A CULTURE-BASED COMPARISON OF SEDIMENT-DWELLING BACTERIA ALONG A TIDAL GRADIENT

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ABSTRACT

Despite hosting an abundant and diverse microbial community, marine sediments are woefully understudied. The goal of this project was to compare sediment-dwelling bacteria across a tidal gradient. To do this, sediments were collected from subtidal, intertidal and littoral zones at Little Sippewisset Marsh, Falmouth, MA in August of 2017. Bacteria were cultured following an enrichment protocol for actinomycetes with both freshwater and seawater-based media. Seawater-based media was found to have a higher number of bacteria colonies compared to freshwater media. Additionally, more similarity in colony morphology was observed between subtidal and intertidal sediment samples when compared to littoral bacteria. Twelve isolates were then grown in six salinity concentrations over thirty-six hours and doubling time determined. All isolates exhibited a significant increase in growth when salts were present in the media. Only isolates from the littoral zones were able to tolerate liquid freshwater-based media. Therefore, it appears that while bacterial communities differ along a tidal gradient, all bacteria seem adapted to growing in salty environments.

INTRODUCTION

Marine sediments host high levels of bacterial diversity yet remain poorly studied (Lozupone & Knight 2007, Zinger *et al.* 2011). While the advent of next-generation sequencing has facilitated our ability to identify bacterial community members in specific samples, we know little about the biogeographic distribution of species or the effects of environmental and biological pressures on sediment communities. Actinobacteria, a phylum of gram-positive bacteria, are considered to be very important ecologically and economically. Actinomycetes, in particular, are prolific producers of secondary metabolites that have been utilized in the pharmaceutical industry, making the discovery of new actinomycetes or insight into their ecology valuable.

Despite major differences between marine sediments and other environments, very few marine obligate genera of actinomycetes have been identified (Jensen & Lauro 2008). It is interesting that the majority of marine species are not evolutionarily distinct enough to be

distinguished as separate genera from freshwater and terrestrial species. Given our poor understanding of microbial communities in the marine sediment environment, I wanted to compare diversity across a tidal gradient, with the hope of specifically selecting for actinomycetes. Sediments from different tidal zones experience different stressors, one of which is variable exposure to seawater. For instance, subtidal sediments are rarely if ever exposed, suggesting that this environment is likely more consistent in its salt concentration when compared to an intertidal region. Littoral zones, in contrast, rarely experience an influx of seawater, but they are in an environment where they are exposed to sea spray and other forms of salt transport. Therefore, it would be expected that bacterial communities present along a tidal gradient are composed of different members, or exhibit different phenotypes, in response to their localized habitat.

Thus, the goal of this study was to assess differences in the bacterial community, and in particular, Actinomycetes, along a tidal gradient. I first aimed to see if the phylum Actinobacteria was detectable from marine sediment samples using fluorescent *in situ* hybridization (FISH). I then took a culture-based approach to enrich for Actinomycetes and grow bacteria from different tidal heights on freshwater and seawater-based media. A subset of isolates was then tested under a range of six different salinities. I hypothesized that we would isolate marine-specific bacteria, predominantly from subtidal regions, since the subtidal environment is consistent in its salt concentration. I also expected to see the most freshwater-based isolates from littoral sediment samples.

METHODS

Sample Sites

Surface sediments were sampled along a tidal gradient at Little Sippewisset Marsh in Falmouth, MA on August 1, 2017. Three sites were sampled during low tide- the main channel that remains subtidal (depth ~30 cm; Salinity 31 ppt; 28°C), an intertidal patch that was exposed during low tide, and a littoral zone that rarely is exposed to the seawater. Samples were taken by shovel and placed in a sealed Tupperware container before being transported back to the Marine Biological Laboratory in Woods Hole, MA. Submerged and intertidal sediments were kept in a seawater table (Salinity 34 ppt; Temp 15°C) with filtered seawater slowly flowing into the containers. Sediment from the littoral zone was kept dry and at room temperature.

CARD-FISH

Approximately 500 mL of sediment from the three sites was added to eppendorf tubes and rinsed with PBS twice to remove seawater. One mL of PBS was then added to the tube and samples were sonicated in intervals of 1 minute for a total of 5 minutes. Samples were left to settle for 5 minutes before 50 uL of supernatant was added to a few mL of PBS and filtered for the purposes of CARD-FISH. CARD-FISH samples were prepared following Ishii *et al.* (2004) using DAPI stain in parallel with either a Eubacteria (EUB338 I-III) or an Actinobacteria (HGC69A) probe.

Culturing and Strain Isolation

Approximately 5 g of sediment from the center of each sample container was taken and placed into a plastic weigh boat. Sediments were then spread out in a thin layer and placed in a 37°C incubator following an adapted protocol from El-Nakeeb & Lechevalier (1962). After 24 hours the dried sediments were broken up and mixed using a clean microspatula before being placed back in the incubator for at least another 24 hours to ensure complete drying. Approximately 1 g of each sediment type was then moved into a new weigh boat and mixed with 1 g of calcium carbonate. Samples were then transferred to sterile petri dishes with a moist filter paper in the lid and were left to sit at room temperature for another 24 hours. Sample mixtures were split in half and added to either freshwater-based 50% A1 media (hereafter FW-A1) or seawater based 50% A1 media (hereafter SW-A1; see Appendix A for media components), vortexed, and allowed to sit for 30 minutes to settle. Corresponding freshwater or seawater-based plates were then inoculated with 50 uL of sample in a dilution series (1x, 1/10x, 1/100x, and 1/1000x). Inoculum was spread using a sterile glass rod. Plates were left to dry and then flipped upside down and placed into a 30°C incubator for 24 hours. Samples were then moved to room temperature and isolates were selected 48 hours post inoculation and re-streaked onto a new plate with the corresponding media.

All images of colonies were taken using a Zeiss Dissecting Microscope.

DNA Extraction

Bacterial isolates were grown in 2 ml of liquid media for 48 hours at 30°C. Following Dabrowska (2015), cultures were then centrifuged at 4000 rpm for 5 minutes. After removing the supernatant, colony pellets were resuspended using 300 uL of lysis buffer (Appendix A) and 100 uL of 20 mg/ml lysozyme. Samples were incubated at 37°C for 30 minutes and then 100 uL of each sample was added to Promega Maxwell RSC Cell Extraction cartridges and inserted into the instrument following the manufacturer's protocol. Final elution of DNA was into 50 uL of molecular grade water.

PCR

For identifying isolates, PCR was performed using GoTaq Green Master Mix, the 16s primers 8F and 1492R and an annealing temperature of 55°C with 1 uL of template (Frank *et al.* 2008). PCR products were run on a 1% agarose TAE gel with Promega 1kb Benchtop Ladder to confirm PCR success. Samples were then cleaned and sent for sequencing.

Salinity Preference/Tolerance

In order to test salinity preference, 12 bacteria isolates were haphazardly selected (Table 1: Isolates 4, 6, 12, 14, 17, 21, 24, 28, 31, 33, 38 & 39) and grown in triplicate in 96 well plates using 50% A1 media at six different sea salt (Instant Ocean) concentrations- 0, 15, 22, 30, 35, 40 g/L. When standardized to the media without salt, the corresponding salinities as determined via refractometer were 0, 11, 20, 25, 29 and 35 ppt. Plates were loaded with 140 uL of media followed by 10 uL of inoculum or blank media (either FW-A1

or SW-A1). The inoculum was prepared by passing a sterile toothpick through an isolated colony and placing it in 1 ml of the corresponding liquid media. Plates were gently shaken and then covered with clear sealing sheets and kept at room temperature with only periodic shaking occurring. A Promega GloMax Discover plate reader was used to determine absorbance at an optical density (OD) of 600 nm 8 times over a 36-hour period. Data was exported from the plate reader into Microsoft Excel where growth curves and doubling times were calculated. Statistics were performed in RStudio Version 0.99.473.

RESULTS

CARD-FISH

Samples from all sites contained high numbers of bacteria seen using a eubacteria probe. In contrast, the Actinobacteria probe revealed few, if any, clear bacteria cells (Figure 1). Green fluorescence seen in overlay photos (Figure 1- row b) was not observed when visualizing with just DAPI, indicating that points of green fluorescence in the Actinobacteria probe samples are likely particulate matter from the sediment.

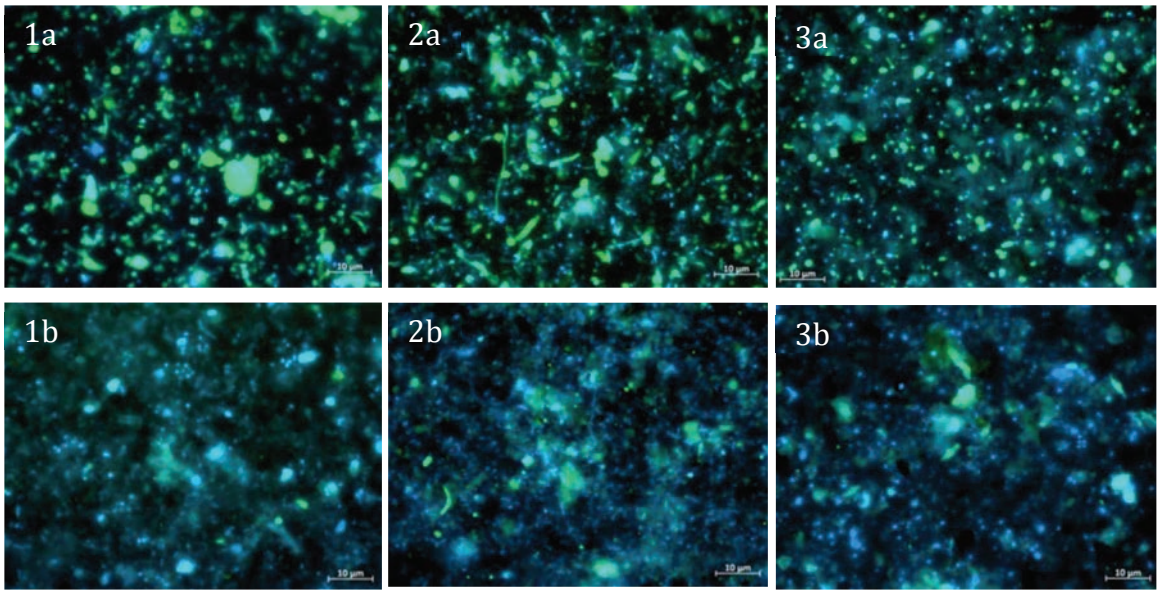


Figure 1. CARD-FISH of Little Sippewisset Marsh Sediments. Sediments from (1) subtidal (2) intertidal and (3) littoral were sonicated in PBS and the supernatant was stained with DAPI. (a) Eubacteria or (b) Actinobacteria probes were then added. Scale bars are 10 um.

Culture Diversity & Isolate Identification

Based on qualitative observations, more bacteria colonies were grown using seawater-based media when compared to freshwater. Subtidal and intertidal samples produced many pigmented colonies, primarily orange and yellow. In contrast, littoral samples were dominated by yellow and cream colored colonies (Figure 2).

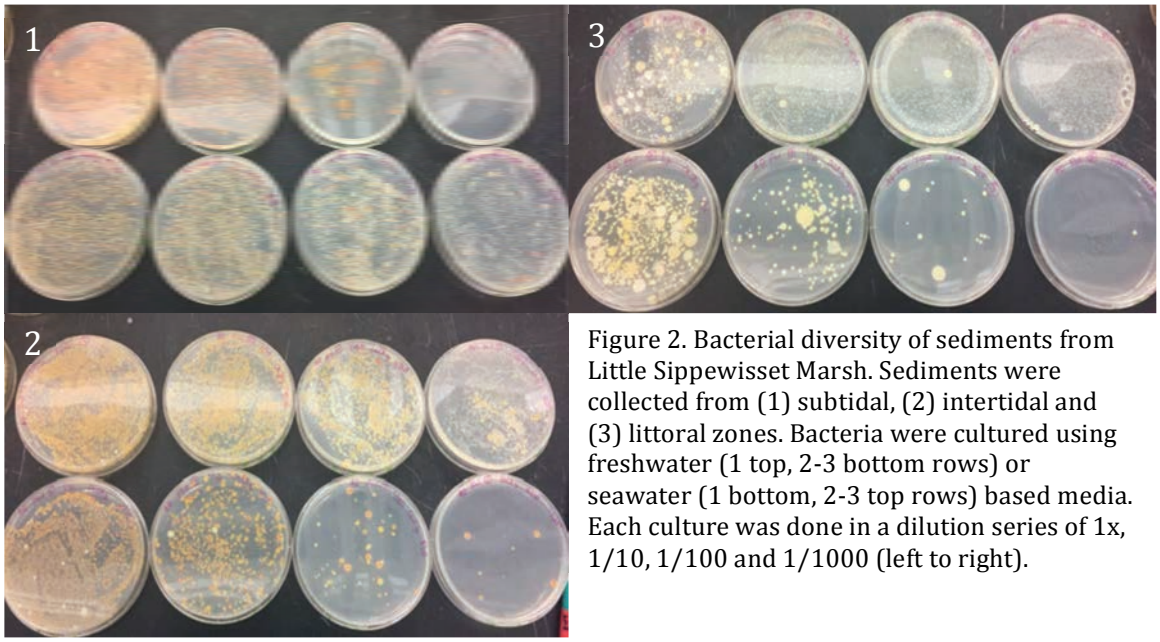
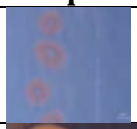
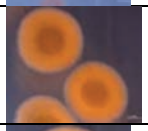
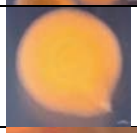
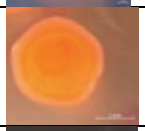
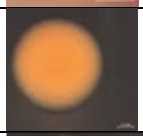
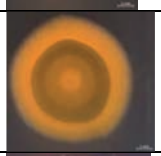
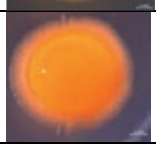
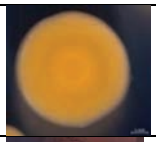
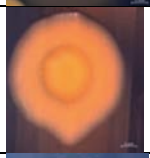
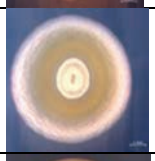
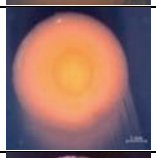
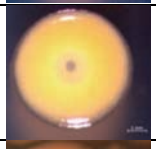
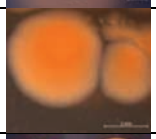
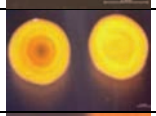
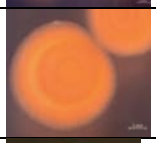
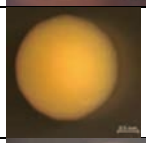
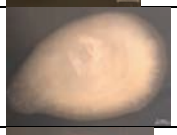
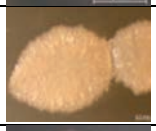
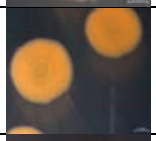
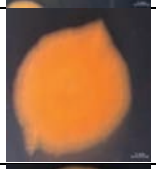
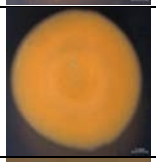
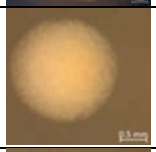
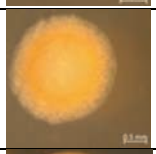
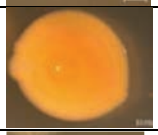
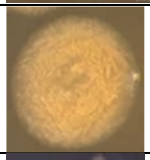
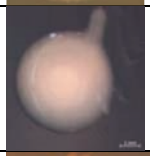


Figure 2. Bacterial diversity of sediments from Little Sippewisset Marsh. Sediments were collected from (1) subtidal, (2) intertidal and (3) littoral zones. Bacteria were cultured using freshwater (1 top, 2-3 bottom rows) or seawater (1 bottom, 2-3 top rows) based media. Each culture was done in a dilution series of 1x, 1/10, 1/100 and 1/1000 (left to right).

Isolate Number	**Colony Morphology**	**Tidal Zone**	**Media**	**16S Identification (Closest BLAST Hit)**	**% ID**
1		Intertidal	FW	NA	NA
2		Intertidal	FW	NA	NA
3		Intertidal	FW	NA	NA
4*		Intertidal	FW	NA	NA
5		Intertidal	FW	NA	NA
6*		Intertidal	FW	NA	NA
7		Intertidal	FW	NA	NA

8		Intertidal	FW	NA	NA
9		Subtidal	FW	NA	NA
10		Subtidal	FW	NA	NA
11		Subtidal	FW	NA	NA
12*		Subtidal	FW	NA	NA
13		Subtidal	FW	NA	NA
14*		Subtidal	FW	NA	NA
15		Subtidal	FW	NA	NA
16		Subtidal	FW	NA	NA
17*		Littoral	FW	NA	NA
18		Littoral	FW	NA	NA
19		Littoral	FW	NA	NA

20		Littoral	FW	NA	NA
21*		Littoral	FW	NA	NA
22		Littoral	FW	*Pseudomonas* sp.	95
23		Intertidal	SW	NA	NA
24*		Intertidal	*SW*	*Aeromonas veronii*	98
25		Intertidal	SW	NA	NA
26		Intertidal	SW	*Ochrobactrum* sp.	99
27		Intertidal	SW	NA	NA
28*		Intertidal	SW	NA	NA
29		Intertidal	SW	NA	NA
30		Intertidal	SW	*Ochrobactrum* sp.	99
31*		Subtidal	SW	*Pseudomonas* sp.	95

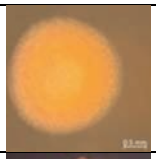
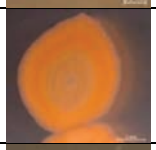
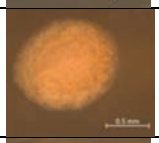
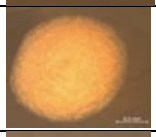
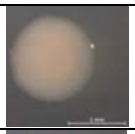
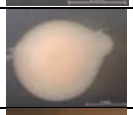
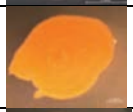
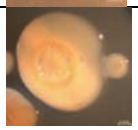
32		Subtidal	SW	*Ochrobactrum* sp.	91
33*		Subtidal	SW	*Pseudomonas*/*Aeromonas* sp.	96
34		Subtidal	SW	*Ochrobactrum* sp.	99
35		Subtidal	SW	NA	NA
36		Subtidal	SW	*Ochrobactrum* sp	99
37		Littoral	SW	NA	NA
38*		Littoral	SW	*Pseudomonas citronellolis*	96
39*		Littoral	SW	*Ochrobactrum* spp.	99
40		Littoral	SW	*Ochrobactrum* sp.	99
41		Littoral	SW	*Pseudomonas* sp.	89
42		Littoral	SW	NA	NA
43		Littoral	SW	NA	NA
44		Littoral	SW	NA	NA

Table 1. Bacteria isolated from Little Sippewisset Marsh Sediments. A "*" indicates the use of that isolate in salinity preference assays. "NA" indicates a lack of 16s sequences for identification purposes.

Salinity Preference

Representative growth curves indicated that time point 1 (4 hrs) and 4 (23 hrs) were appropriate to use in determining doubling time (Figure 3). When doubling times of isolates were averaged based on salinity and media type, an ANOVA of salinity indicated that isolates grown in freshwater had significantly longer doubling times compared to isolates in all other salt concentrations (Figure 4; F=8.73, p= 3.36e-05).

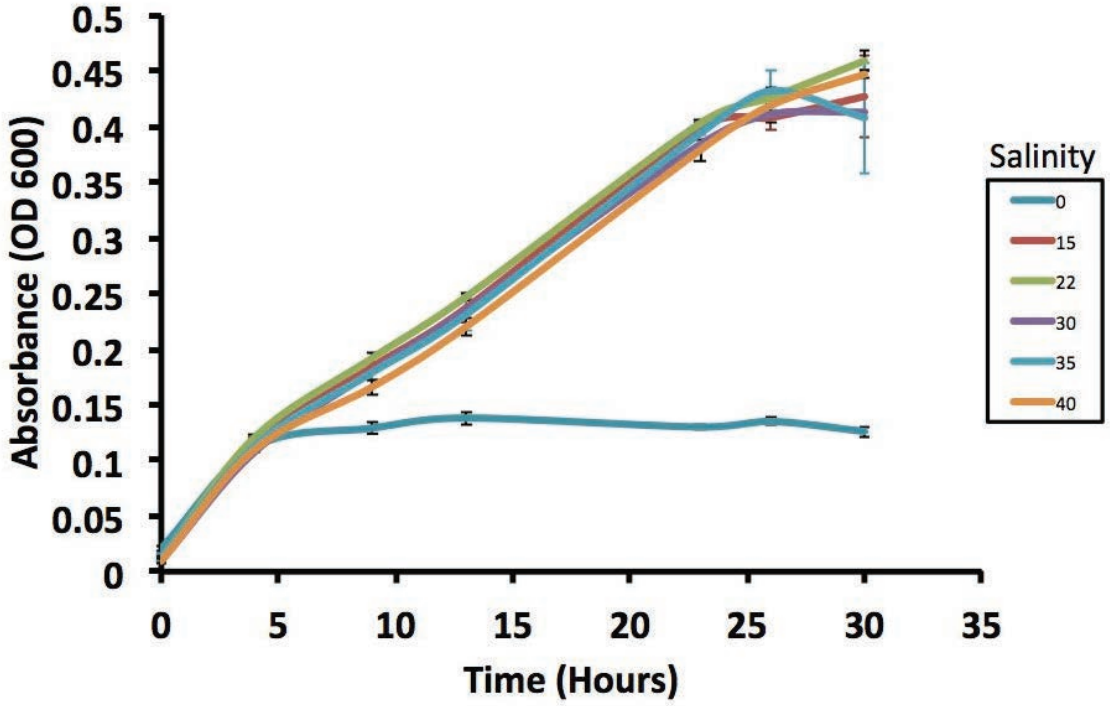


Figure 3. Growth of isolate 12 from Little Sippewisset Marsh when grown under different salinity concentrations (g/L). Error bars represent standard error across three replicates.

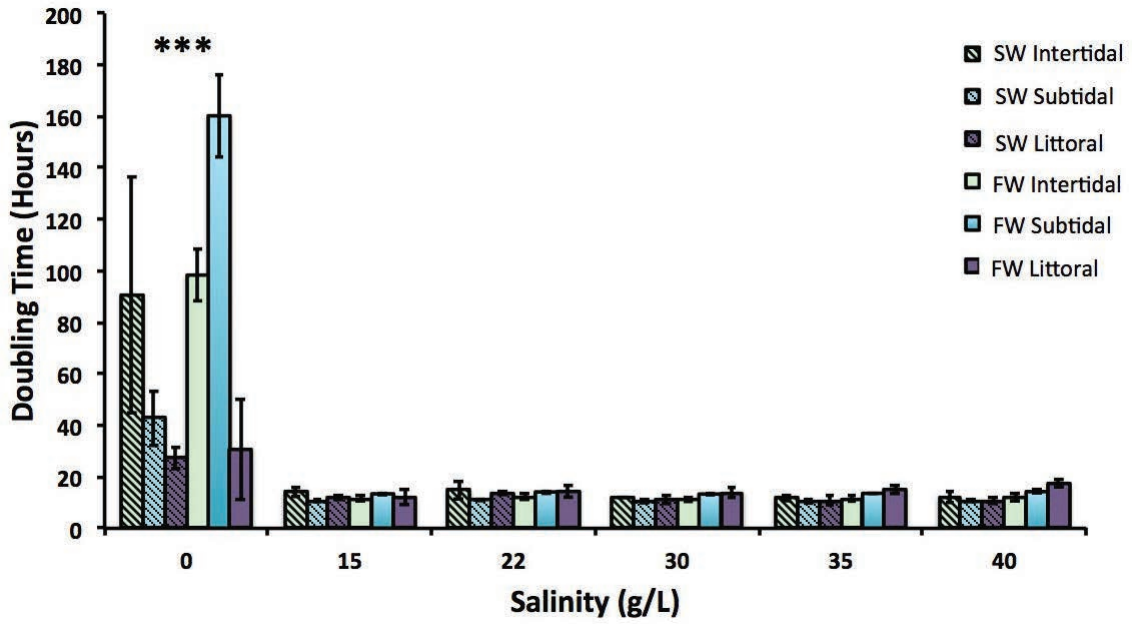


Figure 4. The effect of salinity on average doubling time in bacteria isolates from Little Sippewisset Marsh. Error bars represent standard error across two bacteria isolates per site x media condition. Significance based on a one-way ANOVA of salinity (F= 8.73, p= 3.36e-05).

DISCUSSION

While CARD-FISH results were inconclusive, it is likely that Actinobacteria are present in the community, but are hard to detect. Actinobacteria are known to have relatively low abundances in marine sediments (Bowman & MCCuaig 2003; Demko *et al.* unpublished data; Duncan *et al.* 2014), making them rare and potentially difficult to identify using FISH probes on direct environmental samples.

Growth of a wide variety of bacteria was observed on both freshwater and seawater-based media, although more colonies were qualitatively observed on SW-A1 plates. Even at a dilution of 1/1000, SW-A1 plates were covered in bacteria after 24 hours, making it difficult to quantify the difference between media types. Future work should increase the dilution series so as to dilute to extinction. Interestingly, the more dilute samples on SW-A1 were seemingly dominated by one particular type of bacteria, rather than facilitating the growth of multiple rare isolates. All bacteria were cultured using an actinomycete enrichment protocol that exposed them to high temperature (37°C) and desiccation, making for a selection of particularly tolerant bacteria and/or spore formers. Another interesting observation was that subtidal and intertidal plates seemed to be dominated by colonies with bright orange and yellow pigments. In contrast, littoral plates had colonies exhibiting more muted yellow and cream colors. Despite efforts to identify all isolates, only

a subset was successfully grown and amplified via 16s primers. Based on the colonies that were identified, the enrichment protocol actually selected for the proteobacteria *Ochrobactrum* sp. and *Pseudomonas* sp. rather than actinobacteria (Table 1). Interestingly, multiple isolates were <97% similar to the nearest BLAST hit, suggesting that we may have cultured unique 'species' of *Ochrobactrum* and *Pseudomonas*.

While the enrichment protocol followed was for actinomycetes, the media used was likely too nutrient rich for proper selection. Typically low nutrient media are used to isolate actinomycetes, but it takes them longer to grow. Given the time constraints of this project I opted to do a 50% nutrient media when I should have lowered the nutrients even further. Dabrowska (2015) utilized CHiP media to grow actinomycetes successfully and while I also initially tested that media along with the A1 media, I had a similar pattern of plates being quickly overrun by proteobacteria and it was more noticeable on the CHiP plates. Therefore, I opted to pursue the colorful colonies that were in higher abundance on the A1 media. I did observe the growth of distinct actinomycetes (determined by the presence of aerial hyphae and a distinct soil aroma) on both A1 and CHiP plates, but it was a few days after I selected colonies for isolation. Interestingly, I only observed obvious actinomycetes on plates from littoral sites and I suspect this is because the 'obvious' aerial hyphae and soil aroma are associated with more terrestrial based actinomycetes.

Salinity preference assays indicated that all isolates from Sippewisset Marsh significantly grew better in media containing salts when compared to freshwater-based media. Of all isolates, the only ones that showed even noticeable growth under freshwater conditions were those isolated from littoral sediments, supporting the hypothesis that sediments more likely to be subjected to freshwater (via precipitation) would harbor freshwater tolerant bacteria. Another interesting result was that there was no significant difference among saltwater-based media types. So isolates seemingly grew just as well in media with 15 g/L salt to 40 g/L salt. I suspect that since marsh habitats are highly variable and thus stressful environments, the bacteria present are adapted to tolerate fluctuating conditions. It would therefore be interesting to compare salt preference from marsh isolates to those from a less variable environment, like sediments found in sediments around coral reefs. It would also be interesting to test the isolates from this study in salt concentrations higher than 40 g/L to see at what point, if any, salt concentrations start becoming detrimental to growth.

In conclusion, microbial communities from marsh sediments do seem to differ based upon their tidal exposure. Subtidal and intertidal sediment bacteria seem overall more similar to each other than when compared to littoral bacteria. All marsh sediment bacteria preferred growth media with sea salts present, but littoral isolates exhibited the highest tolerance to freshwater-based media. Future work should aim to further classify the communities present at these different locations and see if the qualitative patterns observed are supported by quantitative approaches.

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APPENDIX

Table A1. 50% A1

Component	**Amount per Liter**
Starch	5 g
Yeast	2 g
Peptone	1 g
Instant Ocean	22 g (omit if making FW based)
Agar	15 g

Table A2. Lysis Buffer

Component	**Amount per 0.2 L**
Sucrose	20.52 g
1M Tris HCL (pH 8)	5 ml
0.5M EDTA (pH 8)	10 ml
RNase	0.1 mg
H2O	185 ml