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Impact of Severe Drought Conditions on Ammonia-Oxidizing Bacterial Communities in a Connecticut Salt Marsh

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Impact of Severe Drought Conditions on Ammonia-Oxidizing Bacterial Communities in a Connecticut Salt Marsh



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Department of Biology
Honors Thesis
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ABSTRACT

Salt marsh ecosystems exist along a boundary between fresh and saline waterways, and their function is regulated by a variety of physical and chemical cycles. Microbial communities hold many important roles as mediators in these cycles, and like other aspects of the ecosystem are sensitive to changes in their environment. Climate change is expected to bring many obstacles for salt marshes around the world, one being periods of sustained drought. In this study we investigate the impact that a severe seasonal drought had on the abundance and community composition of microbes involved in nitrogen cycling, specifically ammonia-oxidizing bacteria (AOB). An undisturbed *Spartina* dominated marsh in Barn Island WMA (Stonington, CT) was sampled across vegetation types, before during and after a period of drought. Community abundance was measured with quantitative PCR, and diversity with DNA fingerprinting (T-RFLP) targeting the ammonia monooxygenase gene (*amoA*). An effect of drought was observed in both abundance and composition for several vegetation types. AOB abundance and diversity increased in *S. patens* plots during the drought, while other vegetation types had a temporary reduction in both abundance and diversity during drought. Potential increases in oxygen penetration from soil aeration could explain the AOB bloom seen in *S. patens*, while in other vegetation types the drought may have selected for more tolerant species. Drought frequency is expected to increase with the continuation of global climate change (GCC), and while these results suggest a tolerance by most vegetation types, with habitat and diversity loss the community remains very susceptible.

INTRODUCTION

Microbial Mediated Nitrification

Nitrogen is a ubiquitous element and is found in every amino acid and nucleotide on the planet, yet most of the nitrogen is biologically unavailable as nitrogen gas. Making abundant atmospheric nitrogen available in the soil is essential for proper plant functioning, and ultimately fostering a biodiverse ecosystem. Nitrogen moves around environments in a complex cycle of compounds, constantly being absorbed and deposited by different organisms. Nitrification is a portion of this cycle that consists of the sequential oxidization of ammonia to nitrite, and then to nitrate. This is often followed by denitrification which returns the nitrogen to the atmosphere as nitrogen gas, making it unavailable again. In coastal systems nitrogen is often a limiting factor (Valiela et al. 1979), giving soil ammonia a regulatory effect on the productivity of the environment.

Plants rely on soil microbes to make atmospheric N_2 bio-available. These tiny organisms generate and house the enzymes which carry out all the reactions of the nitrogen cycle. Ammonia oxidation, the first step of nitrification, is catalyzed by the ammonia monooxygenase enzyme (amo) which is found in ammonia-oxidizing bacteria (AOB) and archaea (AOA). These microbes are ubiquitous in all primarily productive environments and are often studied in order to gain insight on the state of nutrient cycling in an ecosystem (Kowalchuk et al. 2001).

Nutrient cycling is equally important in coastal systems, especially salt marshes where land and water meet. In salt marshes the physiochemical gradient created by

the rapid transition from estuarine to terrestrial ecosystem leads to a high genetic diversity in a relatively small area (Watson, 2009). This is what makes marshes one of ecosystems with highest primary productivity on the planet. One of the major contributions leading to this microbial diversity and productivity is environmental tolerance coupled with nutrient availability.

Salt marsh ammonia oxidizers are aerobic and chemolithotrophic microbes; they oxidize ammonia to nitrate and use that energy to fix carbon dioxide. Some ammonia-oxidizing bacteria (AOBs) that have been successfully cultured are found in two distinct lineages within the gamma and beta subdivisions of Proteobacteria (Woese et al, 1987). Most AOBs are associated with a single monophyletic lineage within Betaproteobacteria, with other species not native to saltmarshes found within the gamma subdivision (Bernhard et al, 2005, Moin et al 2009). *Nitrosomonas* and *Nitrospira* are the two genera comprising all cultured ammonia oxidizers in Betaproteobacteria. *Nitrosomonas* containing the greater known diversity with six lines of descent within the group (Purkhold et al, 2000). Although this study focuses on AOBs, the discovery of ammonia-oxidizing Archaea (AOAs) has expanded the understanding of how ammonia is oxidized in living systems.

Ammonia oxidizers can be targeted by the presence of the gene encoding the alpha subunit of the ammonia monooxygenase enzyme. Novel sequencing of this gene region was done by McTavish et al. (1993), and primers have been developed which target this gene in PCR (Rotthauwe et al. 1997, Nicolaisen et al. 2001). The sequences used to develop these primers works universally for all known AOBs found in the Betaproteobacteria group but does not detect any from the gamma-proteobacterial

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sub-clade. However, gamma-proteobacteria are not found in measurable abundance in New England marshes (Bernhard et al. 2005), and therefore their exclusion for this study shouldn't have a substantial effect on the results.

The established phylogeny of these microbes is incomplete due to limitations with culturing AOBs from natural samples. Therefore, the traditional species classification schema does not accurately represent the diversity of aerobic AOBs found in natural environments (Kowalchuk et al. 2001). Cultured AOB genomes are typically used as references to compare to natural samples, and the observed diversity far exceeds that defined by the taxonomic classification.

Ammonia Oxidizer Response to Environmental Change and Disturbance

Many previous studies have attempted to unearth patterns in AOB diversity and abundance, and the environmental factors that drive population shifts. The majority of studies in salt marshes have indicated that the recently discovered AOA are actually much more abundant than AOB, causing a disruption in the understanding of the nitrogen cycle in marshes. Predictable seasonal variations in microbial abundance have also been well documented. Bernhard et al. (2007) found that seasonal variation in nitrification rates were correlated to relative abundance, with autumn having the lowest rates, potentially due to greater competition for ammonium during microphytobenthic organism blooms, and summer peaking likely because of greater ammonia availability and precipitation. Vegetation type has also been shown to be associated with variation in abundance and in community composition. Moin et al (2009) found AOBs to be more abundant in sites with *S. patens* and *S. alterniflora* (Tall) than in sites with *S. alterniflora*

(Short). They also found AOA and AOB abundances were most closely correlated in vegetation types where AOB was more abundant.

Sediment nutrients play an important role in plant functionality, as well as microbial functionality and are frequently studied to determine if they correlate to population patterns. Nitrate and ammonia concentration as well as rates of production (nitrification and ammonification) in sediment are often used to study the activity level of ammonia oxidizers. Nitrification potentials (Marton et al. 2014) as well as dissolved organic carbon (Seung-Hoon et al. 2016), pH (Xia et al. 2015), salinity (Bernhard et al. 2007, Santoro et al. 2008) and other nutrients (Mosier et al. 2008) have been found to be correlated with AOB abundance. These results however are variable, and no consistent driver has been identified. The strongest explanations of AOB abundances come from multi-linear regression models which factor in multiple variables, ultimately confirming that these systems are complex and governed by a number of factors (Caffrey et al. 2007).

Many aspects of salt marsh ecosystems are governed by the salinity gradient they exist in. The effect of salinity on the salt marsh AOB populations has been studied with various and conflicting conclusions. Many studies have found AOA and AOB to be negatively correlated to salinity, especially when controlling for C:N, which is a known covariant (Mosier et al. 2008, Santoro et al. 2008). Bernhard et al. (2007) found variation across a salinity gradient, with distinct communities in each salinity group. A seasonal salinity effect was also documented by Santoro et al. (2008), who found higher salinity conditions in summer than winter, and subsequent greater AOB abundance in the summer months. While AOA is typically in universally higher

abundance compared to AOB, Santoro et al. (2008) found AOB up to 30X more abundant in higher saline environments. However, this AOB community was markedly less diverse with only two unique groups present in the higher saline samples. Caffrey et al (2007) found conflicting results with salinity not correlated to AOB abundance, but salinity did factor into a multiple linear regression model explaining the overall abundance patterns. Salinity has also been correlated with large variations in diversity, explaining up to 62% of the variation in a Chesapeake Bay study (Francis et al 2003). The salinity of the environment appears to be affecting the abundance pattern and the diversity in most studies, either alone or when considered with other abiotic factors. The mechanism for this impact remains unclear, but has been speculated to involve environmental tolerance and enzyme kinetics (Francis et al. 2003)

Salt Marsh Vegetation Patterns

Marsh vegetation zonation patterns form as a product of tolerance to desiccation and salinity (Watson, 2009). The soil and rhizospheric environments of all of these plant species create unique microclimates which foster a great deal of biodiversity in these habitats (Theodose, 2003). Marsh soil environments are highly complex and variable depending on elevation. Low marsh areas, which flood most frequently, are essentially completely anoxic, while further inland, in the high marsh there is a greater oxic zone. Most areas of the marsh do not hold enough oxygen in root air pockets to have an oxic zone below a few centimeters (Mendelssohn et al. 1981). Effects of anoxic sediment conditions is compounded by waterlogging, which has been shown to negate respiration and productivity rates (Mendelssohn et al. 1981),

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and lead to potentially toxic build-up of hydrogen sulfide in soil (Watson et al. 2016). Waterlogging has also been shown to affect AOB abundance and community composition, with decreased nitrification rates and overall N availability (Nyugen et al. 2017).

Climate change induced sea level rise is unquestionably the largest problem that all salt marshes are facing worldwide (Simas et al. 2001). As the sea levels rise marshes are adapting to the changing conditions by accreting backwards and moving in response. Changes in vegetation occur when accretion fails to keep up with relative sea level (Warren and Neiring, 1993). This adaptation allows them to maintain their zonation patterns driven by the salinity gradient, but makes them highly vulnerable to being squeezed out by infrastructure and developed land. This phenomenon known as coastal squeeze, resulting in irreversible loss of salt marsh habitat, is only one of the ways that anthropomorphic climate change is going to seriously impact salt marshes. Models predicting the response of marshes to sea level rise show a strong resilience capacity of *S. alterniflora* marshes to changing conditions. These models do not account for changes in salinity brought about from climate-induced increased drought frequency, calling into question the true resilience of marshes when confronted with climate change on multiple fronts (Hughes et al. 2012).

Drought Impacts on Microbial and Vegetation Communities

In addition to sea level rise, climate change will induce unpredictable periods of intense drought, which will likely impact many ecological systems (IPCC, 2007). Some researchers have studied the impacts of drought and subsequent inundation on salt marsh vegetation. Wetzel and Kitchens (2007) documented a change in vegetation community during periods of drought. This impact was transient and “patchy”, as it was non-uniform across the marsh and pre-drought conditions were restored relatively fast after the end of the drought (less than four years). Additionally, they documented an increase in sediment salinity with a lack of fresh water inundation. This salinity was correlated to a loss of diversity, possibly from increased level of interspecific competition from this chronic stress. Additionally, Charles and Duke (2009) documented an increase in marsh productivity during drought and warming period. They speculated this was due to a greater volume of inorganic nutrients getting trapped during tidal flushing, which directly contributed to the sediment. Additionally, they speculated that more arid conditions lead to greater secondary growth, and higher photosynthetic rates causing the positive overall effect on growth. Their drought treatment had alleviated waterlogging, causing greater aeration at depth, allowing oxygenation of the rhizosphere, accompanied by increased microbial activity and an associated increase in nutrient availability. Overall, they concluded that salt marshes are resilient to changes in precipitation, but less so to warming fluctuations. The lack of freshwater input, coupled with higher levels of evaporation during drought conditions, causes a marked increase in sediment salinity. Hypersaline conditions have been shown to have an inhibitory effect on nitrification and denitrification rates (Rysgaard et al. 2009). Connecticut College, Department of Biology, 2018

al 1999). Organic carbon production has also been found to be negatively affected by high salinity and/ or sulfides due to interactions with plant and root symbionts communities (Watson et al. 2016). Conversely, increased sediment waterlogging caused a decrease in AOB abundance accompanied by a shift in composition (Nyugen et al. 2017). These changes were attributed to induced physiochemical shifts that result in decreased O₂ and increased pH.

Drought has also been found to affect salt marshes in other ways. Palomo et al (2013) found drought brought on transient oxic conditions, which resulted in significant alterations to sediment geochemistry and microbial pathways. The oxidation of dissolved metabolites leads to lowered sediment pH, lower denitrification activity, and higher metal availability. Drought-related increases in marsh salinity resulted in changes in plant community richness and abundance, especially in the mesohaline regions with *S. patens* (Visser et al 2000). Hughes et al (2012) studied the compounding effect that drought had on marshes experiencing events of rapid vegetation death (Acute Marsh Dieback, AMD) as a result of sea level rise. They determined that an increase in drought frequency will seriously limit a marsh's ability to accommodate sea level rise. Published literature regarding drought tolerance and impact on salt marshes is generally variable. Vegetation communities appear to demonstrate a strong tolerance, with significant impacts being fleeting. Soil chemistry and sediment dynamics appear to be much more significant and lasting as the environment is altered by the lack of freshwater input.

Davis et al. (2018) investigated the impact of drought on nitrogen-fixing bacterial communities (diazotrophs) found in plant rhizosphere. They documented a clear shift in

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pore water (water collected from sediment cores) chemistry, especially for salinity, during the periods of drought, as well as a significant vegetation dieback event. The microbial community, however, demonstrated a strong resilience to the drought conditions, with only some shifts in community structuring, and an increase in diversity post-drought. The diversity increase post-drought was described as a bloom when conditions became favorable again. They hypothesized that long-term droughts could dry out the sediments, especially on exposed high marsh, increase the oxic zones and cause serious impacts on anaerobic bacterial composition. In the short-term, however, the diazotroph population remained stable and resilient when fresh water inundation returned.

Increased oxygen availability could be highly beneficial for aerobic salt marsh microbes like AOBs. In terrestrial drought studies a transient and variable effect on AOB and AOA abundance was observed, which was attributed to potential niche differentiation in the presence of heightened levels of sediment ammonium during drought conditions (Fuchleuger et al. 2014). Drought conditions have also shown to disrupt the correlation between AOA and AOB abundance and nitrification potential (Meyer et al. 2013). This lack of correlation was explained by either incomplete nutrient cycling by these communities, or a larger contribution by other groups of nitrifiers (such as heterotrophs). The observed drought response seen in ammonia oxidizer abundance was variable and short-term, resolving to non-drought (control) like condition in as little as seven weeks after end of drought.

The southeast region of Connecticut experienced a period of decreased rainfall from mid 2014 until the summer of 2017, which culminated with severe drought

conditions in the summer of 2016.



Fig 1: Visualizing the drought, picture taken at low tide, from the same location in Barn island WMA before the drought in 2014 (left) and during the drought in 2016 (right).

These multi-year drought conditions slowly increased in intensity, until ultimately breaking rapidly. The conditions observed in the marsh, and the overall ecological ramifications, are expected to increase globally as weather and precipitation become less predictable with the continuation of climate change. We hope to use the observations made during this period to further understand how marshes will be affected by intensifying fluctuations in rainfall, and ultimately attempt to answer one of many unknown questions regarding the prognosis of global salt marsh ecosystems.

Purpose

In this study, we investigated the impact of regional drought conditions on both the abundance and the community composition of sediment ammonia-oxidizing bacteria. We anticipated, based on previous studies, that the impact will be detectable, short term, and variable among the different vegetation sites sampled.

METHODS

Description of Study Site

The samples for this research were collected at the Wequetequock-Pawcatuck tidal marshes (part of the Barn Island National Wildlife Refuge) in Stonington CT. While most of the marsh system was impounded and later restored, this section of the Headquarters Marsh never saw restriction of tidal flow. This is a marsh dominated by *S. alterniflora* (tall form TSA, short form SSA) and *S. patens* (SP) with some unvegetated microbial mats (MAT). The marsh is fed by the Narragansett Bay and tributaries of the Pawcatuck river. The Headquarters Marsh is an eight-hectare section, which borders the bay, south of the impoundment (Fig. 2). Previous studies looking into ammonia oxidizer communities at impounded areas of Barn Island, have found the dynamic communities to be sensitive to chronic disturbance (Bernhard et al, 2015), with lasting effects of impoundments still detectable more than twenty years after restoration of tidal flow. Sediment sampling for ammonia oxidizer population analysis from this site has been nearly annual since 2014.

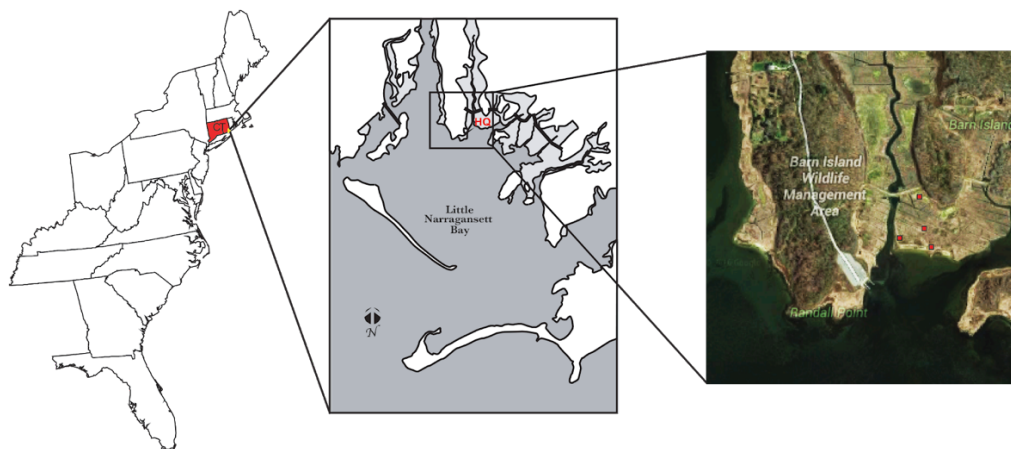


Figure 2: Location of Barn Island WMA, Headquarters Marsh identified in middle pane, and specific sampling sites marked in red on far right pane

Sediment Sampling

Sediment samples were collected from Headquarters Marsh of Barn Island WMA (Stonington CT) at low-tide in late June 2014, July 2016, and late June 2017. Triplicate (6.5 cm diameter) cores were taken from areas dominated by *Spartina patens*, short and tall *Spartina alterniflora*, and microbial mat (microbial biofilm covered unvegetated sites), respectively (exceptions to this methodology occurred in 2016, when only two TSA and SSA cores, and one MAT core were taken). Samples were immediately stored cold (4°C) and dark after collection until processing, less than 24 hours later. Cores were sectioned into different depths and 0.5 gram aliquot samples were taken from the surface (0-2cm) and deep (6-8cm) sections and stored at -80 C. Porewater from each core was obtained from the remaining sediment by centrifugation (5,000×g for 5 min) in 50-ml tubes with 0.45- μM cellulose acetate filter insert (Chrom Tech, Inc., Apple Valley, MN).

Physical and Chemical Sediment Characterization

Salinity was measured from the pore water in each core using a hand-held refractometer. In 2014 salinity was measured on-site rather than from each core. pH was measured from pore water using a pH100 meter with a piercing probe (YSI, Yellow Springs, OH). Nitrate concentrations were collected from pore water nitrate enzyme assay using an NECi kit (NTK-MPLR, NECI Superior Enzymes, Lake Linden MI) and ammonia was measured using the phenol-hypochloride methodology modified for microplate (Strickland and Parsons, 1972). Water content of the sediment was

additionally determined by the change in weight of sediment after drying a 1-2 gram sample from each core at 70°C overnight.

DNA Extraction

DNA was extracted from sediment samples according to manufacturer's instructions using the DNeasy kit (MoBio/Qiagen, Carlsbad CA). Two different editions of this kit were used across the three years of study, and direct comparison of the two kits yielded no significant difference in DNA yield. The only modification made to protocols from both kits was an extended centrifuge time from 30 sec to 5 minutes for the first centrifuge of the extraction. DNA was stored at -80°C and subsequent aliquots used for assays were stored at -20°C. DNA concentration and purity were quantified and confirmed using a NanoDrop Lite (Thermo-Fischer Scientific, Waltham MA) spectrophotometer. Samples with a 260:280 ratio below 1.5 were not used for further analysis, and DNA was extracted from replicate sediment aliquots for those samples.

T-RFLP Analysis

The *Betaproteobacterial amoA* gene was amplified using PCR, with the addition of a FAM labeled forward primer FAMamo-1F and a mix of two reverse primers amo-2R (Rotthauwe et al, 1997) and amo-2RTC (Nicolaisen et al, 2001). The PCR mix consisted of .5 µM of forward primers and .25 µM of each reverse primer, BSA (.01 µM), iQ Master Mix, milliQ water and DNA (1-3 µl, depending on reaction). Amplification protocol had an initial denaturation of 95 for 5 min, then 35 cycles of 95°C for 15s, 57°C for 20 s, and 72°C for 45s and a final elongation at 72°C for 5 minutes. Correct size of amplicons was confirmed using gel electrophoresis (1% agarose), and visualized with GelRed (0.01 %) under UV (ezGel). PCR products were stored at -20°C

until they were digested overnight with *Acil* (New England Biolabs, inc) and “cutsmart” buffer (New England Biolabs, Inc). Following digestion, DNA was cleaned by ethanol precipitation, and the samples were re-suspended in 10 μ l of milliQ water, 0.2 μ l of the internal size standard, GS500-ROX (Applied Bio systems Inc., Fremont, CA), and 10 μ l of Hi-Di Formamide (ABI). Samples were analyzed on an Applied Bio Systems 3730xl DNA Analyzer at the Biotechnology Resource Center at Cornell University (<http://cores.lifesciences.cornell.edu/brcinfo/>). Terminal restriction fragment (TRF) length and relative abundances were estimated using GeneMarker software, v.1.4 (SoftGenetics, State College, PA).

Quantitative PCR of Bacterial *amoA* gene

Betaproteobacterial *amoA* genes were quantified using primers amo-1F and amo-2R/amo-2RTC. All reactions were run in an iCycler or CFX (BioRad) using SYBR Green I master mix (BioRad), forward primers (.2 μ M), reverse primers (.1 μ M), BSA (.01 μ M), and 1 μ l of DNA with the following amplification protocol: 95 °C for 5 min followed by 50 cycles of 95 °C for 15 s, 57 °C for 20 s, 72 °C for 25 s, and 83 °C for 10 s. Fluorescence was measured after the 83°C step to avoid fluorescence from primer dimer formation. Melt curve analysis was also performed after each experimental run to confirm the product specificity. Sample amplification was compared to a standard curve generated in each experimental run using five standards ranging in concentration from 1 pg/ μ l to 0.0001 pg/ μ l. Standards used were isolated from plasmid DNA generated from clone library of *amoA* genes from the same marsh. Only data from runs with efficiencies greater than 85% and standard r-squared values of ~99% were used.

Effects of inhibition during PCR were tested on the same DNA samples after all samples were run undiluted and it was determined that a 1:10 dilution was optimal for amplification with minimal inhibition of some samples (Moin et al. 2009).

Statistical Analysis

Patterns in community composition data (TRFLP analysis) were uncovered using Non-Metric Scaling (NMS, Kuskal 1964) multivariate analyses in PC-Ord v.6 (McCune and Mefford 1999). An arcsine square root transformation was applied to the data to reduce skew, when ordinating the sample using the Sorensen's distance measure. Slow and thorough analysis was done with a dimensionality that minimizes the final stress and maximizes interpretability. Monte Carlo tests were performed to test if the ordinations were significantly different than randomized data. Additionally, the proportion of variance explained by each axis and the cumulative variance explained by the whole ordination were determined by calculating the coefficient of determination between distances in ordination space and distances in the original p-dimensional space. Multi-response permutation procedure (MRPP), a non-parametric statistical test, was used to test for differences between drought conditions, sample years and vegetation types. MRPP is an analysis of similarity and provides a measure of the effect (p value) when testing for differences between two or more groups (McCune and Grace 2002). Significant differences in abundance (Q-PCR) between groups (Years and Vegetation) of samples was determined by an Analysis of Variance test (R).

RESULTS

Patterns in Environmental and Nutrient Variables

Local rainfall totals (Groton, CT) for one month prior to the date of each collection, decreased by 75% from 2014-2016 followed by a 1150% increase into 2017 as the period of decreased rainfall ended (CTDEEP/NOAA) (Table 1). The drought year (2016) also had nitrate and ammonia concentrations which were below the level of detection. This was accompanied by a significant increase in salinity during the drought year (Fig. 3A). In 2017 (post-drought), the salinity levels also decreased significantly to values lower than pre-drought.

Table 1: Environmental and porewater nutrient data collected from study sites. Averages from each vegetation type with both depths combined. Precipitation is amount of rainfall in Groton CT one month prior to date of sampling. Dashed lines indicate no data was collected.

Sample Year	Vegetation	Ammonium ($\mu\text{M-N}$)	Nitrate ($\mu\text{M-N}$)	Precipitation (cm)
2014	TSA	-	-	25
	SSA	-	-	25
	SP	-	-	25
	MAT	-	-	25
2016	TSA	0 (0)	0.04 (0.07)	23
	SSA	0 (0)	0 (0)	23
	SP	0 (0)	0 (0)	23
	MAT	0 (0)	0 (0)	23
2017	TSA	34.6 (19.1)	0.49 (0.40)	40
	SSA	4.2 (2.4)	0.39 (0.20)	40
	SP	3.1 (2.1)	0.13 (0.07)	40
	MAT	35.6 (12.6)	0.08 (0.05)	40

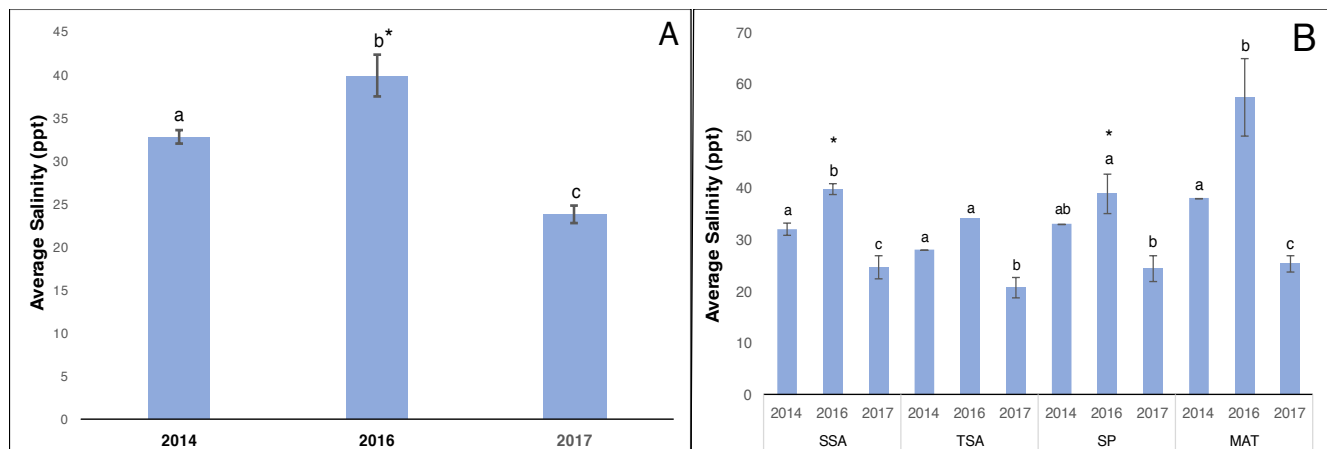


Figure 3: Mean porewater salinity values from all samples (A) and by vegetation type (B), separated by sample year, 2014 (Pre-Drought), 2016 (Drought), and 2017 (Post-Drought). Error bars represent one standard error from the mean. Bars with unique letters indicate significant differences ($\alpha=0.05$).

Salinity also varied consistently between years in all vegetation types, with the drought year always having the highest average salinity, and post-drought the lowest (Fig. 3B).

Abundance of Bacterial *amoA*

AOB abundance increased during the drought when all samples are combined, but the differences were not significant. Analysis of abundance patterns by depth revealed that the change in abundance is greater in the surface samples compared to the subsurface, but still not significantly different. At both depths, the highest abundances are observed during the drought conditions, with a great deal of variability among vegetation types.

The highest abundance in non-drought years was detected in tall form *S. alterniflora* (TSA) (Fig. 4). There was a significant effect of drought in both TSA and *S. patens* (SP) sites. AOB abundance at *S. patens* was 10,000 times higher in 2016 than in the other two sample years, while TSA decreased significantly during the drought. AOB

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abundance also increased in 2016 at the SSA and MAT sites, but the differences were not significant, perhaps due to a low number of replicates.

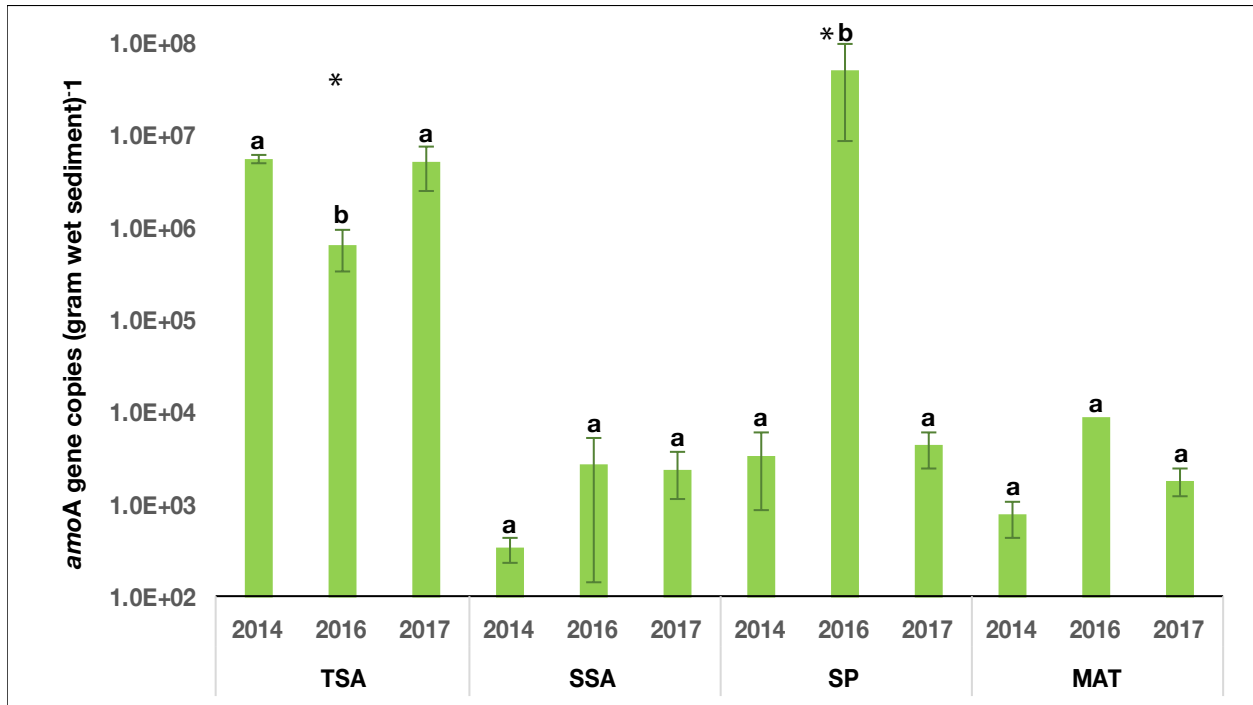


Figure 4: Mean *amoA* gene copies per gram of wet sediment of **surface** (0-2 cm) sample only, separated by vegetation type sampled from; TSA (tall form *S. alterniflora*), SSA (short form *S. alterniflora*), SP (*S. patens*), and MAT (microbial mat, absent of vegetation), as well as sample year; 2014 (Pre-Drought), 2016 (Drought), and 2017 (Post-Drought). Error bars represent one standard error from the mean. Within each vegetation type, unique letters indicate significance ($\alpha=0.05$)

In sub-surface sediments (Fig. 5) no significant effect of drought or year was observed in TSA or MAT samples. SP showed similar patterns as observed in surface sediments, with a significant increase during the drought, although post-drought abundance was higher than during the pre-drought. AOB abundance at SSA sites decreased significantly in 2016, followed by a return to pre-drought abundances in 2017.

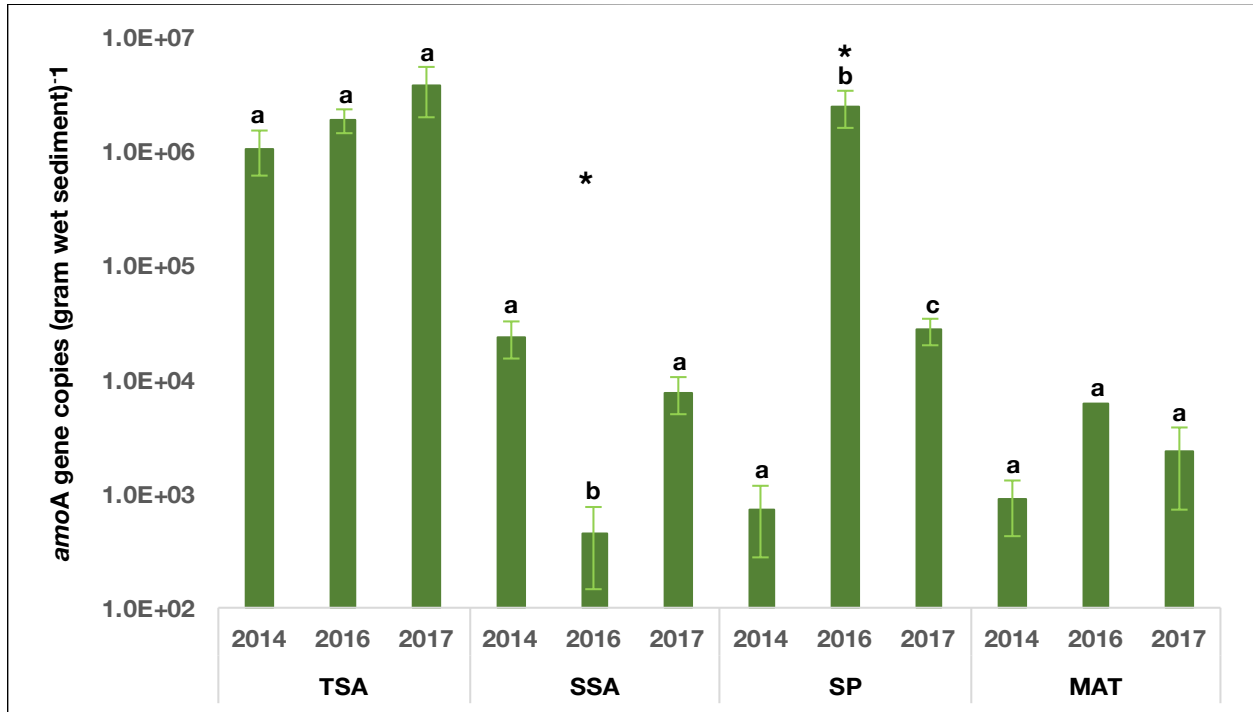


Figure 5: Mean *amoA* gene copies per gram of wet sediment of **sub-surface** (6-8 cm) sample only, separated by vegetation type sampled from; TSA (tall form *S. alterniflora*), SSA (short form *S. alterniflora*), SP (*S. patens*), and MAT (microbial mat) well as sample year; 2014 (Pre-Drought), 2016 (Drought), and 2017 (Post-Drought). Error bars represent one standard error from the mean. Within each vegetation type, unique letters indicate significance ($\alpha=0.05$)

Community Composition from T-RFLP of Bacterial *amoA*

Community composition analysis based on TRFLP of betaproteobacterial *amoA* genes yielded 14 terminal restriction fragments (TRFs). NMS ordination of all samples (Fig. 6) showed significant differences among vegetation types ($p < 0.00005$) and between depths ($p = 0.050$), but no significant effect of year or drought.

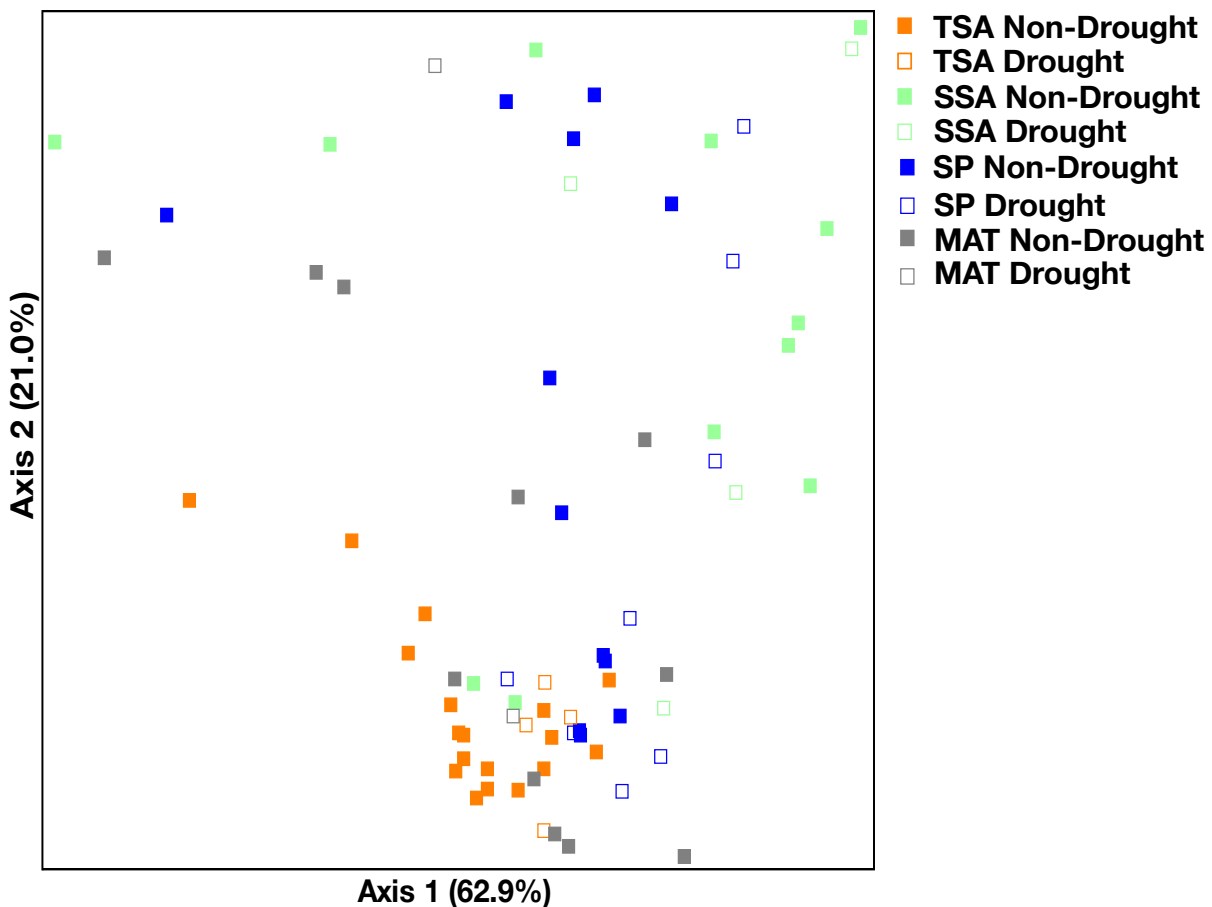


Figure 6: Non-metric multidimensional scaling plot of TRFLP profiles for betaproteobacterial *amoA* genes in surface and deep sediments. Percent variability explained by each axis is shown parenthetically on the axis labels. Filled squares represent samples from non-drought years (2014, 2017) and open squares from the drought year (2016). Colors correspond to unique vegetation types. Final stress on ordination was found to be 7.80 after 57 iterations.

Patterns in variation among sample years become more apparent at the vegetation level when separated by depth category.

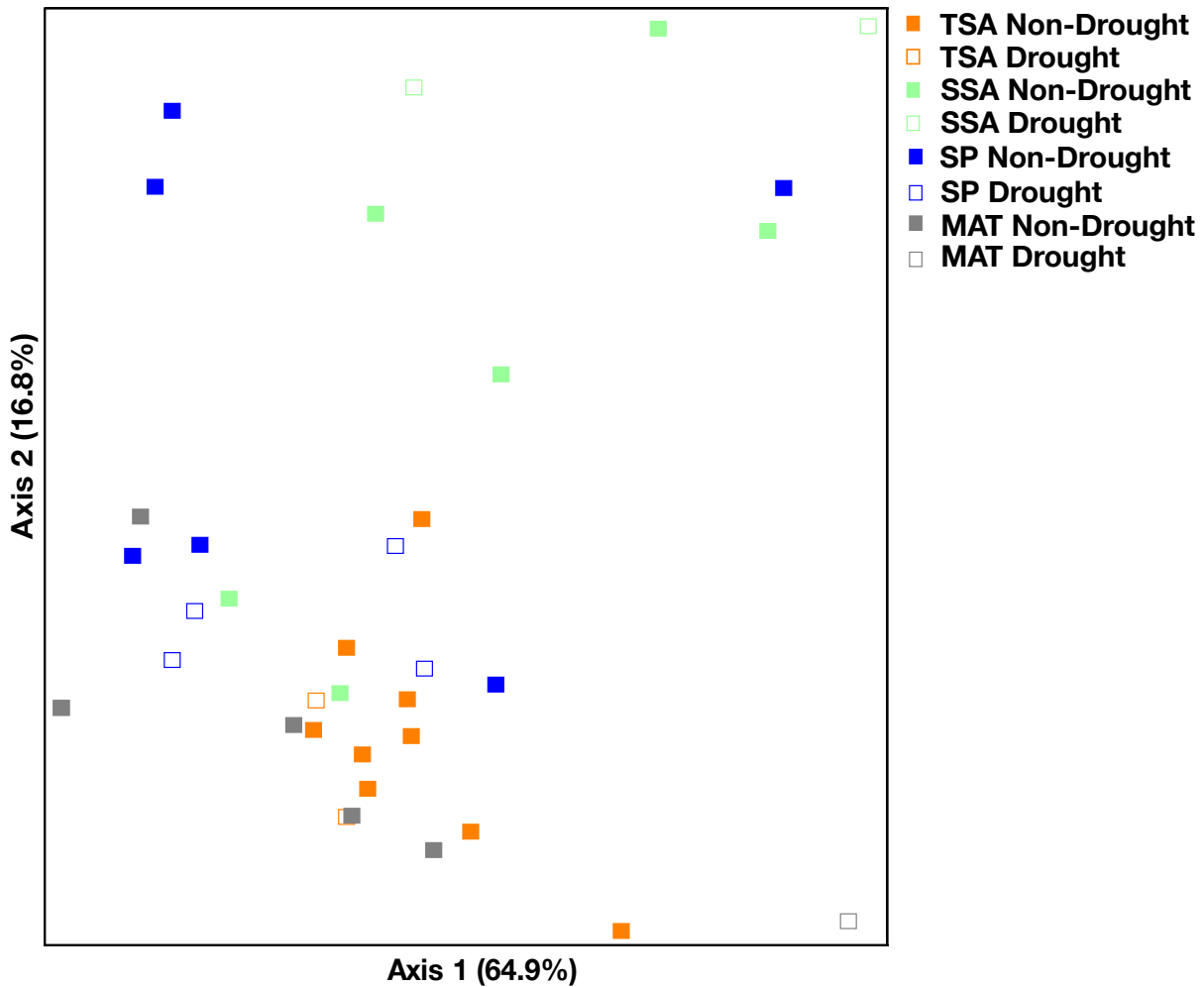


Figure 7: Non-metric multidimensional scaling plot of TRFLP profiles for betaproteobacterial *amoA* genes in **surface** sediments. Percent variability explained by each axis is shown parenthetically on the axis labels. Filled squares represent samples from non-drought years (2014, 2017) and open squares from the drought year (2016). Colors correspond to unique vegetation types. Final stress on ordination was found to be 7.71 after 64 iterations.

Similar ordination analysis of surface samples alone (Fig. 7) yielded a non-significant overall drought effect with all vegetation sites combined. There was, however, a significant drought ($p=0.045$) and year effect ($p=0.022$) within TSA surface samples and a drought effect ($p=0.050$) in *S.patens* samples.

Surface AOB communities differed among vegetation types and all showed significant variations in relative TRF abundance among sample years (Fig. 8).

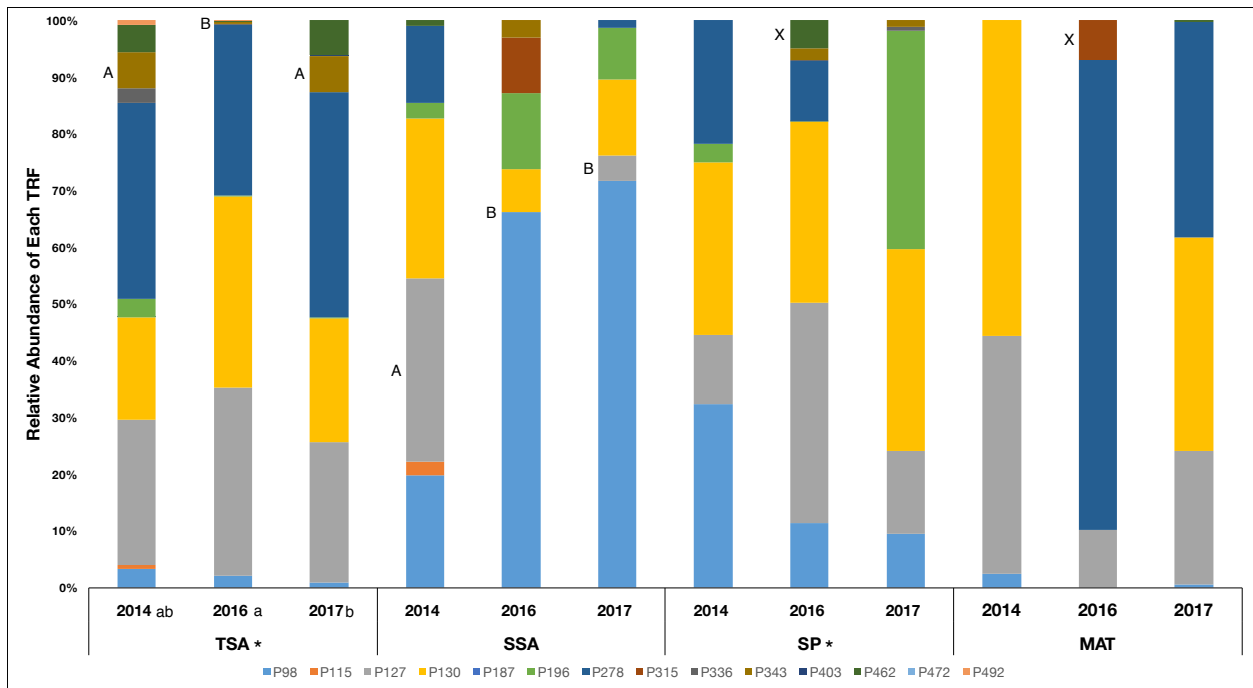


Figure 8: Mean relative abundance of individual TRF's of surface samples only, separated by vegetation type across the three years of study. Unique uppercase letters indicate significant differences between TRF abundances within a vegetation group. Xs indicate the TRF is only present in that sample year, and thus significantly different than the other two years. Lower case letters next to sample years indicate significantly different communities from MRPP analysis of ordinations ($p < 0.05$). Finally asterisks next to vegetation types indicated significant drought affect from MRPP ordination analysis ($p < 0.05$)

SP and MAT samples both show significant changes, with TRF's only present during the drought (P462 and P315, respectively), and had continued community fluctuation post-drought. TSA had significant changes which revert to pre-drought conditions in 2017. While, SSA had a significant shifts in the drought year which persisted into 2017.

Sub-surface samples (Fig. 9) alone had no effect of drought or year in TSA and SP, but a significant effect of year ($p = 0.005$) in SSA sub-surface samples, with 2017 being significantly different from 2014 and 2016.

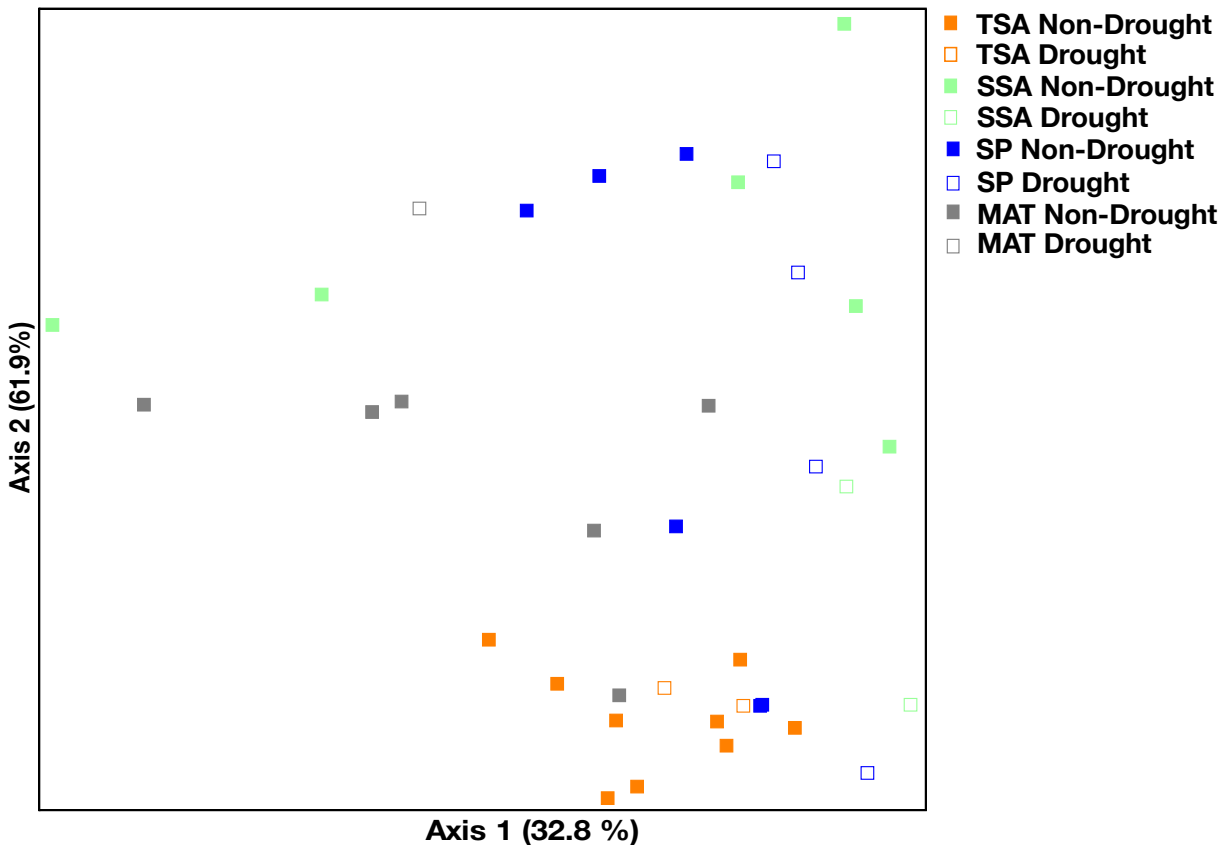


Figure 9: Non-metric multidimensional scaling plot of TRFLP profiles for betaproteobacterial *amoA* genes in **sub-surface** sediments. Percent variability explained by each axis is shown parenthetically on the axis labels. Filled squares represent samples from non-drought years (2014, 2017) and open squares from the drought year (2016). Colors correspond to different vegetation types. Final stress on ordination found to be 9.76 after 37 iterations.

Sub-surface relative abundance community analysis (Fig. 10), found no significant change among the sample years in TSA samples. Similarly, MAT samples also had no significant effect but did see a reduction in TRF diversity during the drought which returns somewhat in post-drought.

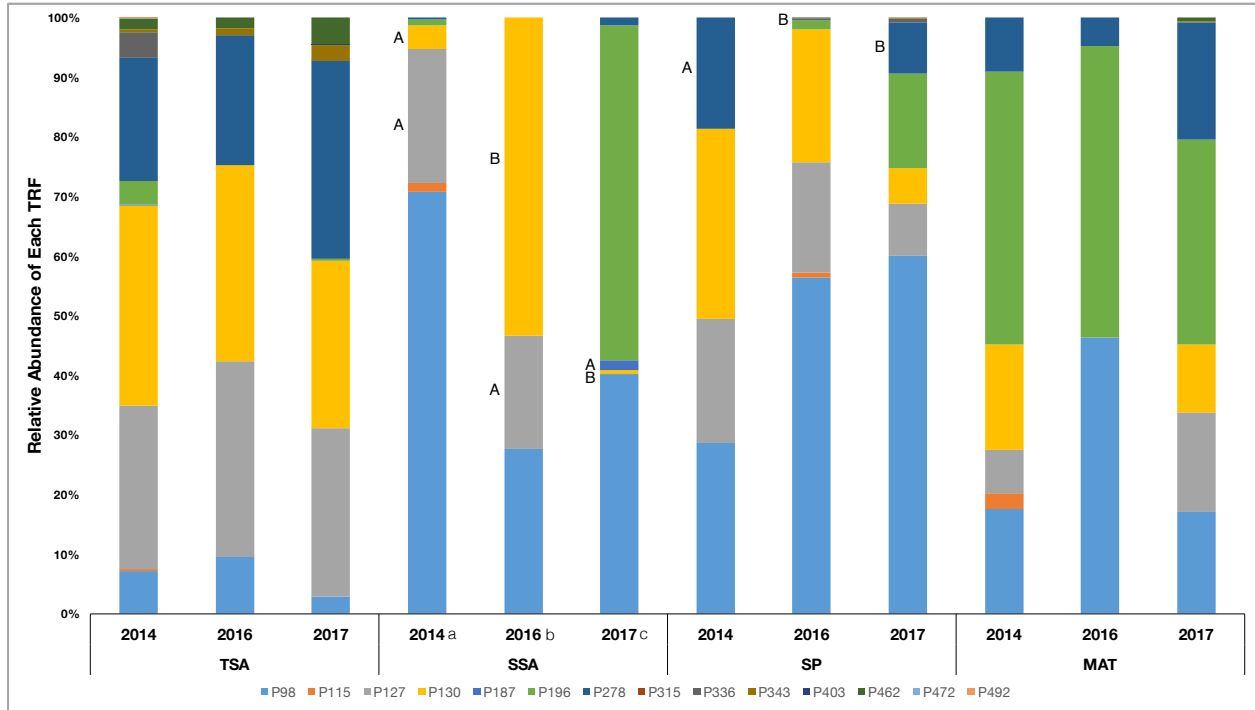


Figure 10: Mean relative abundance of individual TRF's of sub-surface samples only, separated by vegetation type across the three years of study. Unique letters indicate significant differences between TRF abundances within a vegetation group. Xs indicate the TRF is only present in that sample year, and thus significantly different than the other two years. Lower case letters next to sample years indicate significantly different communities from MRPP analysis of ordinations ($p < 0.05$).

SSA and SP however, have multiple TRFs which vary significantly among the three years. These groups also have some sites whose drought effects revert post-drought and others that continue to change. This suggests a less clear pattern or drought reaction, but clearly a period of instability for these communities.

Relationship to Total Microbial Community

Quantitative-PCR assays of bacterial communities, based on 16S rRNA genes, were conducted for all samples and used to place AOB abundance into a more complete environmental context. During the drought, the ratio of AOB to total bacterial abundance (Fig. 11) was significantly higher, indicating AOBs made up a higher proportion of the total microbial community during the drought year. In 2017 the ratio decreased to lower than pre-drought conditions, indicating an surge of growth from the total bacterial community after the break in drought.

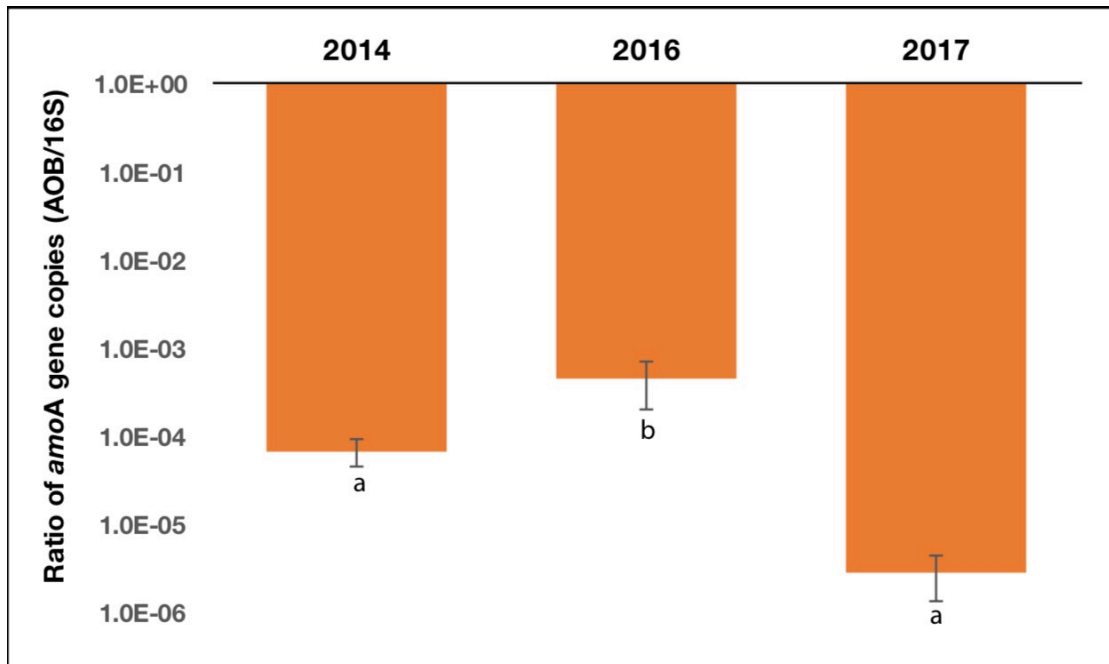


Figure 11: Mean ratios of QPCR total bacterial abundance based on bacterial 16S assays for each sampling year. Error bars indicate one standard error from the mean, and unique letters indicate significance ($p < 0.05$) within each figure.

In surface samples (Fig. 12A) the ratio of AOB to total bacterial abundance in TSA and SSA showed no significance or pattern, but increased significantly in both SP and MAT in 2016. In sub-surface (Fig. 12B) ratios significantly increased during the drought year

in TSA and SP samples. These increases indicate the bacterial community is shifting to have a higher relative proportion of AOBs during drought.

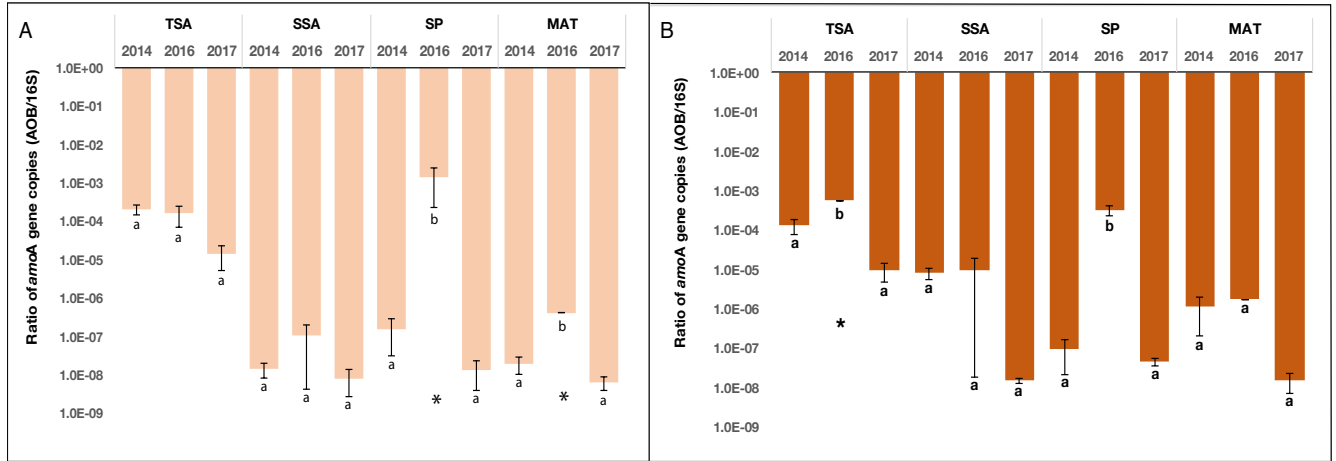


Figure 12: Mean ratios of AOB to total bacterial abundance based on bacterial 16S assays for surface samples (A) and sub-surface samples (B) separated by vegetation and year. Error bars indicate one standard error from the mean, and unique letters indicate significance ($p < 0.05$) within each figure. Asterisks indicate significant drought affect in that vegetation group.

Log AOB abundance values do not significantly correlate to any of the abiotic environmental variables (Fig. 13). Total microbial abundance had a highly significant positive correlation to precipitation, as well as moderate positive correlations to ammonia and nitrate (Fig. 13). Total bacterial communities demonstrated a moderate negative correlation for both pH and salinity. As expected there was a strong negative correlation of salinity to all nutrient variables, and positive interactions among ammonia, nitrate and precipitation.



Figure 13: Compound correlation plot showing scatterplot outlines, correlation coefficient values and significance indicator of log (10) value of microbial abundance and quantitative environmental variables. Corresponding correlation interactions are represented at the interaction of the row and column of the variable. Scatterplot outlines showed with spread size and color intensity indicating R-squared value, with value provided on mirrored side. X markings over the boxes indicate non-significant relationships ($p > 0.05$) based on interaction. Plot generated in R (cran) using packages Car and Corrplot (cor.mtest, corrplot.mixed).

Salinity was significantly correlated to the first axis of the NMS ordination of deep samples only (fig. 14). The highest salinity values, represented by the largest squares are found in the drought year and especially in MAT and SP samples. This suggests that salinity is an important driver of the observed differences among vegetation groupings.

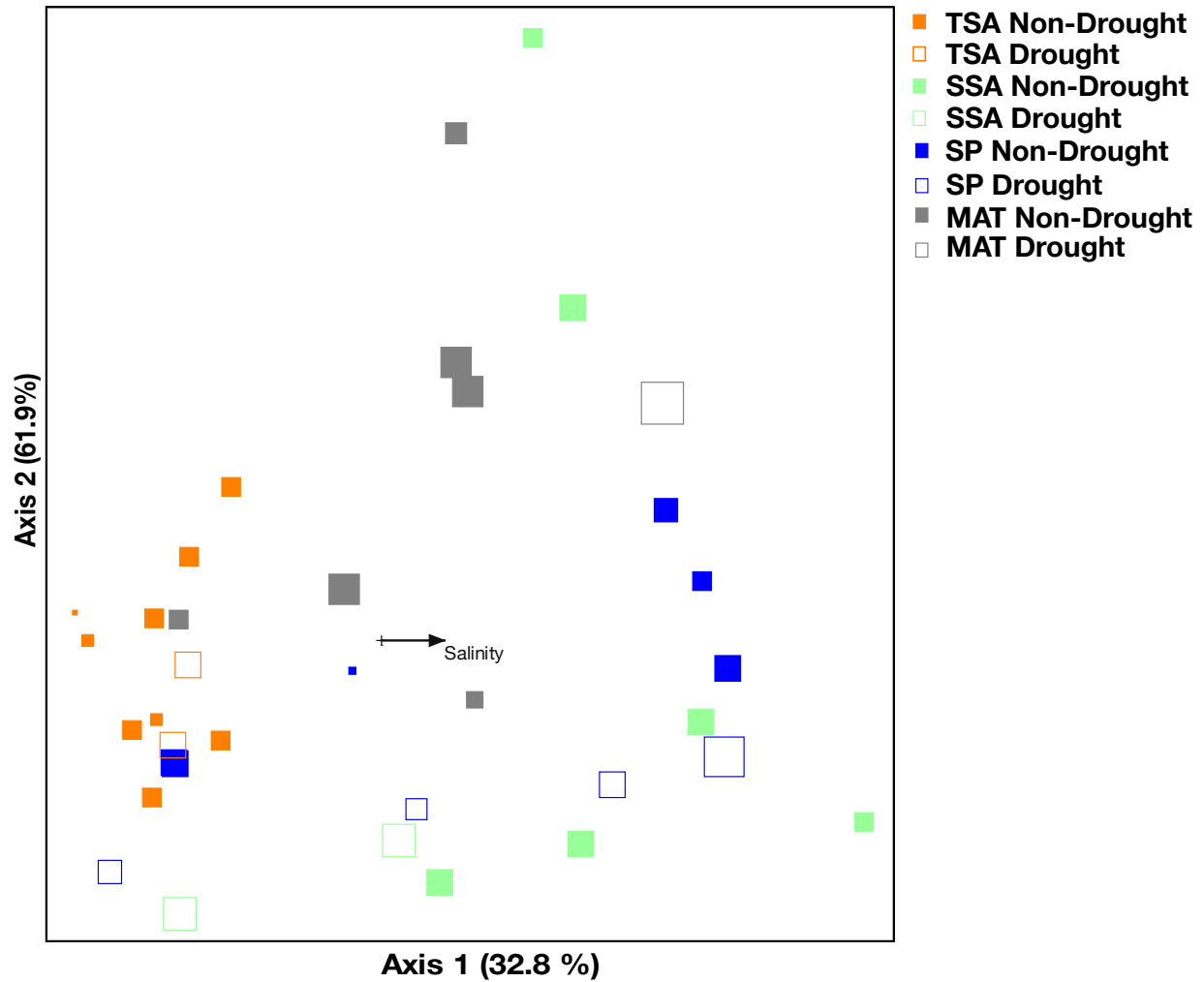


Figure 14: Non-metric multidimensional scaling plot of TRFLP profiles for betaproteobacterial *amoA* genes in deep sediments with size indicating corresponding salinity value of that sample. Axis 1 had a significant correlation to salinity values ($r = 0.326$, $p = .037$), intensity indicated with central arrow. Percent variability explained by each axis is shown parenthetically on the axis labels. Filled squares represent samples from non-drought years (2014, 2017) and open squares from the drought year (2016). Colors correspond to unique vegetation types. Final stress on ordination found to be 9.76 after 37 iterations.

DISCUSSION

The conditions brought about during the period of drought had a significant effect on both abundance and community composition of ammonia-oxidizing bacterial communities. This effect varied greatly between vegetation types, community depth, and post-drought recovery patterns. While some communities (TSA Surface and SSA deep) declined in abundance during the drought year, the SP communities at both depths significantly increased. This difference in community response is matched by significant changes in community composition during the drought, which suggests independent drought responses among microbial communities associated with different vegetation types.

Inter-annual abundance patterns suggest that during the drought, conditions were altered in a manner that caused growth in some communities (SP) and negative effects to others (SSA and TSA). Drought conditions have been shown to have a positive impact on marsh vegetation communities, with increased photosynthetic rates in warm weather, and plant stress alleviated by a lack of waterlogging and increased aeration (Charles and Duke 2009). Decreased soil moisture from drought causes greater nutrient trapping during tidal flooding, and overall greater nutrient availability (Charles and Duke, 2009). Increased oxygen soil penetration has been shown to stimulate and increase nitrification and denitrification rates (Dollhopf et al. 2004), suggesting an increase in microbial activity. Some AOB microbial populations could have benefited from these changes, with greater access to available nutrients to metabolize (Palomo et al 2013) and increase oxygenation allowing the aerobic AOBs to

function deeper in the soil. The variation in conditions between high marsh (SP/MAT) and low marsh (TSA and SSA) ecosystems could have fostered communities with different responses to drought. Greater aeration from sediment drying, and warmer surface conditions in the high marsh could have been especially advantageous for microbial growth in the SP communities.

Conditions at SP sites had similar salinity levels and patterns in annual variation to other sites, especially SSA. Increased salinity has been shown to have an inhibitory effect on nitrification (Marton et al. 2014) and our data show a strong negative correlation of nitrogenous compounds and salinity. Differences in AOB abundance and community composition observed between TSA and SSA could be a result of differences in tolerances of the microbial community to above average salinities. Salinity has also been shown to have a negative correlation with AOB and AOA abundance (Moin et al. 2009), and we also found that salinity was negatively correlated to total bacterial abundance (Fig. 14). During core collection in 2016, a distinct lack of pore water was observed from many sampling sites. This lack of sediment water confirms assumptions about sediment drying and also explains increases in sediment salinity. The lack of porewater could have led to the formation of salt crystals, which could have affected how salt in the soil interacted with the microbial community, potentially lessening the impact of salinity on microbial function.

This observed lack of pore water complements the physical signs of desiccation seen in other areas of the marsh (Figure 1). The drought conditions caused sediment drying to the point of developing fractures in the surface, which could have promoted greater aeration and oxygenation (Charles and Duke, 2009). In the high marsh, the

heightened oxygenation of the soil and rhizosphere could explain a bloom of AOB observed in SP during the drought. Increases in oxygen consuming nitrogen fixers have been seen during drought conditions (Davis et al. 2018), which was explained by an increase in the oxic zone of the soil, allowing expansion of aerobic colonization. This supports the explanation offered for the SP bloom and indicates a common response between different microbial communities under drought stress. Additionally, the abundance decrease in 2017 in most vegetation sites could be a by-product of waterlogging conditions. A spike in rainfall before collection in 2017 could have led to waterlogged soil conditions with decreased O_2 and increased pH, which have been shown to decrease abundance and cause a shift in microbial composition. (Nyugen et al. 2017).

The impact of the drought on abundance was, in all cases, transient, with post-drought conditions, one year later, returning to comparable abundance to pre-drought levels in both surface and deep sediments at sites where there was a drought effect. This rapid return mirrors the transient drought effect observed on vegetation documented in several other studies (Wetzel and Kitchens 2007, Paloma et al. 2013, and Fuchsleuger et al. 2014). The resiliency of the marsh with regards to AOB abundance does not appear to be affected by vegetation, with all significantly affected vegetation types showing post-drought returns. This recovery pattern holds for vegetation types that saw positive and negative growth during the drought conditions. This rapid resiliency with respect to population recovery post-drought, is consistent with other AOB response patterns (Fuchsleuger et al. 2014)

The AOB response and recovery to drought appears to be independent of the rest of the microbial community. In drought conditions, AOBs made up a significantly greater proportion of the total microbial community than in pre/post-drought. Total bacterial abundance decreased significantly in 2016, making the population blooms of AOB in SP account for a larger proportion of the bacterial community, especially in 2016. The ratio of AOB to total bacteria increased at TSA sites during the drought, which is surprising given the AOB abundance decreased during the drought. This suggests that TSA drought conditions had a strong negative impact on all bacterial populations, and even though AOBs were less abundant, they still showed greater resistance than the rest of the microbial community. There is no overall effect of drought on the ratio of AOB abundance to total bacterial abundance with all vegetation sites combined because of strong differences between pre and post drought. After the drought, AOBs made up a much smaller subset of the community than in 2014. This could be due to lesser drought-like conditions seen in 2014 causing a general decline in abundance. In 2017 there was a strong end to the drought and above average rainfall conditions, and therefore an increase in microbial abundance is expected as conditions improve accordingly

The TRFLP analysis revealed significant changes in the communities, which may have had a significant effect on abundance. This combined effect of abundance and community diversity demonstrates a very strong impact of drought in SP, TSA surface and SSA sub-surface . AOB diversity has been shown to shift in response to physiochemical or vegetation shifts and community stress (Xia et al. 2015). The drought impact on community composition resulted in a subtle shift between surface

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and subsurface and large differences between the vegetation types. While abundance consistently returned to pre-drought conditions in 2017, changes in community composition persisted one year later at SSA and SP sites especially. All surface vegetation types had significant changes in TRFs during the drought year. Generally, most types showed strong changes in diversity structuring, especially in the drought year, which either carries over or continues to change. Drought conditions could have caused a shift to more drought tolerant species. Then the return to normal conditions in the post-drought could have allowed a non-tolerant TRF to outcompete the drought tolerant microbes and take hold in the available niche. The unique community shifts seen in 2017 could be a result of selective pressure from sediment waterlogging, which has been shown to cause community change in AOBs (Nyugen et al. 2017). Shifts in salt marsh nitrogen fixing communities have been reported in drought, and were especially strong in the post-drought period (Davis et al. 2018). Environmental factors contribute to compositional changes in salt marsh AOB communities (Bernhard et al. 2007). However, there is no clear indication of a common TRF unique to all drought populations, suggesting that community response to drought tolerance is more complex than a simple shift in genotypes, and may be specific to different vegetation sites and sediment conditions.

The observed community shifts at SP and TSA sites could be the result of opposing response in abundance shifts. SP saw a large increase in abundance at both depths during the drought year, while TSA had a significant decrease in abundance in surface sediments. As stated, the spike seen in SP could be attributed to positive ecosystem alterations, such as increased oxygen aeration, that came about as a result

of the drought. This produced an enlarged oxic zone in the soil which allowed for greater AOB colonization at depth. Since this new niche became available with the increase in oxygenation depth, a greater quantity and diversity of microbes would be able to colonize the depth, as is observed in SP samples. AOB abundance at the TSA sites, however, experienced a negative effect of drought, where the drought likely selected for drought-tolerant AOB. TSA had a significant decrease in TRF343 abundance in 2016, which could represent a non-drought tolerant TRF which was unable to survive. A similar loss of diversity has been attributed to estuarine sediment microbial communities that are found in higher saline environments (Bernhard, 2005). The correlation of community variation to salinity specifically further reinforces drought as the driving force causing the change in 2016, leading to different responses in different vegetation sites. This effect on community composition coupled with abundance, demonstrates a distinct complex response to drought that varies by marsh zones.

The maintenance of a continuous and strong cycling of nitrogen through an ecosystem is crucial for the health and survival of all members of the community. We have shown that drought can manipulate not only the abundance of nitrogen cycling microbes, but also the community diversity. It has been established that changes in the abundance and community structure of AOBs, can impact the nitrification rates, with higher abundances correlating to the highest rates (Wankel et al. 2001). The impacts of decreased nitrification, produced by unfavorable fluctuations in the microbial communities, could have large impacts on the productivity and viability of salt marsh

habitats. This problem could even be further amplified if the frequency and duration of drought events increases with global climate change.

Droughts are a phenomenon common in most ecosystems, and as habitats and populations evolve, so too does their ability to handle the variability in the conditions of their native range. A diverse community of nutrient cyclers is more likely to have the ability to withstand a stress event. The microbial response observed during the drought year is the product of evolution and an adaptation to their ecosystem. Microbial communities may not, however, be able to tolerate an increase in drought frequency brought on by global climate change. Acute marsh dieback events (AMD), which are brought about in large part as a result of sea level rise from climate change, will only become more frequent as the trend in atmospheric carbon dioxide concentration increases (Hughes et al. 2012). *Spartina*-dominated marshes have been shown to be very tolerant to these events, and sea level rise in general (see review by Hughes et al. 2012). However, current model-based predictions do not account for AMDs which are caused by or intensified by drought events (Hughes et al. 2012). Increased frequency of hypersaline pore water has been correlated to increased occurrences of AMDs and can even serve as a predictor (Hughes et al. 2012). The effect that increased drought and salinity fluctuations will have on the microbial community's ability to react remains to be fully elucidated. The non-significant or positive drought effect on AOB abundance in some vegetation areas is a sign of resilience from some vegetation types. Salt marshes however, are being threatened on many fronts. As marsh habitat continues to decrease, so too does the microbial diversity which is the key to tolerances to stressors like drought.

FUTURE STUDIES

This study uncovers the relationship between drought conditions and the ammonia oxidizer communities of multiple salt marsh vegetation types. It was determined that this response varies greatly among the different vegetations. Future studies hope to delve deeper into one vegetation sediment community, specifically *S. patens*. SP AOBs had a bloom in abundance and altered community composition during the drought year, that warrants further analysis from other available Barn Island headquarters marsh samples. More samples could make the observed patterns clearer and make the significance more robust, creating a more confident message.

Phylogenetic analysis of these communities would also be an asset to the characterization of the drought interaction. Using sequence analysis to observe shifts in the genetic composition of the microbial communities before, during and after the period of drought, would be a valuable contribution to completing the story.

Additionally, further investigation into how other nitrogen cycling bacterial groups (i.e. N-fixers and denitrifiers) were impacted by the period of drought could expand the overall understanding of the drought's impact on nitrogen in the salt marsh.

Future studies of this nature should additionally attempt to analyze and quantify an effect of drought on microbial function, in the form of nitrification or denitrification rates. Larger scale studies of drought impact could also be conducted using similar methodologies, which include samples collected from a diversity of marshes, from New England and otherwise. Adding a geographic component to the understanding of the

bacterial drought interaction would help determine if the observed response is a product of the location rather than the ecosystem.

Many aspects of ammonia-oxidizing microbial communities response to disturbance remain uncharacterized. Careful analysis of the impact of drought on these communities from a variety of ecosystems could help elucidate the drivers of these populations, as well as how they may be impacted by a changing climate.

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