Long-term oil contamination yields novel phylum of bacteria by 454 pyrosequencing

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Abstract:

Long-term oil contamination has significant impacts on plant and animal communities, but little is known about the long-term effects on sediment microbial communities. My study examined the effects of residual oil contamination on sediment communities 40 years after an oil spill. I found minimal effects of oil contamination on microbial communities, but did find a novel phylum of organisms resistant to environmental toxins and radiation. Further exploration into this question is warranted to determine the function of these organisms in contaminated sediment.

Introduction:

Four major oil spills have occurred since January of 2011 across the world. These spills have immediate, catastrophic consequences such as loss of habitat, loss of wildlife, and large impacts on overall ecosystem function. In addition, the legacy effects of these spills may persist for decades in ecosystems. Little research has been done to understand the long-term effects of oil contamination on bacterial communities responsible for degrading the chemicals found in oil.

Polycyclic aromatic hydrocarbons are the main remnants of un-degraded oil in ecosystems, a large percentage of which are alkylated naphthalene compounds. Naphthalene is a known human carcinogen, and can have drastic effects on native plant and animal life within ecosystems. Naphthalene degradation can occur both aerobically and anaerobically in ecosystems.

In 1969, the *Florida* barge spilled 700,000 L of oil into Buzzards Bay off the coast of Falmouth, Massachusetts. This spill had immediate effects on the

native wildlife, such that all plant and animal life within the spill zone died. 40 years later, research has shown that significant long-term effects are evident. Researchers have shown levels of total petroleum hydrocarbons (TPH) at depths of 10-20 cm below the surface that are equivalent to what was seen at the surface level in the early 1970s. This contamination has decreased stem density and above- and below-ground biomass of Spartina alterniflora resulting in increased erosion and decreased sediment stabilization (Culbertson *et al.*, 2008). In addition, behavioral studies conducted with U. pugnax from contaminated areas found that crabs exposed to the oil avoided burrowing into oiled layers, suffered delayed escape responses, lowered feeding rates, and achieved lower densities (Culbertson *et al.*, 2007).

Because these long-term ecosystem level effects still persist, my research aimed to examine how TPH contamination altered the sediment microbial community. My research was focused to answer two main questions; 1. What effect does long term TPH contamination have on the microbial community, and 2. Do sediment communities that have evolved in the presence of TPH harbor distinct community members capable of degrading one portion of TPH, naphthalene? I hypothesized that community shifts would be evident based on TPH contamination, and that novel community members would arise capable of degrading naphthalene in areas of contamination.

Materials and Methods:

Sample Collection and Processing:

Sediment cores were collected (5 cm diameter, 20 cm depth) in the low

marsh at two locations in Buzzards Bay, Wild Harbor and Great Sippewissett (n = 2; Figure 1). Wild Harbor contains measurable levels of TPH contamination, while Great Sippewissett is naïve to these compounds. These areas are comparable in other physical properties such as sediment texture and pH thus making them ideal for comparing bacterial communities.

Sediment cores were returned to the laboratory and sectioned from 0-10 cm depth and 10-20 cm depth to allow comparisons between depths. Each depth was homogenized per core by mixing sediment.

DNA Extraction:

DNA was immediately extracted at each depth from each of the cores using the MoBio Power Soil DNA kit. Approximately 0.25 g of sediment was used in each extraction. The manufacturer protocol was followed with two slight modifications. First, two minutes of bead beating was used in place of the 10 minute vortexing step, and the DNA was eluted in 50 ul instead of 100 ul. DNA was frozen at -20 C and used for subsequent 454 sequencing of the 16s rRNA gene.

454 Pyrosequencing:

Initial DNA samples were sent for 454 pyrosequencing of the 16s rRNA gene using primers 8F and 1492R.

Functional PCR:

PCR was performed to assess the naphthalene dioxygenase gene using primers Ac114F (CTGGC(T_A)(T_A)TT(T_C)CTCAC(T_C)-CAT**)** and Ac596R (C(G_A)GGTG(C_T)CTTCCAGTTG). PCR amplification was carried out in 50 ul

reaction volumes, with primer concentration of 0.3 **u**M and a cycling regime of 94°C for 5 min (1 cycle); 94°C for 1 min, 56°C for 45 sec, and 72°C for 45 sec (35 cycles); 72°C for 10 min (1 cycle). Due to the inability to amplify products, the PCR reaction was optimized to reduce inhibition and alter annealing temperatures. DNA concentrations tested ranged from 50 ng per reaction to 0.05 ng per reaction. Annealing temperatures were run in a gradient fashion from 45 C to 65 C.

Stable Isotope Probing:

A microcosm incubation was established immediately following sample collection to examine the community actively degrading naphthalene. 20 g of sediment and 20 mL of sterile seawater were added to 100 mL glass jars. 20 ppm of naphthalene (unlabeled control and C13 labeled) was added to each vial resulting in eight discrete samples (2 depths X 2 replicates X 2 treatments). Sediment slurries were incubated for 48 hours at room temperature on a shaker with sediment samples extracted at 12, 24, and 48 hours. DNA was extracted from sediment samples at time points of 12 and 24 hours, as described above, and 500 ng was added to a Cesium chloride gradient with a density of 1.762 g/ml in gradient buffer (15mM Tris–HCL,15mM KCl, 15mM EDTA, pH 8.0). Gradient tubes were spun at 55,000 X g for 68 hours to separate heavy and light fractioned DNA. After centrifugation, density fractionation was done using a syringe pump to dispense mineral oil at a rate of 200 ul / minute resulting in 200 ul fractions DNA. The refractive index of each density fraction was measured immediately after collection using an AR 200 digital refractometer. DNA from

fractions 1-15 at the 24 hour time point were precipitated with isopropanol, and subsequent analyses were performed on these DNA samples.

Quantitative PCR

The distribution of DNA in CsCL fractions was determined using quantitative PCR in conjuction with SYBR green and primers Bact 519F and Bact 907R. Reactions were carried out in 25 ul volumes using primers at a concentration of 1 uM. 1 uL of DNA was added to each reaction. Thermocycler parameters are as follows: 50 C hold for 2 min and a 95 C hold for 10 min followed by 40 cycles of 15 s at 95 C, 60 s at 60 C (Buckley *et al.*, 2008).

Clone library construction:

PCR was performed on DNA fractions at a density of 1.72 g/ml (unlabeled DNA) and 1.75 g/ml (labeled DNA) to assess the bacterial community actively taking up the labeled carbon substrate. PCR was performed using bacterial primers 8F and 1492R and Ac114F and Ac596R. PCR products were gel purified, cloned into TOPO vector and transformed in E.coli to generate clone libraries for community analyses.

Statisitical Analyses:

454 pyrosequences were aligned and clustered using qiime (Caporaso *et al.*, 2010). Rarefaction analysis was also conducted in qiime to assess the extent to which the community was sampled. Unifrac analysis was conducted in qiime to assess bacterial community similarity across all samples and replicates (Knight *et al.*, 2006).

Results:

454 Pyrosequencing:

454 pyrosequencing yielded greater than 1000 sequences per sample, resulting in approximately 27 unique phyla across all samples (Figure 1). Rarefaction analysis showed the sampling effort was not extensive enough to fully saturate the microbial community (Figure 2). Closer inspection of the phyla evident across all samples yielded one phylum unique to the Wild Harbor 0-10 cm depth samples, *Deinococcus-thermus* (Figure 3). UniFrac analysis of the community showed that replicate samples closely resembled one another, followed by grouping based on sample depth (Figure 4).

Functional PCR and clone library construction:

PCR of the functional gene nahA yielded one sample, Great Sippewisset 0-10 cm depth replicate 1, that produced an amplicon of approximately 500 base pairs. All other samples did not amplify after alteration of the annealing temperature and concentration of DNA template. A clone library was constructed from this one positive sample. Sequences yielded from this clone library showed no positive match in the NCBI database after blasting. Also, these sequences did not match the documented sequence of the nahA gene.

Stable isotope probing, quantitative PCR, and clone library construction:

Quantitative PCR on the fractions of DNA extracted from the stable isotope probing yielded low and erratic DNA concentrations (Figure 5). Due to time constraints, the qPCR reaction could not be further optimized; so all fractions were analyzed using conventional PCR to test for presence/absence of DNA (Figure 6). PCR showed incomplete separation of the DNA, which yielded

presence of DNA in fragments that should have been empty. Unlabeled DNA should have yielded a strong band at approximately 1.71 g/ml density while labeled DNA should have yielded a band around 1.75 g/ml density. Even though the presence of DNA was evident in bands surrounding the targeted density, clone libraries of the 16s rRNA gene were constructed. Transformation failed during cloning so no clones were evident after growing on LB plates over night.

Discussion:

Overall, the majority of the experiments for this project failed, so robust conclusions cannot be drawn about the legacy effects of oil contamination on sediment microbial communities. 454 pyrosequencing did not show significantly different microbial communities based on sample site, but instead yielded clusters of microbial communities based on depth sampled. Pyrosequencing did reveal a novel phylum of bacteria, *Deinococcus-thermus*, that has been shown to degrade oil under thermophillic conditions (Hao *et al.*, 2004). This phylum was only evident in two replicate samples from Wild Harbor 0-10 cm samples.

Deinococcus-thermus is a bacterial phylum with members occurring in extreme thermophillic environments and also commonly in subsurface soils/sediments (Hao *et al.*, 2004, Saul *et al.*, 2005). This phylum is well known for it's resilience in environments that experience extreme toxicity and radiation (Gupta & Griffiths, 2007). Occurrence of this phylum in the Wild Harbor sediment may be due to the oil spill and subsequent long-term contamination of this site. Unlike other studies, an increase in proteobacteria capable of degrading oil did not occur in Wild Harbor as expected. Instead, UniFrac

analysis showed that bacterial communities were similar based depth instead of location.

Conclusions:

Although the stable isotope probing incubation was unsuccessful, 454 sequencing yielded results demonstrating the appearance of a novel phylum of bacteria due to TPH contamination. Further research into this question using stable isotope probing and functional gene analysis is warranted to better understand the long-term effects of oil contamination on sediment microbial communities.

Acknowledgements:

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Literature Cited:

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Caporaso Jg, Kuczynski J, Stombaugh J *et al.* (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods,* **7**, 335-336.

Culbertson Jb, Valiela I, Peacock Ee, Reddy Cm, Carter A, Vanderkruik R (2007) Long-term biological effects of petroleum residues on fiddler crabs in salt marshes. *Marine Pollution Bulletin,* **54**, 955-962.

Culbertson Jb, Valiela I, Pickart M, Peacock Ee, Reddy Cm (2008) Long-term consequences of residual petroleum on salt marsh grass. *Journal of Applied Ecology,* **45**, 1284-1292.

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Figure Legend:

Figure 1. Map from Culbertson et al. showing areas sampled in Wild Harbor and Great Sippewissett (Culbertson *et al.*, 2008).

Figure 2. Bacterial and archaeal phyla resulting from 454 pyrosequencing of samples collected at Wild Harbor and Great Sippewissett, Falmouth Massachusetts. Communities were characterized at two depths; 0-10 cm and 10-20 cm.

Figure 3. Rarefaction curves for all samples and depths of the microbial community demonstrating that the sampling effort did not adequately cover all members of the community.

Figure 4. Closer inspection showed that the Wild Harbor 0-10 cm samples harbored a unique phyla, *Deinococcus-thermus*, that is resistant to radiation and environmental hazards.

Figure 5. UniFrac tree depicting similarity between samples. Replicate samples are the most similar, followed by grouping by sample depth.

Figure 6. qPCR results on unlabeled and labeled fractions of DNA from the stable isotope incubation.

Figure 7. PCR results on unlabeled and labeled fractions of DNA from the stable isotope incubation to assess presence/absence of DNA.

Figure 1.

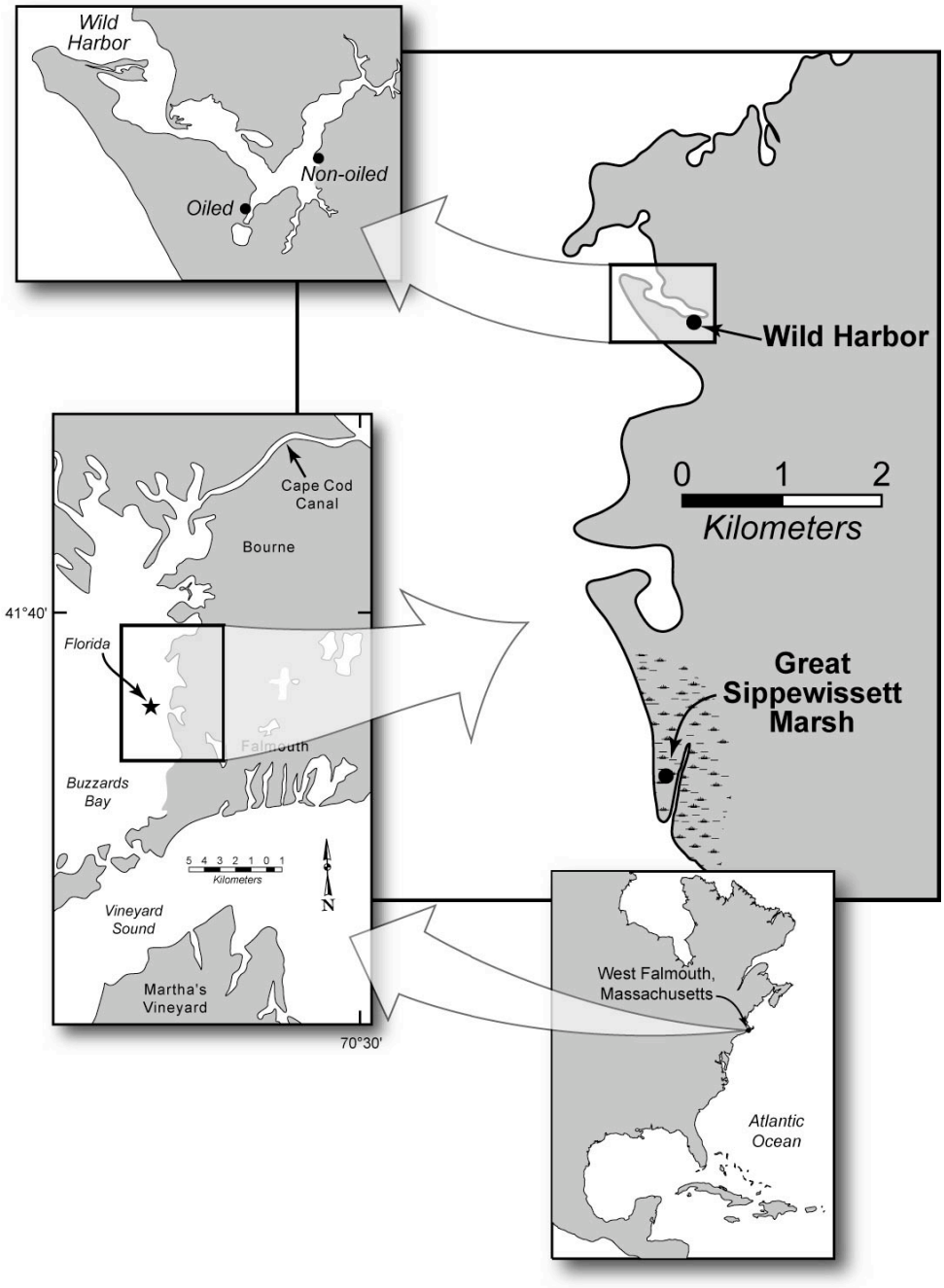

Wild Harbor
Non-oiled
Oiled
Wild Harbor
0 1 2
Kilometers
Cape Cod Canal
Bourne
41°40'
Florida
Great Sippewissett Marsh
Falmouth
Buzzards Bay
5 4 3 2 1 0 1
Kilometers
N
Vineyard Sound
Martha's Vineyard
70°30'
West Falmouth, Massachusetts
Atlantic Ocean

Figure 2.

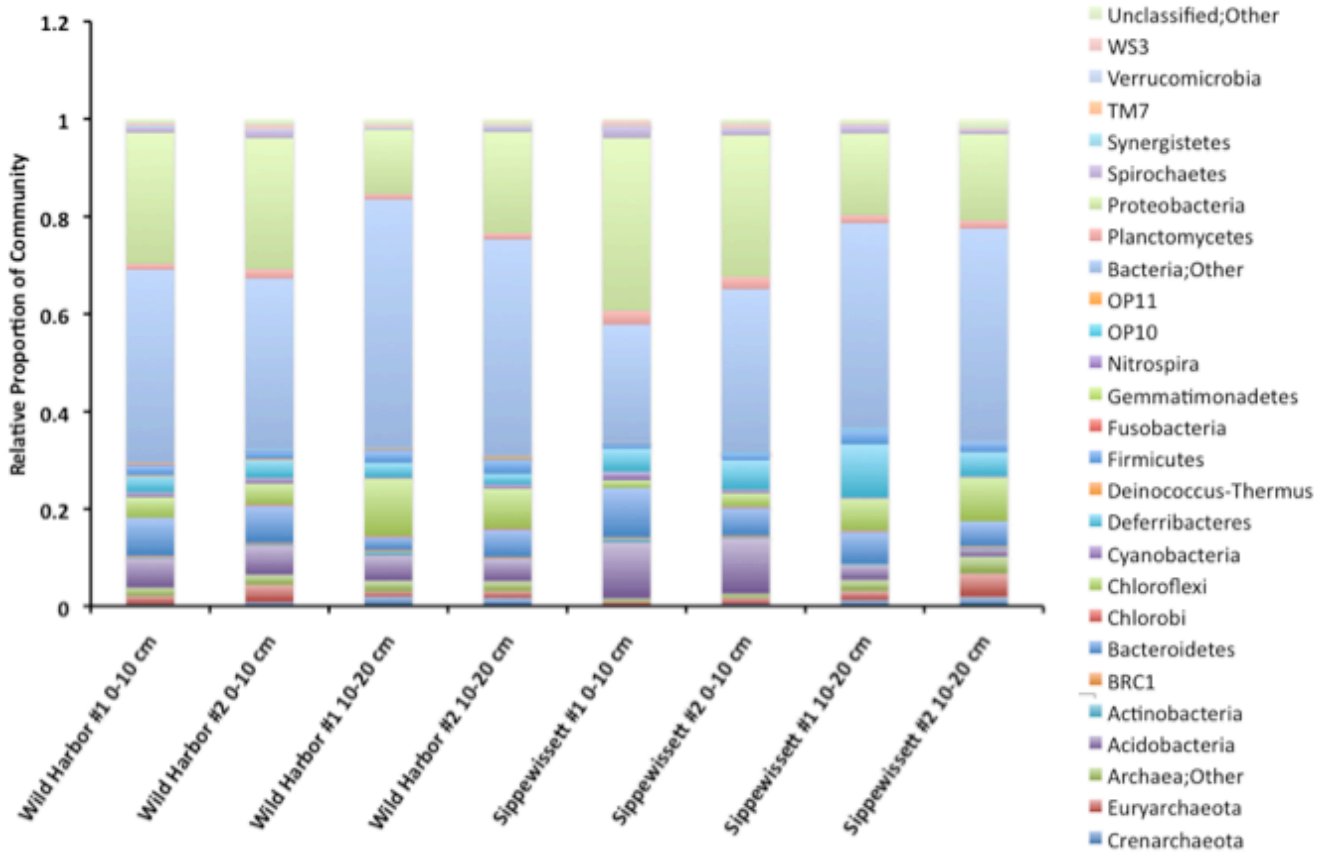
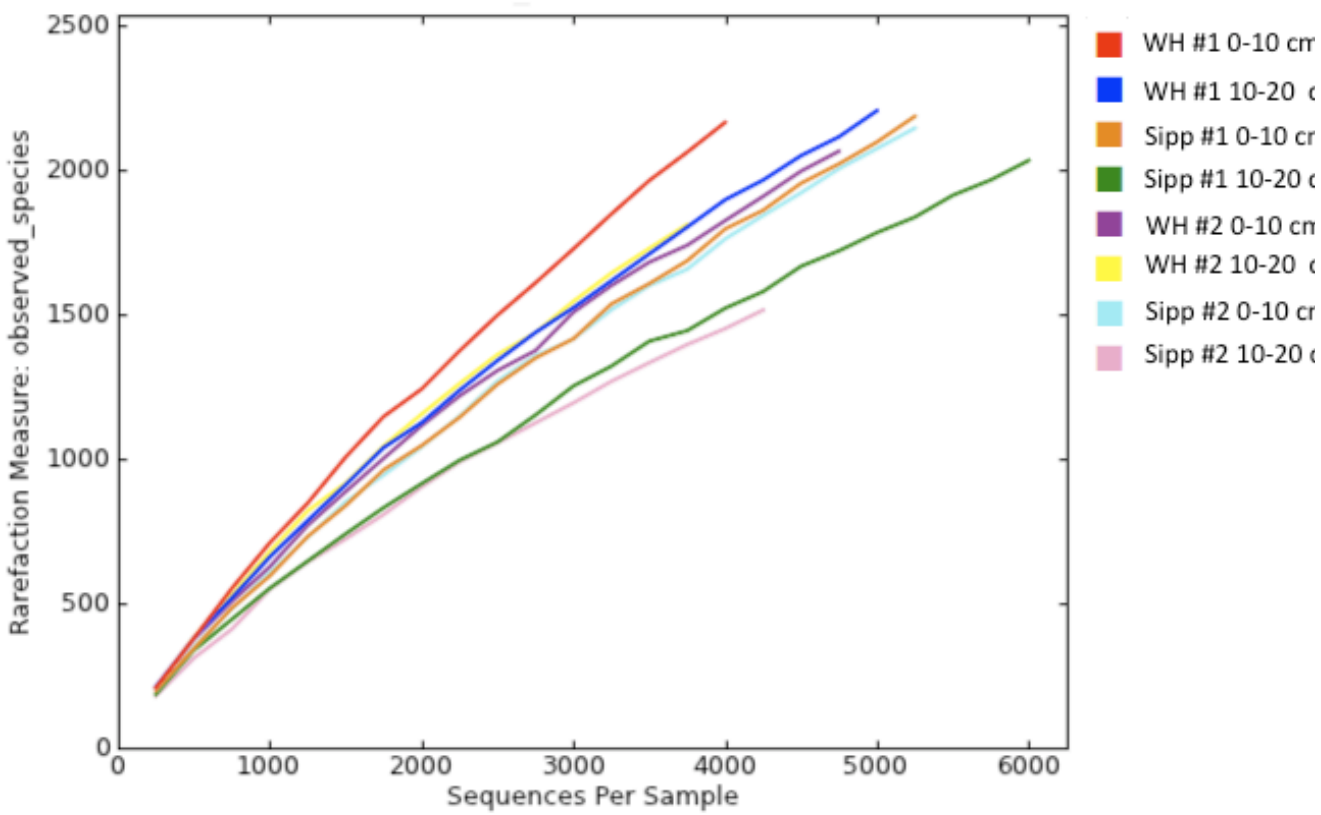

Relative Proportion of Community
1.2
1
0.8
0.6
0.4
0.2
0
Wild Harbor #1 0-10 cm
Wild Harbor #2 0-10 cm
Wild Harbor #1 10-20 cm
Wild Harbor #2 10-20 cm
Sippewissett #1 0-10 cm
Sippewissett #2 0-10 cm
Sippewissett #1 10-20 cm
Sippewissett #2 10-20 cm
Unclassified;Other
WS3
Verrucomicrobia
TM7
Synergistetes
Spirochaetes
Proteobacteria
Planctomycetes
Bacteria;Other
OP11
OP10
Nitrospira
Gemmatimonadetes
Fusobacteria
Firmicutes
Deinococcus-Thermus
Deferribacteres
Cyanobacteria
Chloroflexi
Chlorobi
Bacteroidetes
BRC1
Actinobacteria
Acidobacteria
Archaea;Other
Euryarchaeota
Crenarchaeota

Figure 3.


Rarefaction Measure: observed_species
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2000
1500
1000
500
0
0
1000
2000
3000
4000
5000
6000
Sequences Per Sample
WH #1 0-10 cm
WH #1 10-20 c
Sipp #1 0-10 cr
Sipp #1 10-20 c
WH #2 0-10 cm
WH #2 10-20 c
Sipp #2 0-10 cr
Sipp #2 10-20 c

Figure 4.

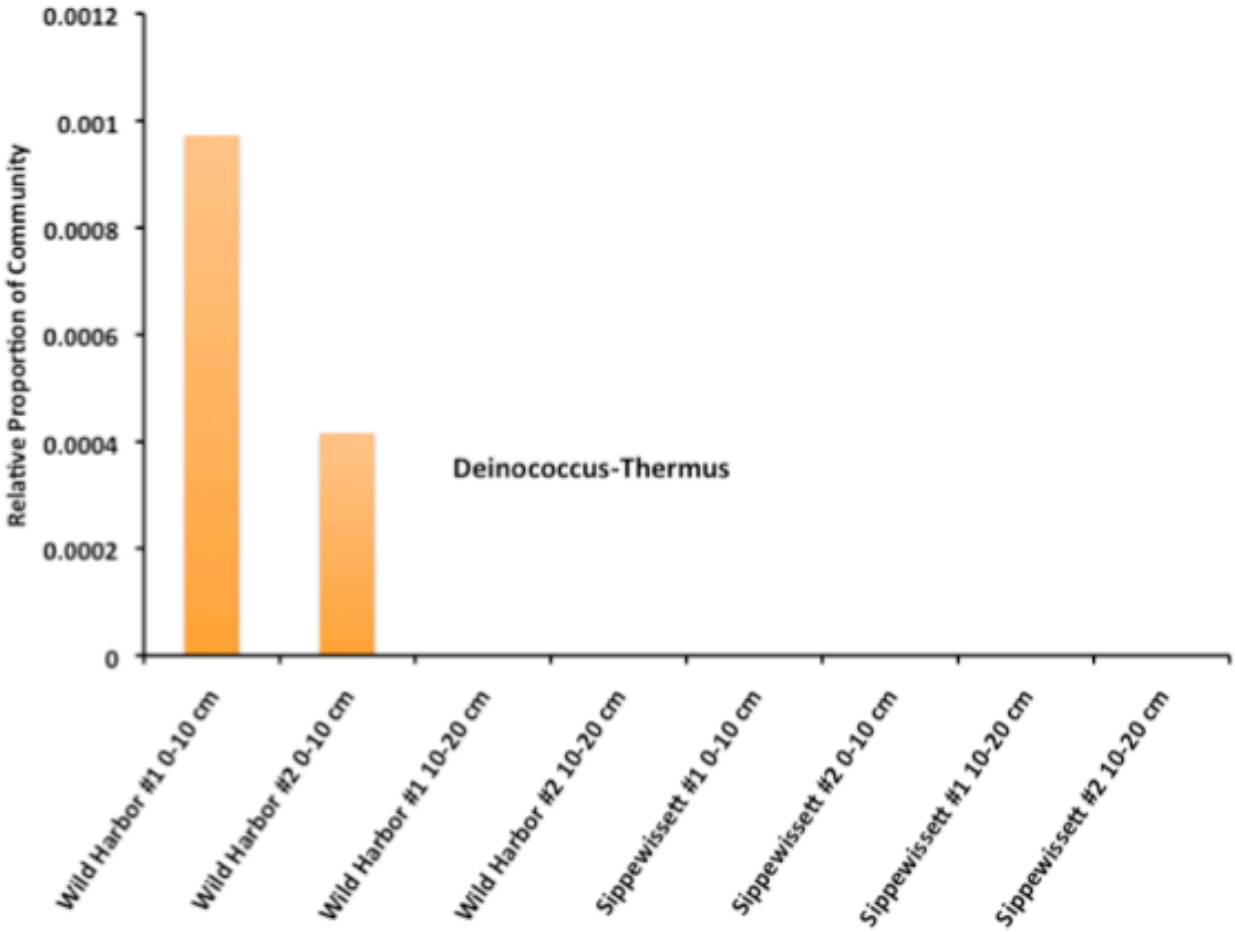

0.0012
0.001
0.0008
0.0006
0.0004
0.0002
0
Relative Proportion of Community
Deinococcus-Thermus
Wild Harbor #1 0-10 cm
Wild Harbor #2 0-10 cm
Wild Harbor #1 10-20 cm
Wild Harbor #2 10-20 cm
Sippewissett #1 0-10 cm
Sippewissett #2 0-10 cm
Sippewissett #1 10-20 cm
Sippewissett #2 10-20 cm

Figure 5.

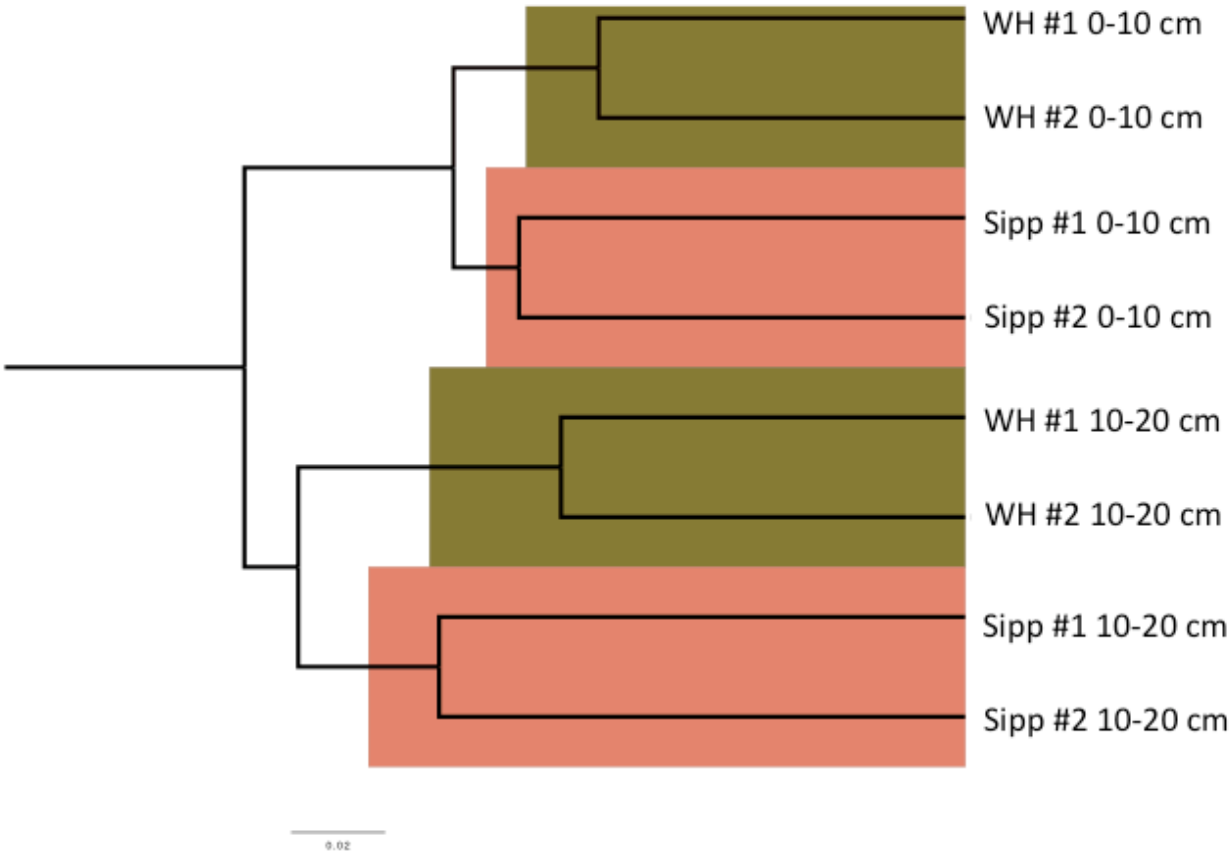

WH #1 0-10 cm
WH #2 0-10 cm
Sipp #1 0-10 cm
Sipp #2 0-10 cm
WH #1 10-20 cm
WH #2 10-20 cm
Sipp #1 10-20 cm
Sipp #2 10-20 cm
0.02

Figure 6.

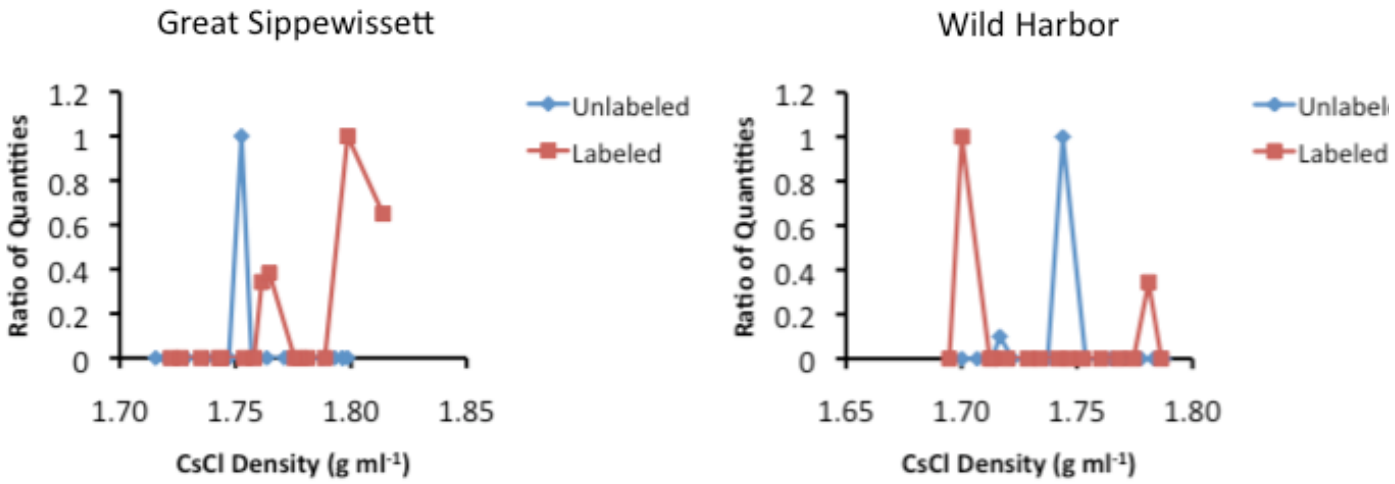
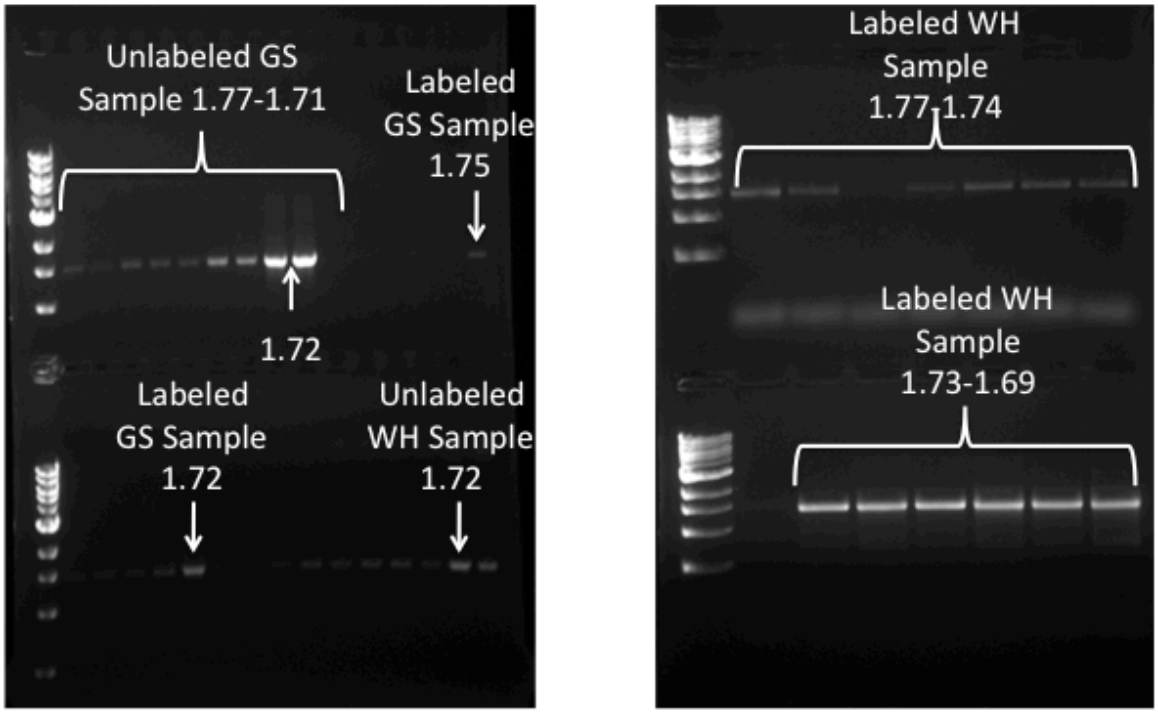

Great Sippewissett
Ratio of Quantities
1.2
1
0.8
0.6
0.4
0.2
0
1.70
1.75
1.80
1.85
CsCl Density (g ml-1)
Unlabeled
Labeled
Wild Harbor
Ratio of Quantities
1.2
1
0.8
0.6
0.4
0.2
0
1.65
1.70
1.75
1.80
CsCl Density (g ml-1)
Unlabel
Labeled

Figure 7.


Unlabeled GS
Sample 1.77-1.71
Labeled
GS Sample
1.75
1.72
Labeled
GS Sample
1.72
Unlabeled
WH Sample
1.72
Labeled WH
Sample
1.77-1.74
Labeled WH
Sample
1.73-1.69