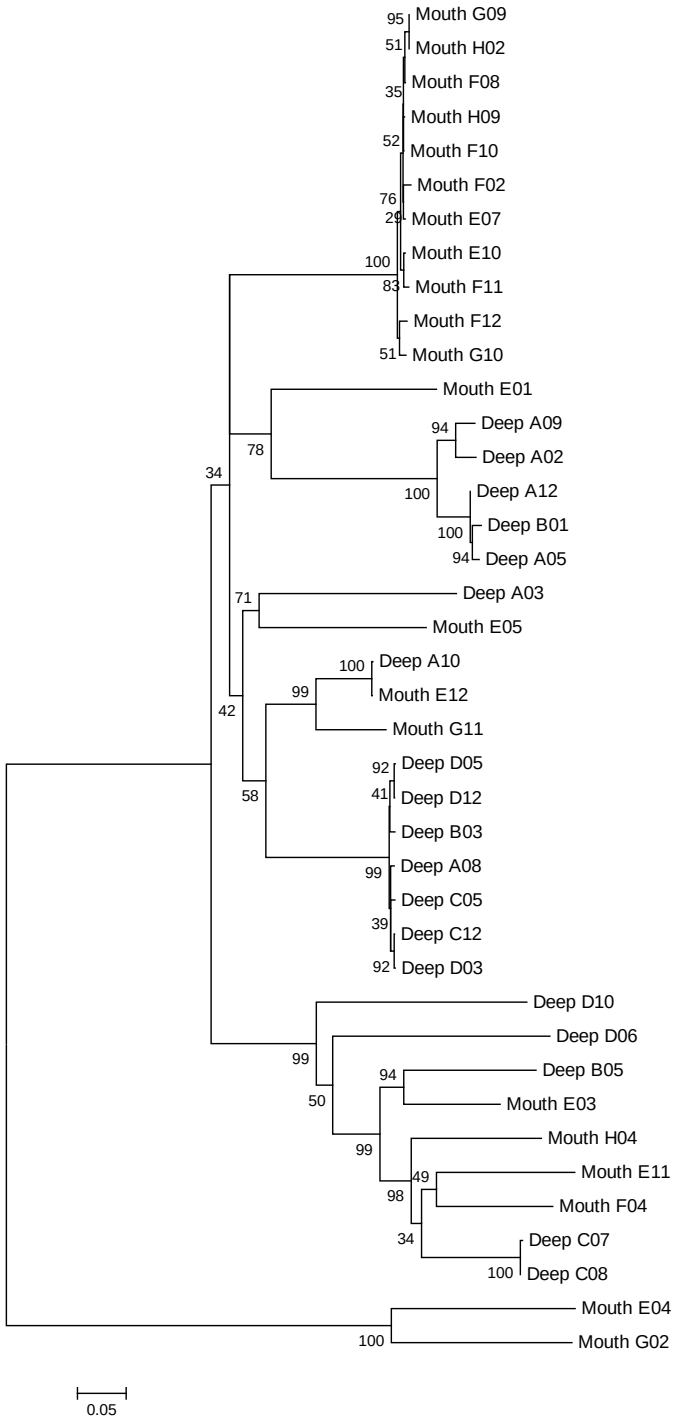

Figure 6. A bootstrap tree of the *soxB* clones showing the distribution of clones from the two sites.

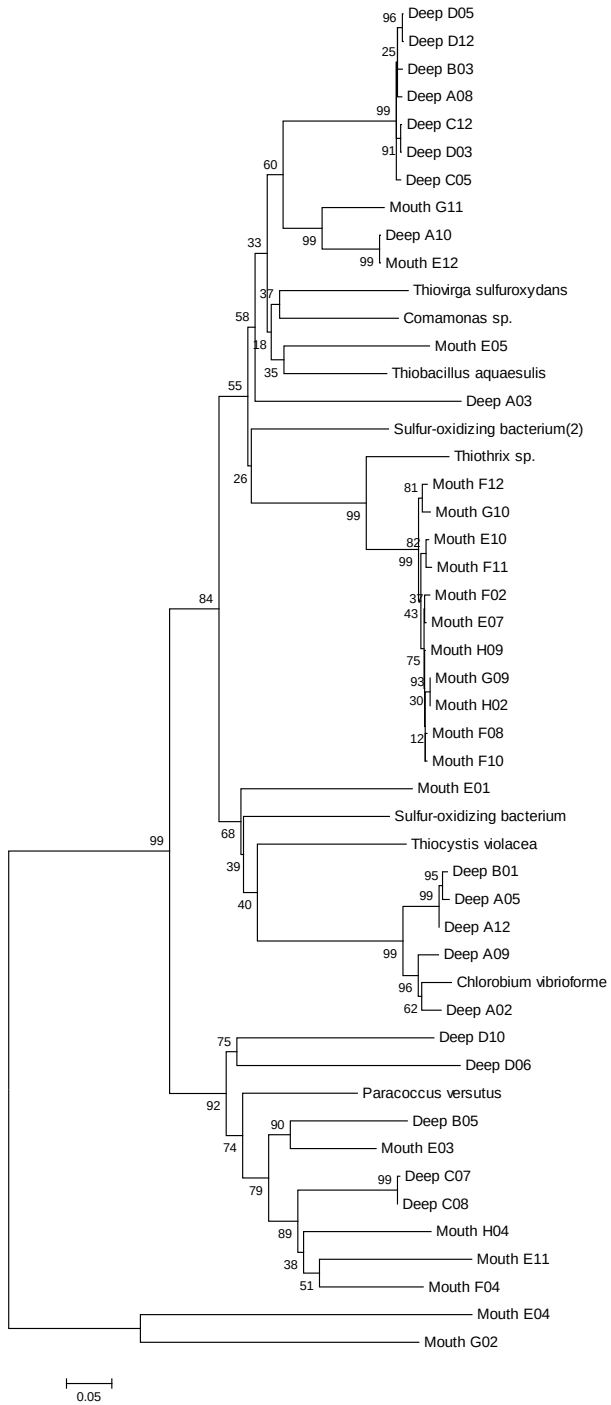

Figure 7. A bootstrap tree of the *soxB* clones that includes BLAST hits of the closest matching organisms for the different clades.

These trees support the hypothesis that the site environment is different between the mouth and the deep which causes their communities to be different. However since some clades have representatives from both sites it shows that the differences between the sites is not great enough to completely exclude members of one community from the other site's community.

Terminal restriction fragment length polymorphism was also used to determine the differences between the communities at the Mouth and the Deep of the Trunk River. Two different restriction enzymes were used and then the two sites were compared by the presence and absence of peaks. DNA extracts of two anaerobic enrichments were also used to see how the community was changing during the enrichments from each site. By comparing the RSAI initial TRFLP it is evident that the peak pattern is different between the two sites, however some of the peaks do match but have different heights (Fig. 8). This supports the clone library tree where some of the clades are mixed. When comparing the initial DNA extractions for each site to the enrichment culture DNA it is evident that the number of peaks are decreasing. This suggest that a specific group of organisms are being enriched for and other organisms are being selected against (Fig. 8).

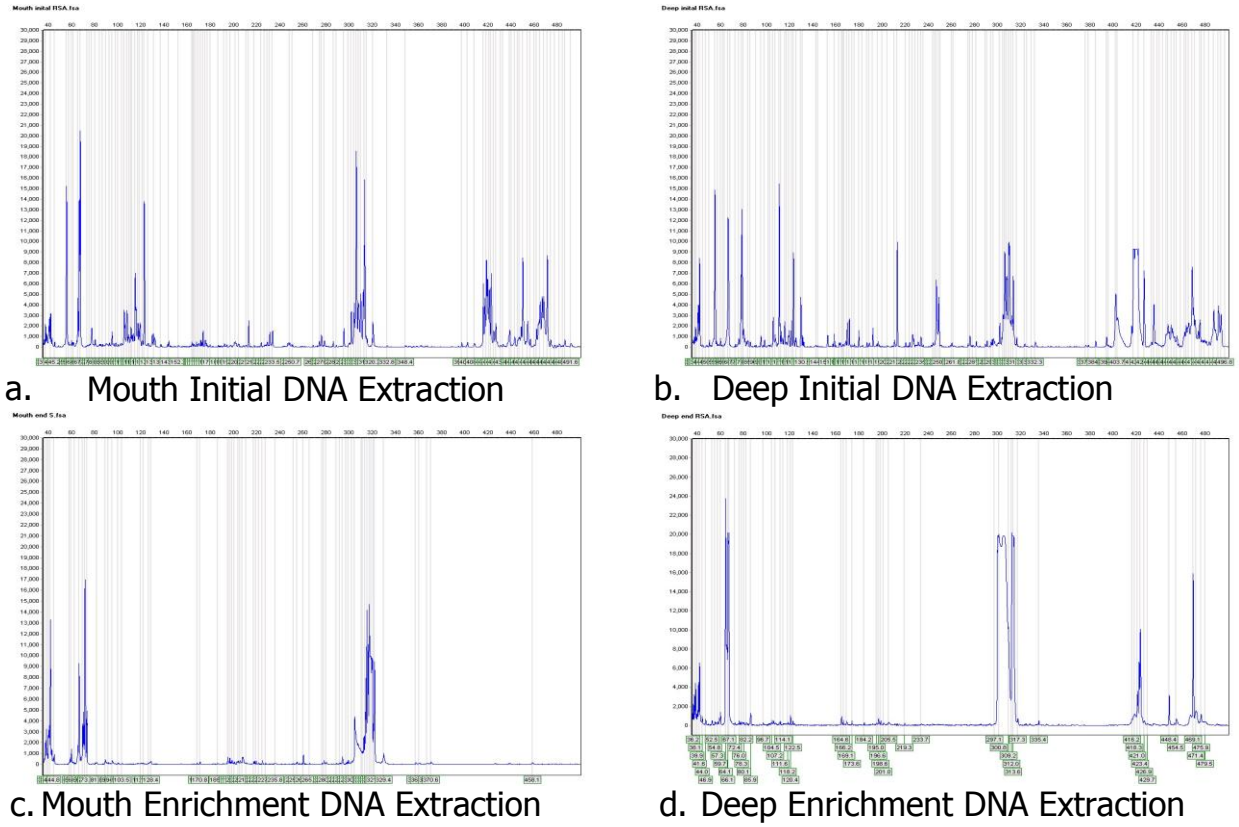

Figure 8. TRFLP electropherogram of the two sites initially and then after enrichment using the RSAI restriction enzyme.

TRFLP was also completed using the SAU96I restriction enzyme. This electropherogram has a different peak pattern than RSAI because they cut the DNA at different sequences (Fig. 9). When comparing the two initial samples the pattern is different again supporting the idea that the sites have different communities. When compared to the later time point the number of peaks had decreased in both

of the enrichments suggesting that as expected a specific subpopulation is being enriched.

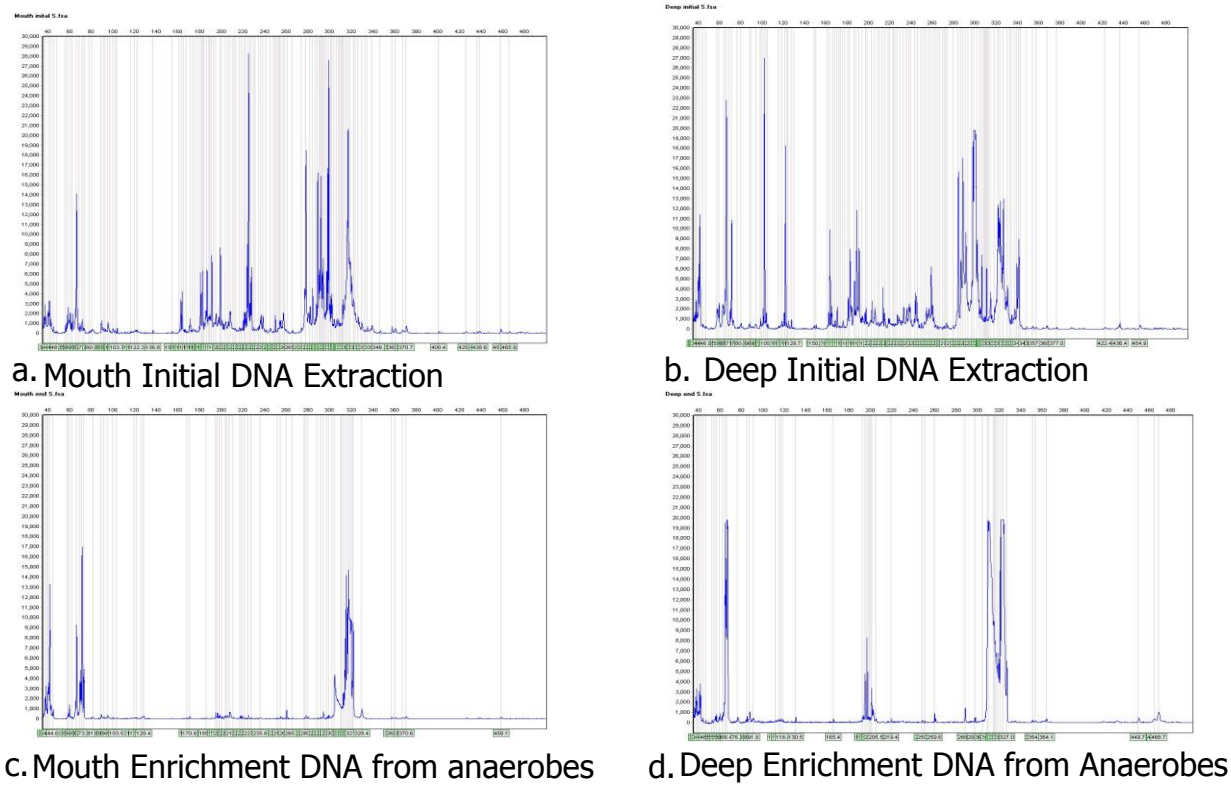

Figure 9. TRFLP electropherogram of the two sites initially and then after enrichment using the Sau96I restriction enzyme.

Through site characterization and molecular techniques it has been shown that there are differences in the two sites and that this has an effect on the microbial community. However since these sites are not extremely different there are still some organisms that can crossover and live in both sites as is shown with the clone library and the TRFLP. It was clear that the enrichments were selecting for a subpopulation of microorganisms.

More diversity was seen with the *soxB* gene clone library than with the enrichment culture isolates which helps to reinforce the idea of culture bias. However some of the organisms in the liquid cultures appeared to be oxidizing the different forms of sulfide suggesting that it would have been possible to isolate more organisms either by making more dilutions then streaking or doing dilution to extinction techniques.

The first future experiment would be to make a 16S rRNA clone library of the two sites and the same anaerobic enrichment cultures so that the peaks on the TRFLP could be identified. This way the organisms that are disappearing or increasing can be determined. It is also important to continue the isolation process in the enrichment cultures so that some of the organisms in the system can be

studied further. It would also be interesting to go to the Trunk River and leave probes for sulfide, oxygen, and pH over several tidal cycles to clearly see what the normal conditions are instead of just a single time point. This will make it easier to determine how different the sites actually are from one another. Additionally the PCR for *soxB* should be further optimized as the primers were amplifying multiple size fragments and also amplifying something in organisms that should not have *soxB* such as *E.coli*.

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