Responses of Leaf Litter Breakdown Rates and Microbial Enzyme Activity to Salinity in North Carolina Wetlands.

By Patrick R. Korn

June, 2014

Director of Thesis: Marcelo Ardón

Major Department: Biology

Sea-level rise and human activities are causing the increase of salinity in coastal freshwater wetlands. Increased salinity in some wetlands has been found to accelerate leaf litter decomposition, an important driver of nutrient availability and carbon sequestration. Research at Timberlake Observatory for Wetland Restoration (TOWeR) and two reference wetlands in eastern North Carolina has documented periods of increased salinity associated with drought. Here, I examined breakdown rates of leaf litter from common wetland tree species (*Nyssa biflora* and *Liquidambar styraciflua*) in TOWeR and two reference wetlands. I also examined macroinvertebrate abundance and microbial enzyme activity on the litterbags. Leaf breakdown and microbial activity was also measured in a microcosm experiment that exposed microbial inoculums from both reference wetlands to high and low salinities. Microbial extracellular enzyme activity for the acquisition of carbon (beta-glucosidase, BG), nitrogen (N-acetylglucosaminidase, NAG, and leucine aminopeptidase, LAP), phosphorus (acid phosphatase, AP), sulfate (arylsulfatase, AS), and the breakdown of phenol groups (phenol oxidase, PO) was obtained using standard fluorometric (absorbance for PO). Leaf litter breakdown rates, as well as BG, NAG, LAP, PO, and AP activity were expected to increase with higher salinity, while the activity of AS and macroinvertebrate

abundance was expected to decrease. During the 29 weeks of the field experiment, salinity incursion was not as prevalent as in previous years; reaching conductivities of 111.59 and 1863.37 µS cm^{-1} for the sites with the lowest and highest respectively. Despite the lack of a large change in salinity, I observed a tripling of field leaf litter breakdown rate (from 0.001±0.0001 d^{-1} to 0.0029±0.0001 d^{-1}) and a linear increase of breakdown rates with increasing conductivity across the sites (R^2=0.84 p=0.027). Microcosm breakdown rates were higher than field breakdown rates (0.0026±0.007 d^{-1} to 0.0033±0.0006 d^{-1}) and did not correspond with salinity but did have a strong negative linear relationship with the amount of dissolved organic carbon (DOC) available (R^2=0.96 p=0.006). Enzyme activity increased in response to increased salinity in the field and microcosm experiment but responses were not consistent between lab and field and overall were low compared to literature values. Macroinvertebrate presence was low, only being present in 49 out of 315 litter bags, and did not correlate to salinity or increased breakdown rates. Overall, the lack of consistent results between field and microcosm suggest that small changes in salinity are unlikely to lead to major changes in leaf decomposition and microbial enzyme activity.

Responses of Leaf Litter Breakdown Rates and Microbial Enzyme Activity to Salinity in North Carolina Wetlands.

A Thesis

Presented To

The Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Patrick R. Korn

June, 2014

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Patrick R. Korn

APPROVED BY:

DIRECTOR OF DISSERTATION:_____
Marcelo Ardón, PhD

COMMITTEE MEMBER:_____
Emily Bernhardt, PhD

COMMITTEE MEMBER:_____
Robert Christian, PhD

COMMITTEE MEMBER:_____
Ariane Peralta, PhD

CHAIR OF THE DEPARTMENT OF BIOLOGY:

_____
Jeff McKinnon, PhD

DEAN OF THE GRADUATE SCHOOL:

_____
Paul J. Gemperline, PhD

Acknowledgements

First and foremost, I would like to thank my advisor, Marcelo Ardón, for all of his hard work, patience, motivation, enthusiasm, and dedication. Without his support and immense knowledge, this project would not have been possible. His guidance helped push me through the research and writing of my thesis. I could not have asked for a better advisor.

Besides my advisor, I thank the members of my committee: Emily Bernhardt, Robert Christian, Ariane Peralta, and Matt Schrenk for their though provoking input and insightful comments.

I would also like to thank my fellow graduate student, Amanda Powell, and lab assistant, Lonnie Jackson, for all of their help in the field and lab. Special thanks are owed to Colleen Rochelle for her help in analyzing water samples and laboratory tutelage, and Katrina Twing for passing along her genetic knowledge.

Many headaches were averted thanks to the ECU Biology Department staff, foremost, Joyce Beatty.

The Society of Wetland Scientists National and South Atlantic chapters also receive my appreciation for their financial support.

Lastly but not least, I would like to thank my fiancée, Ashland Joyner, and the rest of my family for their continual support of my endeavors.

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Introduction

Wetlands are important ecosystems offering many valuable services such as: pollution regulation, food and water provision, flood mitigation, climate regulation, education, recreational, and cultural services (Mitsch and Gosselink 2007). Wetlands also sequester large amounts of carbon due to their high productivity and slower decomposition when compared to upland environments (Collins and Kuehl 2001, Mitsch and Gosselink 2007). Despite the many services provided by wetlands, it is estimated that over 50% of wetland area worldwide has been lost to development, agriculture, damming, and other human activities (Mitsch and Gosselink 2007). While these stressors influence wetlands everywhere in the landscape, the anthropogenic pressure placed on coastal wetlands is particularly large because 23% of the global population lives within 100 km of the coast (Small and Nicholls 2003). In addition to direct anthropogenic drivers of wetland loss, coastal wetlands also face climate change challenges to their structure and function, such as increased frequency and duration of drought, increased intensity of storms, and accelerating sea-level rise (SLR) (Moorhead and Brinson 1995, Sallenger et al. 2012, Pederson et al. 2012). One of the outcomes of these pressures on wetlands in coastal regions is functional change due to increased saltwater incursion.

Many wetlands in the coastal plain of North Carolina are hydrologically connected to the Albemarle and Pamlico Sounds. In the sounds and their sub-estuaries, freshwater flowing seaward mixes with high salinity water from the Atlantic Ocean.

However, the point of mixing is not always stagnant, and can move upstream or downstream in response to wind driven tides, rainfall, drought, storms, and season (Day et al. 2007, Anderson and Lockaby 2011). During the summer, and other periods of reduced precipitation and increased evaporation, the high saline water from the Sounds migrates further inland, causing saltwater incursion into formerly freshwater wetlands (Ardón et al. 2013). Climate change predictions for the southeastern United States of accelerating sea level rise and increased frequency and duration of drought will further exacerbate the frequency and magnitude of saltwater incursion (NCA 2014, Mulholland et al. 1997). In addition to climate and weather, anthropogenic actions such as dredging, coastal development, and water use also increase the risk of saltwater incursion (Newport 1977). Increasing salt concentration in freshwater wetlands can influence many different aspects necessary to a functional wetland. This study focuses on the effect of increasing salinity on leaf litter (organic matter) decomposition and microbial enzyme activity.

Organic matter decomposition is an important process in determining nutrient availability and carbon sequestration in wetlands (Brinson et al. 1981). Controls on the rate of leaf litter breakdown include environmental (salinity, temperature, water availability, flooding regime, nutrient availability) and biotic (leaf litter quality, microbial community structure, microbial enzyme production, and macroinvertebrate presence, Brinson et al. 1981) drivers. The first step of organic matter decomposition is the leaching of soluble compounds when leaves enter the water (Benfield 2006). Many of these soluble compounds are primarily made of carbon (dissolved organic carbon) and organic forms of nutrients that microbes use for metabolism and growth (Strauss and

Lamberti 2002). Different salts have varying effects on the quantity of nutrients leached (Tukey 1970); however, it is unclear if increased salinity can change the leaching of dissolved organic carbon (DOC) from leaf litter or its usage by microbial communities. Regardless of the changes to the leaching of DOC, salinity induced changes in the solubility of DOC or its quality could determine whether this C is mineralized or is buried in the soils. Additionally, research in a restored wetland in the coastal plain of North Carolina has shown that increased salinity can dramatically reduce the export of dissolved organic carbon (DOC) from coastal wetlands (Ardón et al. in preparation).

After the rapid initial leaching, organic matter is then further broken down by microbial communities and macroinvertebrates. Saltwater incursion into former freshwater wetlands can also alter organic breakdown by altering the inputs of leaf litter, changing microbial activity (Craft 2007), and changing the distribution of macroinvertebrates (Kefford et al. 2003). In some situations increased salinity has been found to increase microbial decomposition of leaf litter due to an increase of the electron acceptor, sulfate (SO_4^{2-}, Dierberg et al. 2011, Barendregt and Swarth 2013). In anaerobic conditions, microbes use electron acceptors other than oxygen to break down organic matter. The most common anaerobic electron acceptors are used according to their relative energetic yield, in the order: NO_{3-}, Fe^{3+}, SO_4^{2-}, and CO_2 (Stumm and Morgan 1996, Burgin et al. 2011). Because salinity increases availability of SO_4^{2-} in anaerobic conditions, several studies have found that higher salinities increase organic matter mineralization, as well as microbial activity (Wright and Reddy 2001, Weston et al. 2006, Weston et al. 2011). However, increased salts in the water can make it difficult for freshwater microorganisms to osmoregulate (Oren 2001), leading to a decrease in

metabolic activity and respiration (Rietz and Haynes 2003). However, this decrease in activity is likely temporary and only present when the microbial communities are changing as the ecosystem transitions from fresh to saline water. Saltwater incursions effects on organic matter breakdown depend on both the amount of SO_4^{2-} introduced into the system and the amount of osmotic stress that the microorganisms experience.

In addition to organic matter breakdown, microbial extracellular enzyme production also hinges both the osmotic stress induced by increased salts and the amount of SO_4^{2-} available. Microbial extracellular enzymes are responsible for catalyzing the rate-limiting steps in organic matter breakdown and altering nutrient availability (Sinsabaugh et al. 2008). Enzyme production is controlled by microbial activity as well as substrate composition (Sinsabaugh and Moorhead 1994) and can be interpreted as an indicator of microbial nutrient demand (Olander and Vitousek 2000, Moorhead and Sinsabaugh 2006). Microbes use specific enzymes to acquire or breakdown compounds made of carbon (C), nitrogen (N), phosphorus (P), sulfur (S), and the small monomers in phenol groups. The breakdown of carbon compounds and acquisition of organically bound P, N, and S can be obtained by measuring the microbial enzyme production of: beta-glucosidase, C acquisition (BG); N-acetylglucosaminidase, N acquisition (NAG); leucine aminopeptidase, N acquisition (LAP); Acid phosphatase, P acquisition (AP); arylsulfatase, S acquisition (AS); and phenol oxidase phenol group breakdown (PO). Enzymes are released in greater amounts when nutrients are more difficult to obtain or when the microorganisms are stressed. Extracellular enzymatic activities, such as BG and PO, are important for transforming the C in cellulose or lignin into sugars for microbial metabolism (Sinsabaugh et al. 1991).

Macroinvertebrates are also important drivers of organic matter decomposition. Some macroinvertebrates colonize and shred leaf litter, increasing the surface area and making it easier for microbial breakdown (Hauer and Resh 2006). Conversely, microbial colonization of leaf litter also makes it more nutritious for macroinvertebrate consumption (Barlocher and Kendrick 1975). Macroinvertebrate communities vary in their tolerance to salinity based on their life stage, with many adults being highly tolerant and larvae and eggs being very sensitive to as little as a 5% increase in salinity from freshwater conditions (<0.5 ppt, Kefford et al. 2004).

This study combined a field component and a laboratory microcosm designed to measure the breakdown of organic material and microbial enzyme activity in different salinities that represent the early stages of saltwater incursion. In both components I examined leaf litter breakdown rate and microbial enzyme activity on leaf litter across different salinities. For the field component, I examined the questions 1) *Do leaf litter breakdown rates change across forested wetlands with different salinities? 2) Do the microbial enzyme activity profiles differ across forested wetlands with different salinities?* And *3) Does the abundance of macroinvertebrates decrease with increasing salinity across different wetlands?* I predicted that **1) Leaf litter breakdown rates in the litterbags will increase with salinity, 2) Microbial enzyme activity of PO, BG, AP, NAG, LAP will increase with salinity, while AS will decrease, and 3) There will be fewer macroinvertebrates in higher salinities**. To answer these questions, I used litter bags containing leaves from two of the most common tree species in three wetlands (swamp tupelo (*Nyssa biflora*) and sweet gum (*Liquidambar styraciflua*)). These bags were placed in 5 sites along a salinity gradient: one site in a freshwater

wetland, 3 sites within a restored wetland along a salinity gradient, and one site with brackish conditions.

The laboratory microcosm component was used to examine different microbial community's ability to breakdown leaf litter, take up DOC, and their microbial enzyme activity, at different salinities. This component shared questions 1 and 2 of the field component, and also asked *4) Does salinity affect microbial uptake of dissolved organic carbon (DOC)? 5) Does salinity have a different effect on the microbial enzyme activity and leaf litter breakdown of inoculum that have been previously exposed to salinity or naïve inoculum?* I hypothesized that **4) DOC uptake will increase as salinity increases because of the additional SO_4^{2-} available to the microbial communities and 5) Inoculum that have had previous salinity exposure will have more microbial enzyme activity and faster leaf litter breakdown.** To test hypotheses 1, 2, 4, and 5, I exposed two microbial communities to both high and low salinities in a laboratory setting. The microbial communities differed in their historic exposure to salinity (one "naïve" community from a freshwater wetland with no prior saltwater incursion, and the other "experienced" community from a brackish wetland where salinity can be greater than 5 parts per thousand, ppt).

The information gathered in this study will give the scientific community a better idea of what will happen to organic matter breakdown and microbial enzyme activity as saltwater incursion into freshwater wetlands becomes a more common occurrence.

Site Description

Albemarle Peninsula

All of my sites were located in the Albemarle Peninsula, which is a 5000 km² area found in the coastal plain of North Carolina, where recent acceleration of sea-level rise (SLR) has led to rates that are 3 to 4 times greater than the global average, leading to regional SLR of around 27-48 cm by 2100 (Sallenger et al. 2012). Additionally, climate models and paleohydroclimate records indicate that drought may increase (Pederson et al. 2012). Other factors that can lead to saltwater incursion are human water use, dredging, and coastal land development. These other factors coupled with drought and accelerated SLR are expected to exacerbated saltwater incursion (Mulholland et al. 1997, Barlow and Reichard 2010).

Timberlake Observatory for Wetland Restoration

The Timberlake Observatory for Wetland Restoration (TOWeR) is a 1704.2 ha privately held mitigation bank, within the Great Dismal Swamp Mitigation Bank, LLC, in the Albemarle Peninsula in Tyrrell County, NC (35˚54’22” N 76˚09’25” W) (Ardon et al. 2010)(Figure 1). TOWeR consists of four sections that include a 787 ha forested wetland; a 420 ha mature forested wetland that has never been under agricultural production (PA); a 57.2 ha drained shrub-scrub area; and a 440 ha stream and wetland restoration area that was formerly agricultural fields. The site receives direct inputs of water from the adjacent agricultural field and the forested wetland preservation area, and precipitation. TOWeR drains into the Albemarle Sound, via the Little Alligator River. This drainage pathway is also the route for saltwater incursion from the sound. The three sites historically experienced salinity from 0 to 6 ppt (during an intense drought in

2008-2009 Ardon et al. 2013), depending on the proximity to outflow and drought conditions, with sites closer to the outflow experiencing higher salinities during drought conditions.

Palmetto Peartree Preserve

Palmetto Peartree Preserve (PPT, 35°55'5" N 76°15'0"W) is a 4046 ha area of wetlands established by the Conservation Fund located 5 miles north of TOWeR (Figure 1). Parts of PPT are in active silviculture while others are preserved. This area is in close proximity to the Albemarle Sound and contains areas of higher salinity (more than 5 parts per thousand). My study takes place in an intact stand of baldcypress and tupelo swamp.

Preservation Area

The preservation area (PA, 35˚55'38" N 76˚09'1" W) is a forested wetland that is dominated baldcypress, tupelo, and oak and is located just north of TOWeR (Figure 1, Morse et al. 2012). Due to the high elevation of this site, water levels are precipitation driven and it has very low concentrations of salt (range of 94-333 µS cm^{-1} over the course of this study).

CHAPTER 2

Methods-Field Experiment

I placed litter bags in 5 sites; three along a salinity gradient within TOWeR (sites TL1, TLMP, and TL6), one “low salinity” site in PA, and one “high salinity” site in PPT (Figure 1). Each site had three randomly selected plots in areas that had similar water depth and vegetation cover. Conductivity and temperature values were recorded both continuously with data loggers and at the time of litter bag collection with a handheld YSI multiprobe (YSI, Ohio, US).

Leaf Collection, Litter Bag Construction and Processing

Swamp tupelo (*Nyssa biflora* Walter*,* here after referred to as *Nyssa*) and american sweetgum (*Liquidambar styraciflua* L.*,* here after referred to as *Liquidambar*) are common throughout all sites and were used in litter bags to determine decomposition rates. Originally, *Nyssa* was to be the only species used. However, as there was not enough leaf material available, *Liquidambar* was included in the bags.

All leaves were hand-picked from trees that were beginning to senesce from the TOWeR site. After gathering the leaves, they were returned to the lab to dry (72 hours at 72˚ C) and then separated into 22 x 40 cm coarse mesh (5mm) pecan bags, each containing ~2g of *Nyssa* leaves and 3g of *Liquidambar* leaves (based on tree and litter availability), and reweighed. Twenty-one bags were deployed into each plot within each site, for a total of 63 bags per site, or 189 bags in TOWeR and 63 bags in each reference site. Bags were attached to the ground with PVC piping and fishing line.

Three bags from each site were collected with decreasing frequency (Table 1) in order to obtain the initial changes in enzyme activity and decomposition rate (Benfield 2006).

Once litter bags were collected they were returned to the lab and immediately frozen until they could be processed. The litter bags were left to thaw in a refrigerator overnight before processing. Processing began with rinsing the leaf material over 0.0197 in mesh sieves to remove sediments and macroinvertebrates. Macroinvertebrates were stored in 70% Ethanol and were identified and counted. Using a 13.1mm cork borer, five leaf discs were cut from each tree species for use in ash free dry mass calculations for litter breakdown rate. An additional 0.5 g of wet weight of each species was used for measuring microbial enzyme activity. The remaining material and the ten discs used for measuring litter decomposition were dried at 72˚ C for 24 hours and weighed to 0.001 g. The ten 13 mm diameter leaf disks from each litter bag (five from each species) and a 1 g sub-sample were placed into foil envelopes, weighed, combusted (at 500C for 3 hours), and then reweighed to calculate ash free dry mass (AFDM, Benfield 2006). The leaf disks were used to account for the AFDM of the samples taken from the litter bags, and added to the AFDM of the remaining material to obtain the total AFDM of each litter bag. Breakdown rates for each site were calculated as slope of regression of the natural log of percent AFDM remaining versus time in the field in days (Benfield 2006).

Enzyme assays

The ~1 g of leaf material collected from each litter bag was used as substrate for the microbial enzyme assays. Samples taken on the dates of January 18th, April 19th, and July 26th were used in the assays. The specific enzymes that were measured are:

beta-glucosidase, C acquisition (BG); N-acetylglucosaminidase, N acquisition (NAG); leucine aminopeptidase, N acquisition (LAP); acid phosphatase, P acquisition (AP); arylsulfatase, S acquisition (AS); and phenol oxidase, phenol group breakdown (PO). Standard fluorometric assays (Jackson et al. 1995, Allison and Vitousek 2005, Jackson and Vallaire 2009) were used to measure fluorescence (absorbance for PO). To measure the activity of BG, NAG, AP, AS, PO, and LAP I used the following substrates: 4-MUB-β-D-glucopyranoside, 4-MUB-N-acetyl-β-D-glucosaminide, 4-MUB-phosphate, 4-MUB- sulfate, L-3-(3,4-dihydroxyphenyl)alanine (L-DOPA), and L-Leucine-7-Amido-4-Methylcoumarin respectively. An 96 well assay column consisted of 50 µL of substrate solution added to 200 µL of sample suspension. Sample control wells received 50µL of acetate buffer in addition to the 200 µL of sample suspension. Substrate control wells received 50 µL substrate solution plus 200 µL of acetate buffer. Quench standard wells consisted of 50 µL of standard (4-methylumbelliferone or 7-amino-4-methylcoumarin) added to 200 µL sample suspension. Reference standard wells received 50 µL of standard (4-Methylumbelliferone or 7-amino-4-methylcoumarin) and 200 µL acetate buffer. Following incubation (in the dark at 20°C for 3 h), 10 µL of .5 M NaOH was used to raise pH above 7.5 to stop the reaction and florescence/absorbance was measured with a microplate reader.

Macroinvertebrate Identification and Counts

All macroinvertebrate samples were examined under a dissection microscope, counted, and identified to the taxonomic levels used in Hauer and Resh's chapter 20 in *Methods of Stream Ecology* (Hauer and Lamberti 2006).

Methods-Microcosm

Experimental Design

The microcosm component of this study exposed *Nyssa* leaves and inoculum from a wetland that has experienced saltwater incursion (PPT) and a wetland naïve to salinity incursion (PA) to conductivities of 88 and 8021 µS cm^{-1} (L and H salinity treatments, salinities of 0 and 4.7 ppt respectively) to measure the DOC uptake, leaf litter decomposition, and microbial enzyme activity of each microcosm. Twenty 300 mL glass jars were filled to capacity and used as individual microcosms. Ten jars received filtered (0.7 µm GF/F glass microfiber) and autoclaved water from TOWeR that had a measured conductivity of 88 µS cm^{-1}. The other ten jars received an artificial seawater mixture composed of: 11.972 g NaCl, 2.005 g K_2 SO_4^{2-}, 0.010 g $NaHCO_3$, 0.005 g KBr, 0.014 H_3BO_3, 0.002 NaF, 28.527 mL $MgCl_2$-6 H_20, 5.604 mL CaC_2-$2H_2O$, 0.456 mL $SrCl_2$-$6H_2O$ and diluted to 8021 µS cm^{-1} with the same filtered TOWeR water. About 1 g wet weight of soil from PA or PPT was used as inoculum in each microcosm (1g wet weight was equal to 0.05 g dry weight for both inoculum). Five jars from each salinity treatment received PA inoculum (LPA or HPA) and 5 jars from each treatment received inoculum from PPT (LPPT or HPPT). *Liquidambar* leaves were collected from senescing trees in the TOWeR site and cut into 13mm disks using a cork borer. The leaf disks were then dried at 72˚ C for 24 hours, weighed, and divided equally into each microcosm. Microcosms were sealed and placed in a light excluding box and kept at 22 ± 0.5˚ C. Samples of leaf material and water were repeatedly collected from each microcosm on days 0,2,8,15,30,65,85, and 99. AFDM, as described in the field experiment methods, was calculated for each sampling date using 5 discs from each

microcosm. Twenty leaf discs per microcosm per sampling date were also used for microbial enzyme assays of BG, AS, AP, NAG, LAP, and PO, also as described in the field experiment methods. Using 0.7 µm glass microfiber filters and a syringe, 10 mL of water from each microcosm was taken every sampling day, filtered, and immediately frozen until analysis for non-purgeable organic carbon and total nitrogen on a Shimadzu TOC-L machine (Shimadzu Scientific Instruments, Columbia, Maryland, USA). A YSI 5000 (YSI, Yellow Springs, Ohio, USA) was used to measure dissolved oxygen percent, dissolved oxygen mg L^{-1}, and temperature on each sampling date for each microcosm.

Dissolved Organic Carbon and Total Nitrogen

In order to obtain the dissolved organic carbon present in each microcosm, a Shimadzu TOC-L machine with an additional Total Nitrogen Module was used to measure the dissolved organic carbon (measured as non-purgeable organic carbon) and total nitrogen (TN). Water samples were filtered (0.7 µm GF/F glass microfiber) and diluted (20 fold) before analysis.

Methods- Statistical Analysis

I used a one-way ANCOVA followed by a *post-hoc* Tukey's HSD to determine differences in mass lost over time between field sites in the field or treatments and inoculum in the microcosm. An ANOVA followed by a *post-hoc* Tukey's HSD was also used to determine differences in average enzyme activity between sites in the field. An ANCOVA was used to determine differences in average enzyme activity between treatments and inoculum in the microcosm. A repeated measures ANOVA was conducted for mean field enzyme activity to compare the changes within each site, over

time, and the interaction of the two. Breakdown rates for both the field and microcosm experiment were linearly regressed against average conductivity. Field breakdown rates were also linearly regressed against mean field measurements of dissolved oxygen, redox potential, temperature, and pH. Microcosm breakdown rates were linearly regressed against the mean values for temperature, dissolved organic carbon, total nitrogen, and dissolved oxygen. Mean field enzyme activity was also linearly regressed against average conductivity. An ANOVA and *post-hoc* Tukey's HSD was used to determine the dissolved organic carbon differences within and between inoculum, treatment, and sampling day. The macroinvertebrate differences between sites over time were also measured using an ANCOVA and *post-hoc* Tukey's HSD. An ANOVA was used to determine differences in macroinvertebrates between sites and total abundance at each site was linearly regressed against conductivity.

Results-Field Experiment

Leaf Litter Breakdown

After rapid leaching, initial breakdown was very similar across the sites. After temperatures increased in April, PPT began to experience much faster mass loss than the other sites, which were fairly closely grouped throughout the course of the experiment (Figure 2). There was a difference in mass loss between sites ($F_{9,325}$=105.64, P<0.0001) with PPT losing mass more quickly than all other sites (Figure 2). Over the course of the experiment, rainfall led to lower levels of saltwater incursion than had been experienced in years past. Average conductivities ranged 111.59 to 1863.37 µS cm^{-1} (0.057 to 0.944 ppt, Table 2) and were much lower than

values recorded during the 2008-2009 drought in the region (up to ~11,000 µS cm^{-1}). Although salinity levels were low, a tripling of leaf litter breakdown rate from 0.001±0.0001 d^{-1} to 0.0029±0.0001 d^{-1} was measured (Table 2). Conductivity explained 84% of the variation in breakdown rate (p=0.018, Figure 3), while relationships between breakdown rate against temperature (R^2=0.51, p=0.17), pH (R^2= 0.07), percent dissolved oxygen (R^2=0.44, p=0.22), and redox potential (R^2=0.59, p=0.13) were insignificant.

Microbial Enzyme Activity

A linear regression of mean enzyme activity against conductivity indicated that beta-glucosidase (BG), arylsulfatase (AS), leucine aminopeptidase (LAP), and acid phosphatase (AP) all increased with conductivity, while phenol oxidase (PO) and N-acetylglucosaminidase (NAG) showed no change (Figure 4). Temperature had no effect on average enzyme activity, while dissolved oxygen effected only PO (R^2=0.88, p=0.02). BG (R^2=0.85, p=0.01), AS (R^2=0.87, p=0.02), LAP (R^2=0.82, p=0.03), and PO (R^2=0.83, 0.03) were affected by pH. The oxidation reduction potential effected BG (R^2=0.85, p=0.03), AS (R^2=0.92, p=0.01), and PO (R^2=0.85, p=0.03, Appendix B Supplemental Table 3). Activity of BG at Palmetto Peartree (PPT) was greater than at the rest of the sites, there was no difference in activity at the other sites (Table 2). AS and AP activity at PPT and TLMP were similar, with the other sites being having lower activity. PPT had greater LAP activity than the Preservation Area (PA) while all TL sites fell in between the two. NAG activity at PA was lower than all other sites. PO levels were lowest in PA, TL6, and TL1, slightly higher in PPT and TL6, and highest in TLMP. All groupings were determined by one-way ANOVA and *post-hoc* Tukey's HSD pairings

(Table 2). Mean enzyme activity was significantly influenced by both the time in the field and the site they were located (repeated measures ANOVA, Table 3).

Macroinvertebrates

Of the 315 leaf litter bags deployed, only 49 bags had macroinvertebrates present upon collection and totaled 102 individual macroinvertebrates. 39 were found at TLMP, 20 at PPT, 27 at TL6, 13 at TL1, and 4 at PA. A linear regression of macroinvertebrate presence against conductivity showed no preference for lower or higher salinities ($p=0.484$). Bags with macroinvertebrates present on collection day against bags with no macroinvertebrates showed no difference in mass loss (ANOVA $F_{1,333}=0.2391$, $p=0.6252$). The organisms present were: Anisoptera (infraorder), Oligochaeta (subclass), Hirudinea (subclass), Amphipoda (order) , Tipulidae (family), Corydalidae (family), Dytiscidae (family), Simuliidae (family), Zygoptera (suborder), and Coleoptera (order).

Results-Microcosm

Leaf Disk Breakdown

As with the field experiment, the leaf material experienced a rapid initial leaching phase where 30% to 35% was lost between days 0 and 2. Overall, there was no difference in mass lost across all treatments and inoculum ($F_{3,156}=0.8$ $p=0.49$). Specifically, when including inoculum classification, there was no variation between mass loss rates of the salinity treatments (62% and 58% mass loss in the low salinity treatments and 56% and 52% in the high salinity treatments).There was also no difference in average mass loss between the two inoculums, independent from salinity

treatment (57% AFDM remaining, Figure 5). When omitting inoculum classification, high salinity treatments experienced insignificantly greater mass loss than low salinity treatments and ended with an average of 54% AFDM remaining (Low salinity treatments ended with 60%, Figure 5). The leaves in the microcosm experienced more mass loss than all field sites other than PPT. Breakdown rates (k) per day were also higher than all field sites except PPT ($k=0.0029\ d^{-1}$), the low salinity naïve inoculum and experienced inoculum (LPA and LPPT) were $0.0027\ d^{-1}$ and $0.0026\ d^{-1}$ respectively. The high salinity treatment with naïve inoculum (HPA) was also less than the field PPT breakdown rate ($k=0.0028\ d^{-1}$), but the high salinity treatment experienced inoculum (HPPT) breakdown was greater than field rates ($k=0.0033\ d^{-1}$, Table 4). There was no correlation between breakdown rates and conductivity (p=0.22), dissolved oxygen (0.55), temperature (0.40), or total nitrogen (p=0.68). Breakdown rates decreased as the amount of dissolved organic carbon increased ($R^2=0.98$ p=0.006, Figure 6).

Microbial Enzyme Activity

Microbial enzyme activity in both inoculum responded inconsistently to differences in salinity. Overall enzyme activity in the microcosm was lower than that observed in the field study for all enzymes except acid phosphatase (AP). I used an ANCOVA to compare the average enzyme activity of each enzyme within each salinity treatment and inoculum type (Table 4). Beta-glucosidase (BG) was significantly higher in the Palmetto Peartree (PPT) inoculum than in the Preservation Area (PA, $F_{3,136}=2.75$ p=0.009), but the difference disappeared in the high salinity treatments ($F_{3,136}=2.752$ p=0.56, Figure 7A). There were no significant differences for salinity or inoculum type for arlysulfatase (AS) or leucine aminopeptidase (LAP) ($F_{3,136}=1.27$ p=0.29, $F_{3,136}=1.39$

p=0.25, Figure 6B,C). Salinity decreased AP activity just for the PA inoculum ($F_{3,136}$=2.93 p=0.005), but did not affect the PPT inoculum (Figure 6D). N-acetylglucosaminidase (NAG) was significantly higher in microcosms with the PA inoculum than the PPT inoculum under both salinity treatments ($F_{3,136}$=6.54 P<0.0004, Figure 6E). Salinity decreased phenol oxidase (PO) activity in the PA inoculum ($F_{3,136}$=10.76 P<0.0001), while slightly increasing it in the PPT inoculum (Figure 6F). PO activity was significantly higher for the PPT inoculum compared to the PA only under high salinity ($F_{3,136}$=10.76 p=0.018, Figure 6F).

Dissolved Organic Carbon and Total Nitrogen

Overall, the high salinity microcosms had less dissolved organic carbon (DOC) than the low salinity treatments. In the high salinity treatments PPT inoculum had less DOC than the PA inoculum ($F_{1,78}$=4.33, p=0.041) while the amount of DOC in low salinity treatments was not affected by inoculum ($F_{1,78}$=0.69, p=0.41, Figure 8 A and B). The DOC amount in the microcosms with PA inoculum were not affected by salinity treatment ($F_{1,78}$=0.036, p=0.85) while there was less DOC present in the microcosms containing PPT inoculum with the high salinity treatment ($F_{1,78}$=8.33, p=0.005, Figure 8 C and D). Inoculum type omitting salinity treatment classification had no effect on DOC levels ($F_{1,158}$=0.702, p=0.4, Figure 8 E). The high salinity treatments, omitting inoculum classification, had less DOC than the low salinity treatments ($F_{1,158}$=5.19, p=0.024, Figure 8 F). DOC for all microcosms was incredibly high (averaging 900 mgC/L), while Total nitrogen (TN) was also very high (average 1.5 mgN/L). TN decreased with time ($F_{4,152}$=2.29 p=0.008) but did not vary with treatment (0.52) or inoculum (p=0.15, Appendix B, Supplemental Table 5).

CHAPTER 3

Discussion

Overall, my results indicate that relatively low increases in salinity may alter microbial enzyme activity and increase leaf litter breakdown rates. Varying results between field and laboratory experiments suggests that decomposition and enzyme profiles can be influenced more heavily by other variables and there is not a unidirectional effect that corresponds with salinity. Due to this, it will be difficult to forecast the effect of increased salinization of wetlands. Salinity is likely to increase in coastal areas of North Carolina due to both changes in the frequency and intensity of droughts and sea-level rise, making it important to better understand the responses of wetland ecosystem processes to salinization.

The breakdown rates I measured were similar to other studies conducted in nearby wetlands (Brinson 1977, Day 1982). However, for many of the sites in this study, the experiment ended with approximately 70% organic matter remaining. These studies show that it can take over a year for litter to lose more than 50% of its organic mass in anaerobic wetland soils. My litter packs experienced breakdown rates of 0.0010 to 0.0029 g d^{-1}. These breakdown rates are lower than the 0.0052 g d^{-1} rate reported in a study by Brinson (1977) for *Nyssa* only litter bags. His study took place in a swamp forest located 15 km upstream from the Pamlico Sound in the Tar River flood plain. Salinity data was not reported in his study, but Division of Water Quality monitoring data from a sampling station in close proximity to the study site recorded salinity levels ranging 0 to 6.89 parts per thousand (ppt) during the 1988- 2014 period (NCDENR).

This suggests that salinity levels may have been higher than those of my study, potentially contributing to a higher breakdown rate. Additionally, a study conducted further north in the Great Dismal Swamp reported a *Nyssa* only litter bag breakdown rate of 0.0018 g day^{-1} (Day 1982), which is within the range of my breakdown rates. Another study in conditions similar to my own reported *Liquidambar* breakdown rate of 0.002 g day^{-1} (Shure et al. 1986). My study was conducted over a 203 day period, while the previously mentioned experiments continued for at least 365 days. This temporal difference may have impacted the breakdown results because my litter packs were collected in the middle of the warmest time of the year when decomposition is typically highest.

The microcosm breakdown rates (0.0026 to 0.0033 d^{-1}) were, on average, greater than the breakdown rates seen in the field (0.0010 to 0.0029 d^{-1}). This is most likely due to the seasonal temperature fluctuations present in the field experiment (ranging from 4.4 to 24.7˚C and averaging 14˚C) while the microcosm was maintained at 22±0.5˚ C. The field study was started in winter and ended mid-summer, so not only were the microbial communities exposed to very cold temperatures, they were also not allowed to continue breaking down the leaf packs for the majority of the summer season when temperatures would have been warmer. When standardized by the average temperature experienced over exposure time, breakdown rates for the microcosms are faster than the Timberlake and Preservation Area sites but slower than the breakdown rate measured in Palmetto Peartree (PPT, Figure 9). Lack of difference in microcosm breakdown rates between salinity treatments and inoculum types could be due to the abundance of dissolved organic carbon (DOC) and total nitrogen (TN) in the water

column making it unnecessary for microbial communities to breakdown the leaf material as quickly. This is also supported by the strong negative correlation between breakdown rates and DOC (R^2=0.98, p=0.006, Figure 6) which suggests that microbial communities do not need to breakdown as much material when there is more DOC available. Additionally, the quantity of inoculum added to each microcosm may not have contained equal microbial biomass, which would lead to differing breakdown ability of each microcosm. My sampling method of repeatedly sampling the same microcosm may have also contributed to homogenized results. Regardless, the slower breakdown of organic matter in the high salinity treatments when compared to the breakdown measured in PPT suggests that salinity is not the strongest driver of variation in organic matter breakdown. The lack of a consistent trend between breakdown and salinity also indicates that the effects of salinity on organic matter breakdown can be variable depending on other environmental factors.

Most enzymes in the field study increased with salinity, except for N-acetylglucosaminidase (NAG) and phenol oxidase (PO). Arylsulfatase (AS) activity was expected to decrease with salinity due to the increase of SO_4^{2-} with saltwater. Contrary to expectations, AS activity increased. I hypothesize that AS levels increased because overall microbial enzyme activity increased, even though SO_4^{2-} was more readily available. However, in the microcosm experiment, AS showed no increase with higher salinity. In a study of 36 aerobic environments PO was found to be as high as from 1000 μmol gdm^{-1} h^{-1} and average 76.47 μmol gdm^{-1} h^{-1} (Sinsabaugh et al. 2008). The low phenol oxidase (PO) values (3.6 to 26 μmol gdm^{-1} h^{-1}) in both the field and microcosm experiment agree with our low measurements of dissolved oxygen (Freeman et al.

2011). However, the low values from TL1 might have been due to a drying event for an unknown duration between 08-March and 31-May sampling days. Water levels in PA were also very low, but a complete drying event did not occur. This drying event is likely to have had an impact on decomposition and enzyme activity results. Mean enzyme activity was comparable to other studies performed in the same salinity range, but was intermediate between lower activity reported from soils (Beta-glucosidase, BG: 0.1-1.4 μmol gdm $^{-1}$ h^{-1}, arylsulfatase, AS: 0.08-0.45 μmol gdm $^{-1}$ h^{-1}, acid phosphatase, AP: 0.22-0.87 μmol gdm $^{-1}$ h^{-1}, and phenol oxidase, PO: 31-365 μmol gdm $^{-1}$ h^{-1}) and higher activity reported for leaf litter (BG: 0.42-11 μmol gdm $^{-1}$ h^{-1}, AS: 0.9-2.7 μmol gdm $^{-1}$ h^{-1}, AP: 2-60 μmol gdm $^{-1}$ h^{-1}, NAG: 3-8 μmol gdm $^{-1}$ h^{-1}, PO: 20-100 μmol gdm $^{-1}$ h^{-1}) depending on the enzyme (Morrissey et al. 2014, Rejmankova and Sirova 2007, Neubauer et al. 2013, Alvarez and Guerrero 2000). This variation in activity is likely due to the nutrient content of the environment. TOWeR receives direct pumping from agricultural fields (Ardón et al. 2010) so labile nutrients could potentially be higher than the wetlands described in the literature, decreasing the amount of enzymes the microbes need to produce in order to meet their nutrient requirements.

The low levels of enzyme activity present in the microcosm experiment are also likely due to an abundance of nutrients and low microbial biomass. Due to the large amount of leaf material relative to the amount of water, the microcosms were incredibly rich in dissolved organic carbon and total nitrogen (Appendix B, Supplemental Table 5) but confined in space and likely had low microbial biomass when compared to a natural plot of the same size. I hypothesize that the rich nutrient composition coupled with the low microbial biomass allowed the microbial communities to obtain nutrients without

having to produce many enzymes. Additionally, the low microbial biomass in each microcosm could have led to a low amount of microbial enzyme activity because there were not as many microbes producing enzymes. The lack of a need to produce enzymes also helps to explain the why there was no microbial enzyme activity distinction seen between salinity and inoculum types.

As stated above, the dissolved organic carbon (DOC) levels were very high in my microcosms, ranging from 50 to 1700 mg C per liter and averaging 900 mg L^{-1}. These values are much greater than the 16.3-61.1 mg L^{-1} concentrations reported from five years of field samples collected from the TOWeR site (Ardon et al. in preparation). The elevated level of dissolved organic carbon could be due to excessive DOC leaching from leaf material that had not been given enough time to senesce before harvesting. Another possible explanation is that there was not enough water in each microcosm proportionate to the amount of leaf material. Between inoculum in the high salinity treatment, Palmetto Peartree (PPT) inoculum consumed more DOC (Figure 6). This relationship could be explained by the experienced inoculum microbial communities being better adapted to use the higher amount of SO_4^{2-} available in the high salinity treatment. Additionally, the increased salinity can increase flocculation causing DOC sink to the bottom (Ardón et al. in preparation). This salt induced increase in flocculation can also decrease the amount of DOC. Overall, the high salinity treatments had lower DOC than the low salinity treatment which supports my original hypothesis that DOC consumption by microbial communities will increase as salinity increases because of the additional SO_4^{2-} available to microbial communities. However, the constant decline of total nitrogen over time suggests that both inoculum types were processing nitrogen at

the same pace. This indicates that both inoculum types may not have been either stressed, or benefited from salinity or lack thereof, and were more responsive to the amount of DOC in the water.

My field experiment hypothesis that macroinvertebrate presence would decrease as salinity increased was not supported. Macroinvertebrate densities did not vary between sites and were low across all sites and dates. Although other studies have shown increased salinity to decrease species richness and abundance (Kefford et al. 2004, Stewart et al. 2009), the low levels and small range of salinity could have been contributing factors in the lack of change between sites. One survey of freshwater ecosystems across Australia (see Williams 1988 for reasons why Australia has global typical limnological conditions) documented that macroinvertebrates did not experience stress at conductivities under 1500 µS cm^{-1} (Hart et al. 1991), and the highest average conductivity experienced was only slightly greater than that at ~1800 µS cm^{-1}(Table 2). Overall I found very few macroinvertebrates in all leaf packs. Tipulidae are known for being particularly voracious shredders of leaf litter, however, even the bags containing them did not show increased mass loss. Furthermore, they were present across all sites so any undocumented effect that they could have should be equal. In several studies in blackwater streams similar to my wetlands, leaf shredders have been found to contribute little when compared to the microbial communities (Smock et al. 1985, Smock and Roeding 1986). In one study, Rader and others (1994) also determined that the microbial community was responsible for the vast majority of leaf decomposition in a blackwater stream system. The early year start time of my study could have had an impact on the low presence of macroinvertebrates. Additionally, there are occasionally

low levels of macroinvertebrates found in wetland substrates due to their anaerobic nature (Murkin et al.1994).

At the relatively low concentrations of salinity measured in the field section of this study (111.59 to 1863.37 µS cm^{-1}, or 0.057 to 0.944 ppt), I observed increased decomposition and enzyme activity (of beta-glucosidase (BG), arylsulfatase (AS), acid phosphatase (AP), and leucine aminopeptidase (LAP)) at the site with the highest salinity. Although my field results show an increase of microbial activity and faster breakdown rates with salinity, it is mostly driven by the Palmetto Peartree (PPT) site alone. Even when salinity is omitted, PPT is very different from the other sites, having denser vegetation, much thicker peat deposits, a visibly higher amount of particulate organic carbon, as well as many other variables. It is not clear that salinity was the driving factor influencing faster breakdown rates and greater microbial enzyme activity. Additionally, the microcosm experiment results did not support the trends seen in the field experiment, so it cannot be said with certainty what effect salinity has on organic matter decomposition and microbial enzyme activity, though it appears that other factors influence them more heavily.

Other studies conducted in relatively low salinities (≤ 2 ppt) have reported increased enzyme activity and faster decomposition in response to small increases in salinity (Craft 2007, Morrissey et al. 2014). However, in full seawater (34 ppt) organic matter breakdown happens at a lower rate than is observed in freshwater (Rejmankova and Houdkova 2006, Neubauer 2011). A study by Chambers and others (2013) found that freshwater microbial communities increase their activity and decomposition of organic carbon in response to pulses of salinity (13 ppt), while brackish (~11 ppt) and

salt marsh communities (~24 ppt) experienced increases in response to pulses of freshwater (<0.5 ppt). These results suggest that freshwater microbes might be stimulated by relatively small salinity increases (<10 ppt) due to the added abundance of SO_4^{2-}, while brackish and salt marsh microbial communities benefit from freshwater pulses that alleviate the stress caused by high salt content. When omitting other environmental variables, I hypothesize that when salinity concentrations reach a point where the microbial community has difficulty osmoregulating, organic matter breakdown will slow because of the osmotic stress (Figure 10). This effect may be more pronounced in the early stages of saltwater incursion because microbial communities may adapt to higher salinities when given enough time (Bouvier and del Giorgio 2002). However, when other environmental variables are considered this hypothetical effect of salinity may be rendered null due to the strength of influence from those other variables.

Nevertheless, as salinity incursion becomes more prevalent in coastal North Carolina wetlands due to climate change, anthropogenic pressures, and accelerated sea-level rise, the importance of understanding the effects of salinity on wetland ecosystem process rises. Although, it will be difficult to forecast the effect of increased salinization in individual wetlands, my results indicate that relatively low increases in salinity can have varying effects on organic matter breakdown and microbial enzyme activity. More research is needed on organic matter breakdown and microbial enzyme activity along salinity gradients to tease apart the effects of salinization with respect to other biotic and abiotic variables.

Tables

Table 1. Collection date and number of days in the field of each sampling period. 9 litter bags were collected from each site on sampling days. * this date represents the deployment date of all litter bags.

Date	Days Exposed
11-Jan-13*	0
18-Jan-13	7
25-Jan-13	14
08-Feb-13	28
08-Mar-13	56
19-Apr-13	105
31-May-13	147
26-Jul-13	203

Table 2. Mean field enzyme activity (beta-glucosidase (BG), arylsulfatase (AS), leucine aminopeptidase (LAP), acid phosphatase (AP), N-acytelglucosaminidase (NAG), and phenol oxidase (PO)), breakdown rate, and mean conductivity in the different sites. Letters under the enzyme values represent differences among enzyme activity between different sites as determined by one-way ANOVA ($p<0.05$) and *post-hoc* Tukey's HSD analysis. All variation is represented as one standard error from the mean.

Site	Conductivity (µS/cm)	Breakdown Rate (day^{-1})	Mean Enzyme Activity (µmol h^{-1} gdm^{-1})					
			BG	AS	LAP	AP	NAG	PO
PPT	1863.4 ± 598.0	0.0029 ± 0.00014	2.49±0.75 a	0.065±0.041 a	0.489±0.322 a	2.11±0.78 a	0.535±0.298 a	21.2±12.3 ab
TL1	466.0 ± 258.1	0.0010 ± 0.00014	1.29±0.36 b	0.031±0.015 b	0.369±0.324 ab	1.45±0.39 b	0.463±0.157 a	12.2±6.9 c
TLMP	276.0 ± 99.7	0.0015 ± 0.00010	1.65±0.89 b	0.059±0.029 a	0.380±0.327 ab	2.05±0.83 a	0.538±0.352 a	26.0±8.4 a
TL6	111.6 ± 27.1	0.0012 ± 0.00014	1.64±0.56 b	0.035±0.013 b	0.330±0.237 ab	1.13±0.44 b	0.455±0.331 a	17.8±7.8 bc
PA	216.5 ± 54.9	0.0015 ± 0.00009	1.50±0.36 b	0.026±0.015 b	0.235±0.146 b	1.48±0.33 b	0.225±0.113 b	12.4±10.1 c

Table 3. Repeated measures analysis of mean field enzyme activity over time between sites and the interaction between site and time. Site by time interactions were measured with the Wilks' Lambda test.

	BG		AS		LAP		AP		NAG		PO	
	F ratio	P value	F ratio	P value	F ratio	P value	F ratio	P value	F ratio	P value	F ratio	F ratio
Site	158.6	<0.0001	162.1	<0.0001	21.3	<0.0001	93.6	<0.0001	71.1	<0.0001	77.2	<0.0001
Time	186.7	<0.0001	604.9	<0.0001	242.2	<0.0001	16.0	<0.0001	279.5	<0.0001	11.8	<0.0001
Site*Time	23.9	<0.0001	57.7	<0.0001	29.6	<0.0001	69.2	<0.0001	71.3	<0.0001	83.4	<0.0001

Table 4. Mean microcosm enzyme activity (beta-glucosidase (BG), arylsulfatase (AS), leucine aminopeptidase (LAP), acid phosphatase (AP), N-acytelglucosaminidase (NAG), and phenol oxidase (PO)), breakdown rate, and salinity treatment of the different microcosms. Letters under the enzyme values represent differences among enzyme activity between different sites as determined by one-way ANCOVA ($p<0.05$) and *post-hoc* Tukey's HSD. All variation is represented as one standard error from the mean.

Inoculum	Salinity Treatment	Breakdown Rate (day^{-1})	Mean Enzyme Activity ($\mu mol\ h^{-1}\ gdm^{-1}$)					
			BG	AS	LAP	AP	NAG	PO
PA	L	0.0027 ± 0.0006	0.28±0.17 b	0.0027±0.0014 a	0.139±0.072 a	2.27±0.49 ab	0.069±0.034 a	5.7±4.6 bc
PPT	L	0.0026 ± 0.0007	0.37±0.16 a	0.0023±0.0016 a	0.128±0.062 ab	2.36±0.61 a	0.053±0.028 bc	8.7±6.1 ab
PA	H	0.0028 ± 0.0007	0.32±0.18 ab	0.0026±0.0015 a	0.128±0.069 ab	2.03±0.45 b	0.059±0.022 ab	3.6±2.0 c
PPT	H	0.0033 ± 0.0006	0.36±0.06 a	0.0022±0.0008 a	0.109±0.045 b	2.06±0.63 b	0.042±0.011 c	10.6±7.7 a

Figures

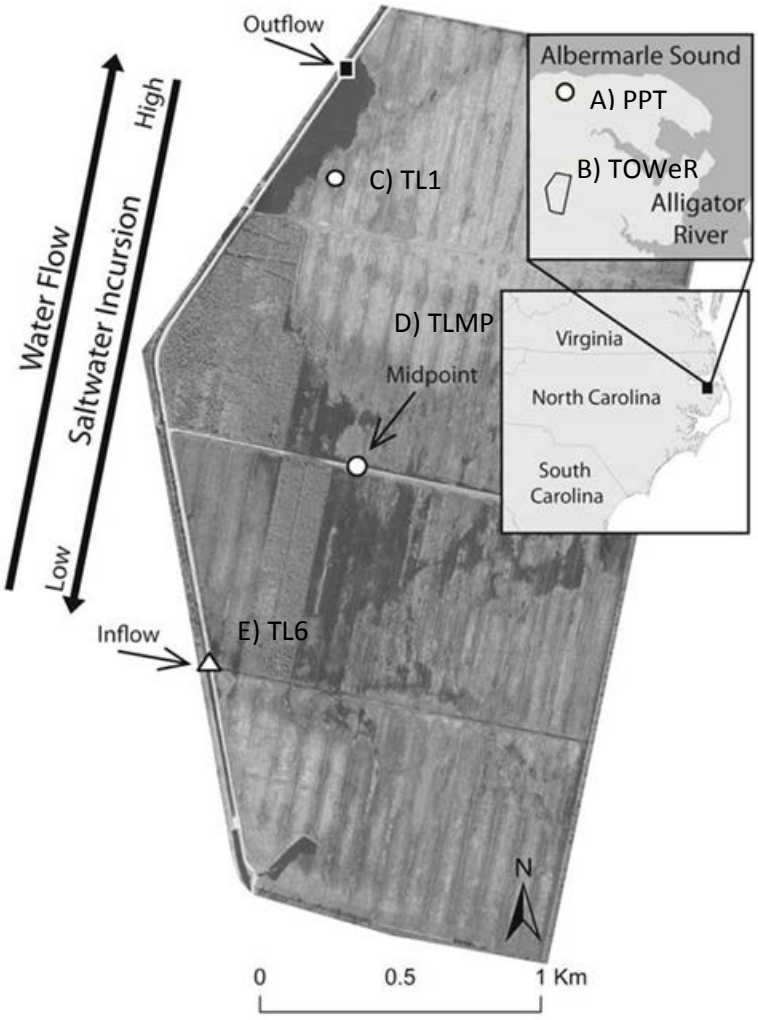

Figure 1. Location of A) PPT and B) TOWeR on the Albemarle Peninsula (inset is the location within NC). Within TOWeR, sampling sites C) TL1, D) TLMP, E) TL6, as well as water flow and saltwater intrusion directions through TOWeR. Also depicted are the upstream inflow, the downstream outflow, and the midpoint locations. Not pictured is site PA, which is located immediately north of TOWeR.

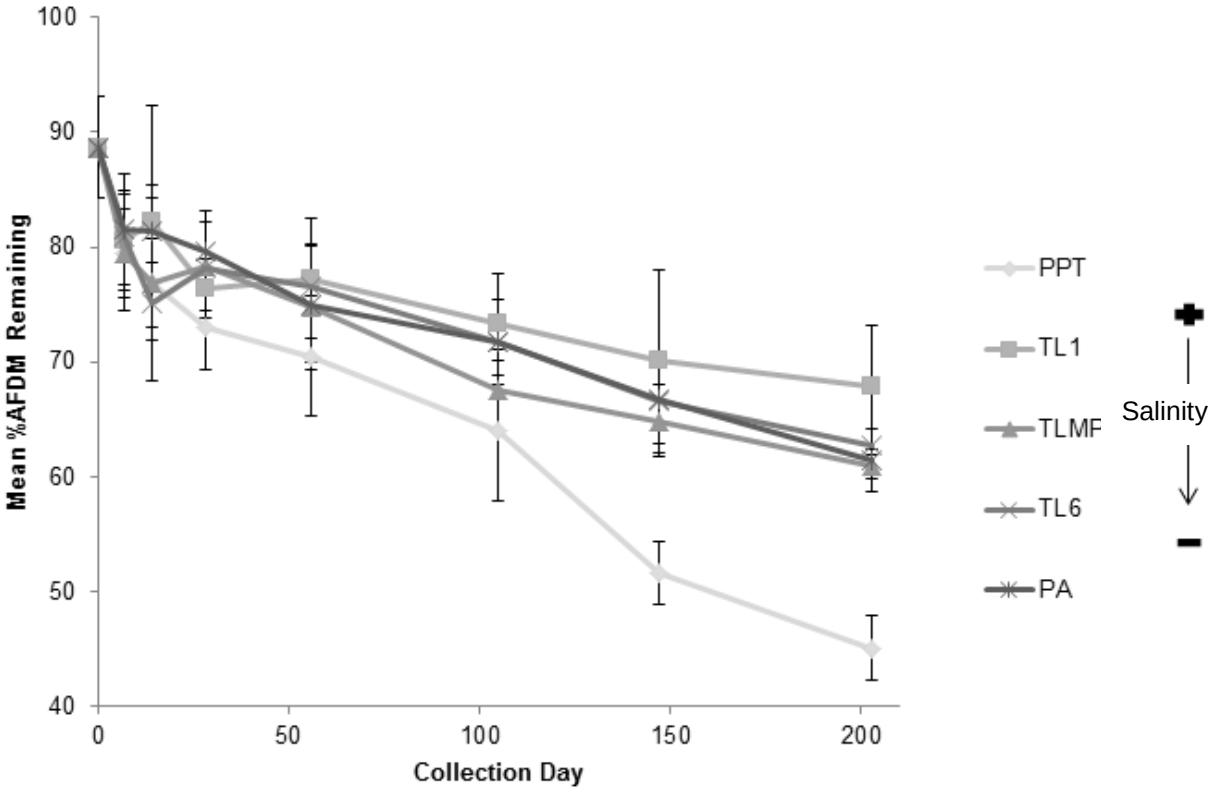

Figure 2. Mean percent ash free dry mass (AFDM) remaining on collection day of all five sampling sites. Error bars represent 1 standard error from the mean.

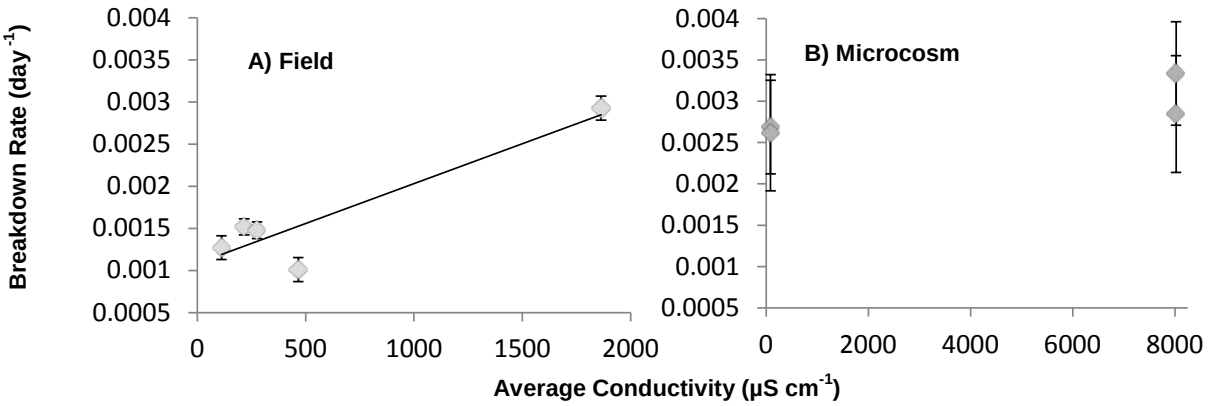

Figure 3. Breakdown rates of field sites (A; R^2=0.84, p=0.027) and microcosm treatments (B; R^2=0.61, p=0.22) versus average conductivity. Error bars represent 1 standard error from the mean.

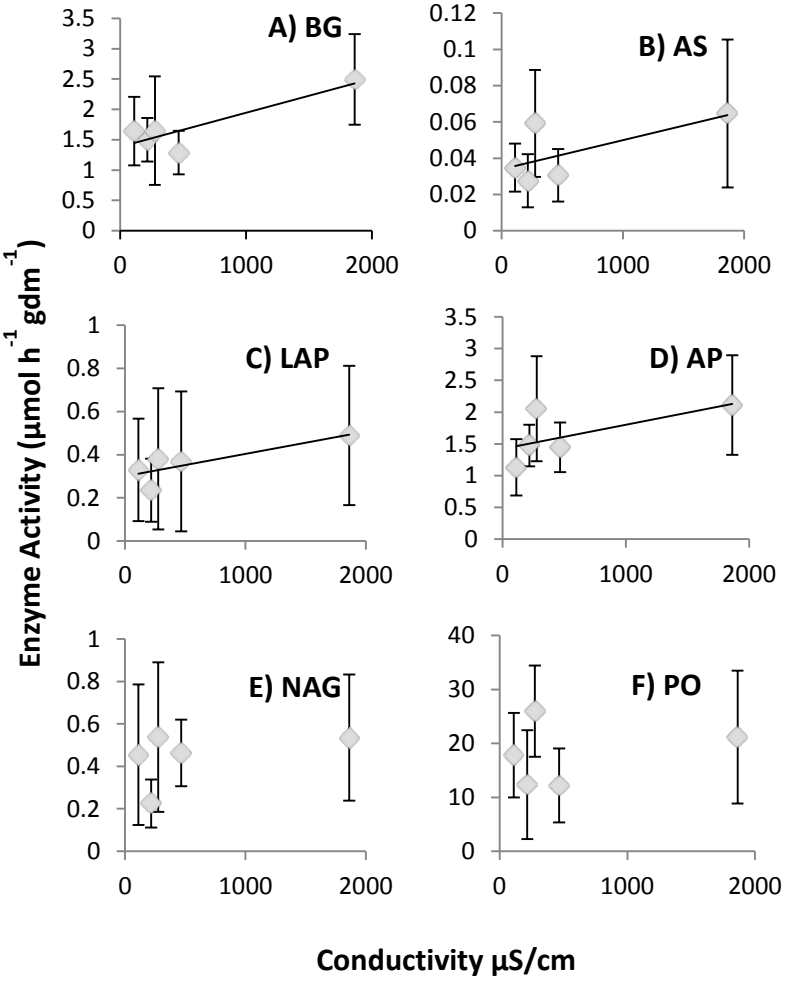

Figure 4. Mean field enzyme activity of A (BG),B (AS), C (LAP), D (AP), E (NAG), and F (PO) regressed against the mean conductivity at each site. A-B were significantly related to conductivity (A; R^2=0.24, p<0.001, B; R^2=0.13, p<0.0001, C; R^2=0.05, p=0.006, D; R^2=0.13, p<0.0001) while E and F did not (p=0.05 and 0.13 respectively). Error bars represent 1 standard error from the mean.

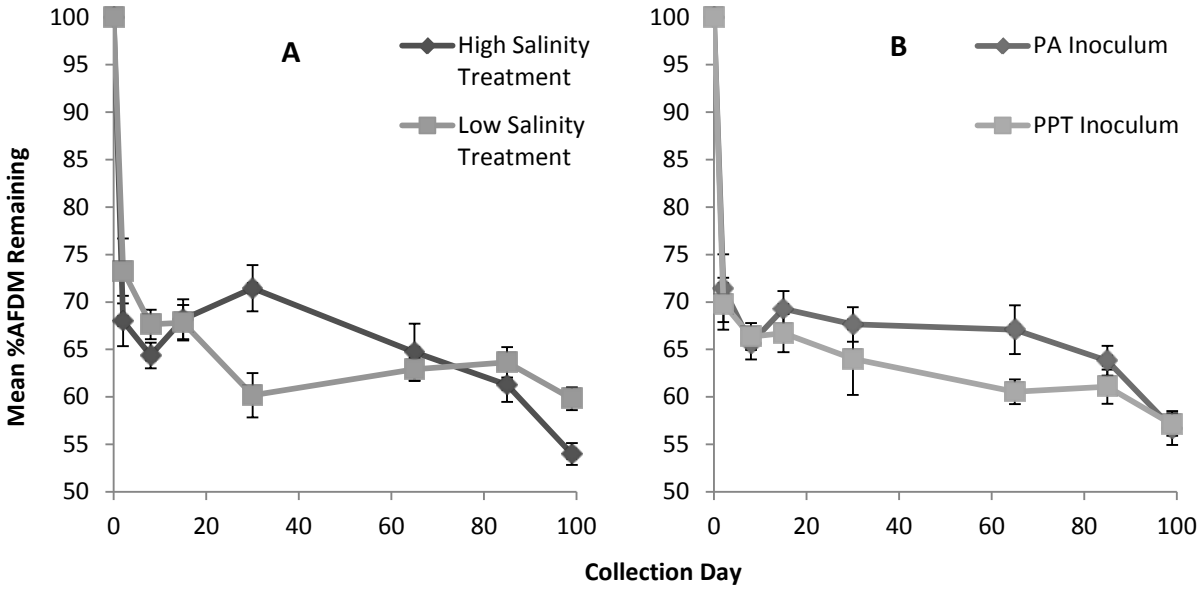

Figure 5. Mean percent ash free dry mass (AFDM) remaining of the microcosm against the collection day. (A) represents the average of AFDM remaining of each treatment, omitting inoculum distinction, while (B) represents the average of each inoculum, omitting treatment distinction. An ANCOVA test revealed that neither the treatment, inoculum, nor the combined effect of treatment and inoculum significantly altered percent AFDM remaining ($F_{7,152}$= 10.73 p=0.82, 0.28, and 0.14 respectively). Error bars represent 1 standard error from the mean.

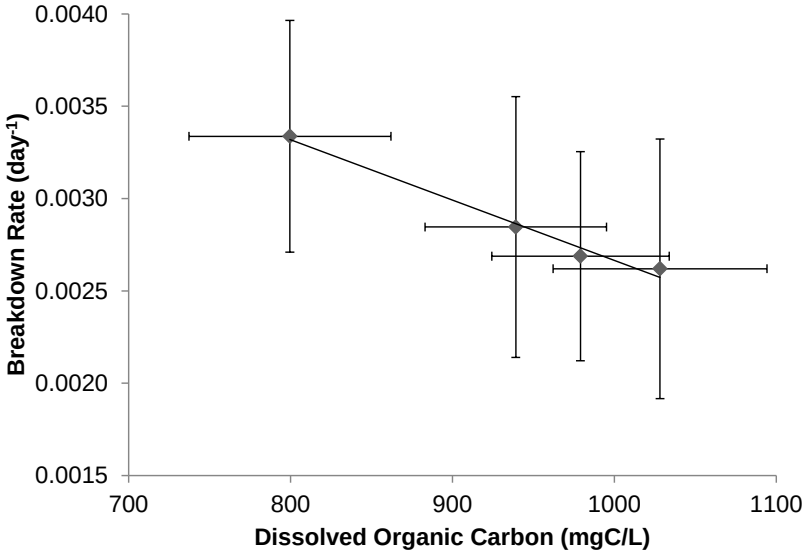

Figure 6. Breakdown rate of microcosm treatment and inoculum types against mean dissolved organic carbon (R^2=0.98, p=0.006). Error bars represent 1 standard error from the mean.

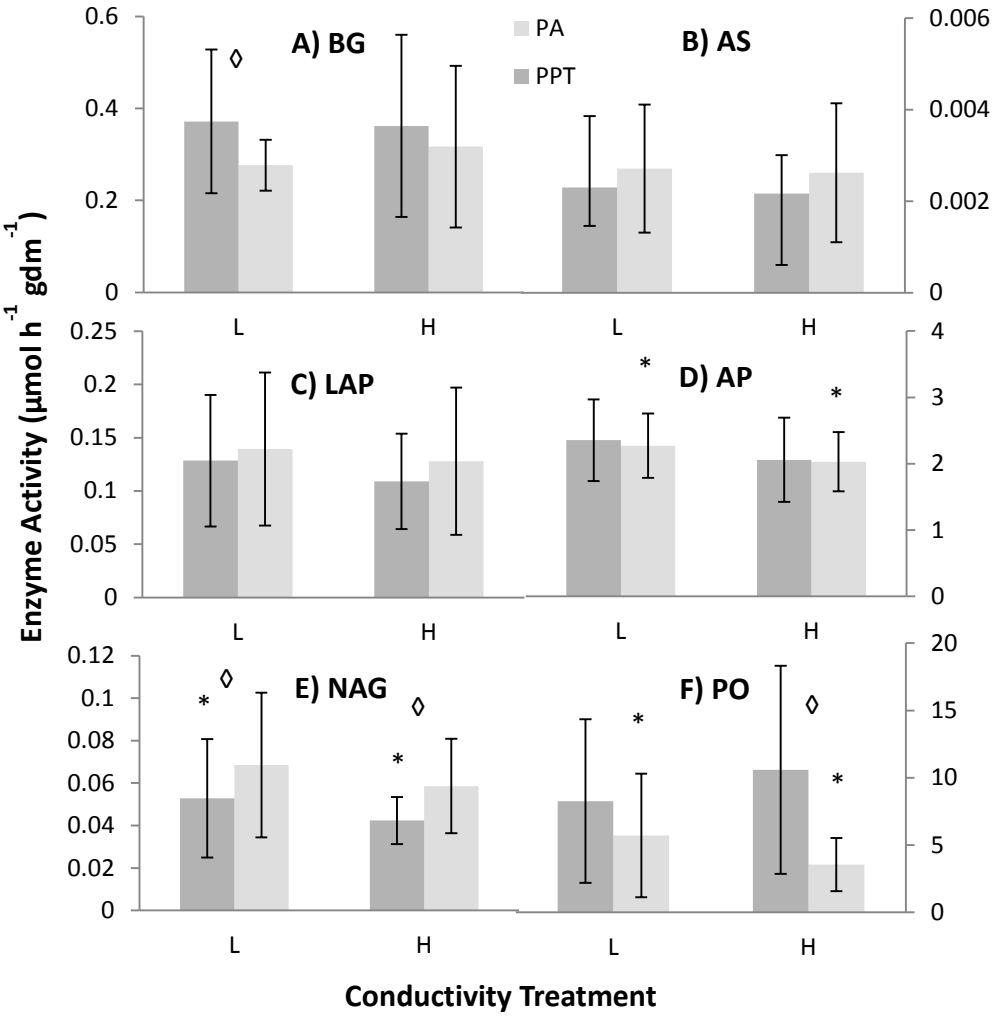

Figure 7. Mean microcosm enzyme activity of A (BG), B (AS), C (LAP), D (AP), E (NAG), and F (PO) separated by microcosm salinity treatments (H= 8021 µS cm-1, L=88 µS cm-1) and classified by inoculum. The * Represents significant differences between treatments and ◊ represents differences between Inoculum, reported from Tukey's HSD (all $P<0.05$). Error bars represent 1 standard error from the mean.

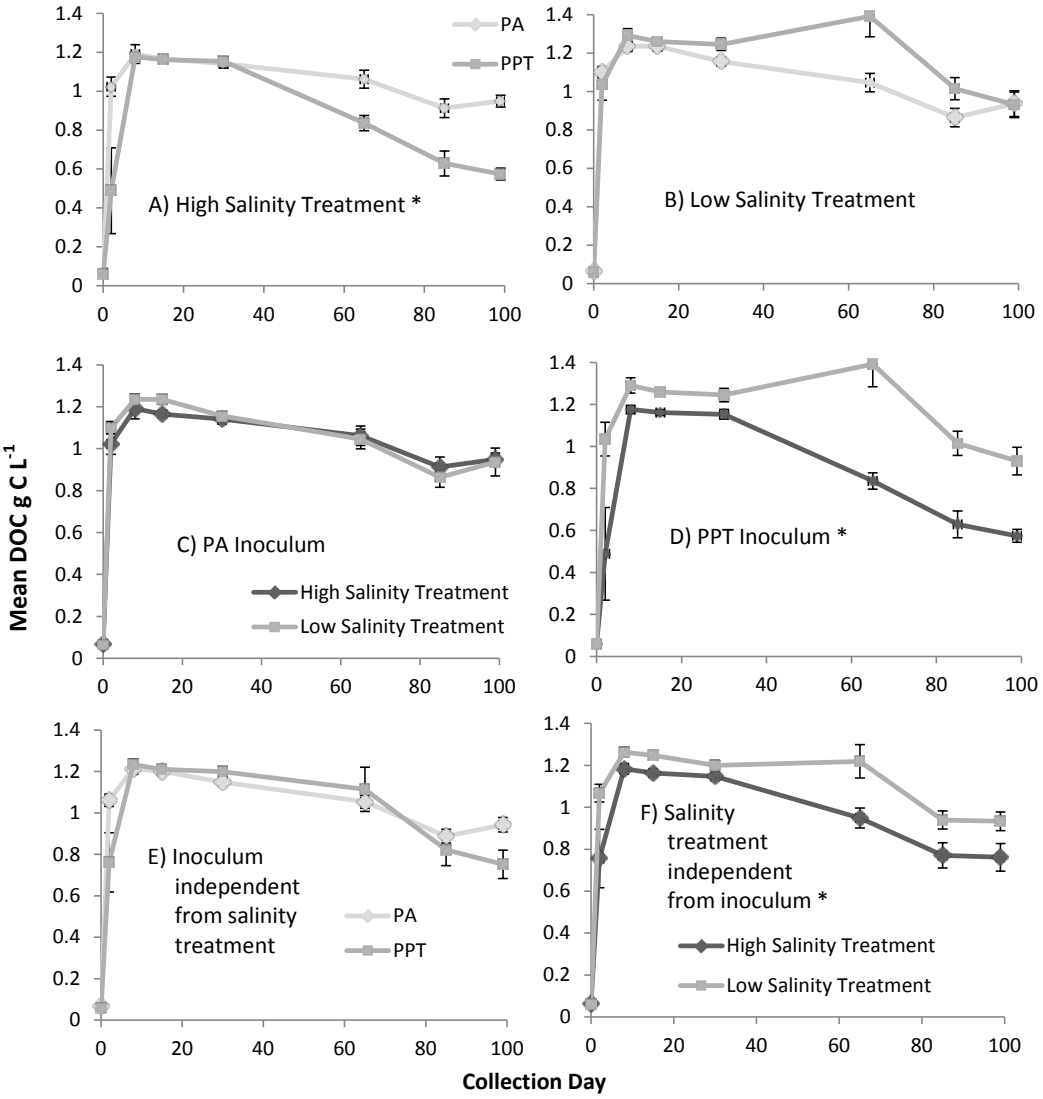

Figure 8. Mean dissolved organic carbon (DOC) of (A) high salinity treatment with both inoculum types, (B) low salinity treatment with both inoculum types, (C) PA inoculum with both salinity treatments, (D) PPT inoculum with both salinity treatments, (E) both inoculum types with no distinction between salinity treatment, and (F) both salinity treatments with no distinction between inoculum. The * represents significant differences reported from a one-way ANCOVA. Error bars represent 1 standard error from the mean.

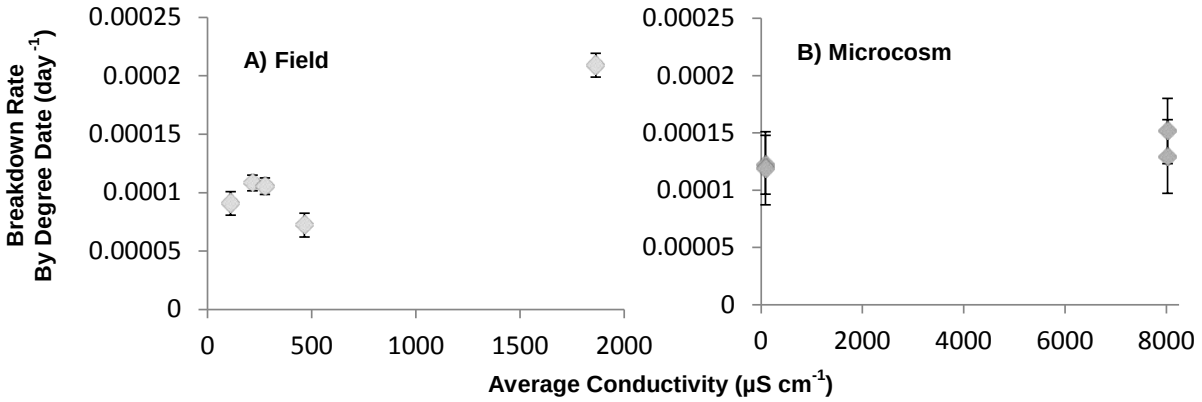

Figure 9. Breakdown rates by degree date of field sites and microcosm treatments and averaged conductivity. Error bars represent 1 standard error from the mean.

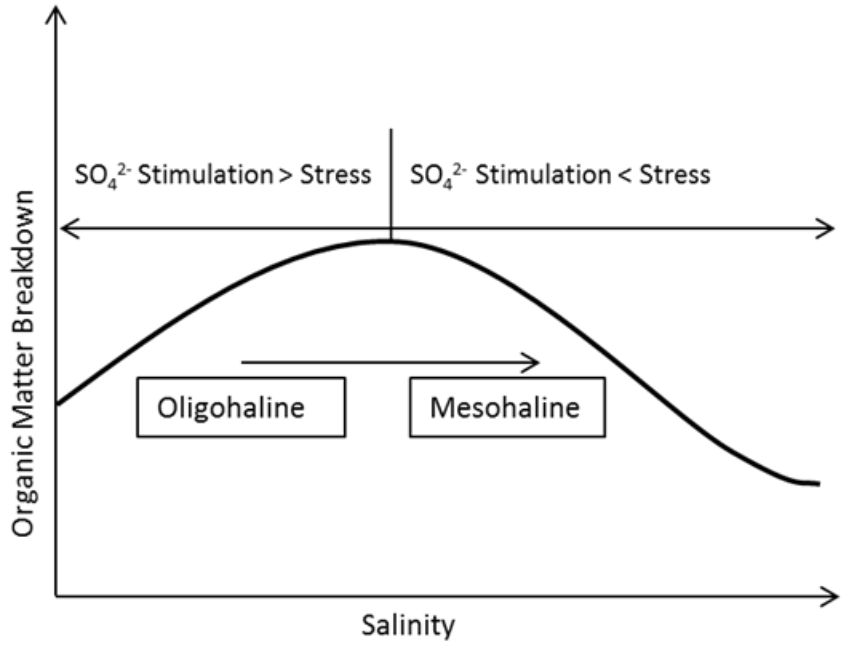

Figure 10. Hypothetical response of organic matter breakdown to salinity. Organic matter breakdown increases due to an increase of the electron acceptor SO42-. Breakdown begins to decrease once the pressure from osmotic stress becomes greater than the positive effect of an increase in SO42-. The degree to which salinity effects organic matter breakdown depends on the resistance of the microbial community to salinity.

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APPENDIX A

Microcosm Diagram

Low Salinity Treatment Naïve Inoculum

Low Salinity Treatment Experienced Inoculum

High Salinity Treatment Naïve Inoculum

High Salinity Treatment Experienced Inoculum

Each Microcosm was non-destructively sampled on days 0,2,8,15,30,65,85, and 99 for leaf material, water samples, and physiochemical characteristics.

Low Salinity Treatment = 88 µS cm^{-1}

High Salinity Treatment = 8021 µS cm^{-1}

Naïve Inoculum = Preservation Area (PA)

Experienced Inoculum = Palmetto Peartree Preserve (PPT)

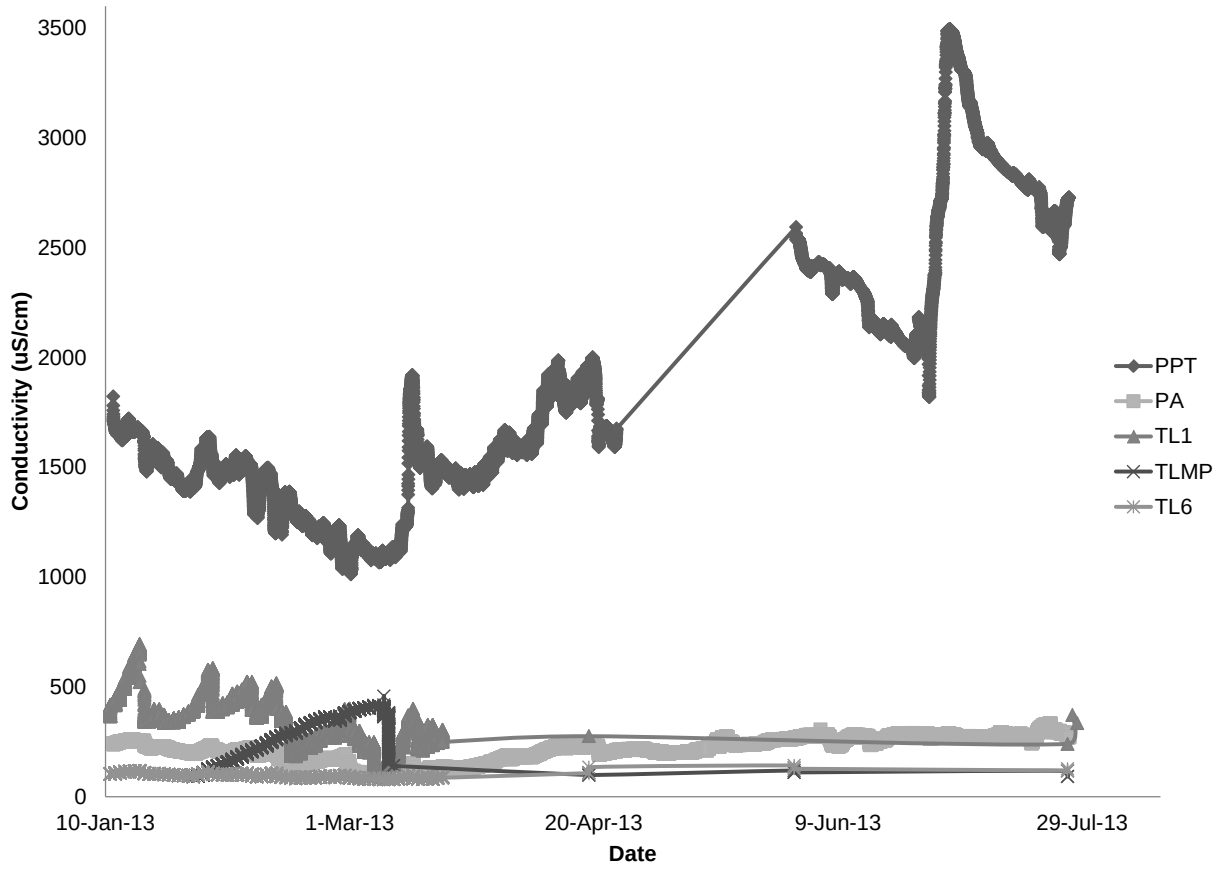
Each Microcosm Received:

- 300 ml water (High Salinity or Low Salinity)
- 2.34 g dw of leaf material (*Liquidambar*)
- 1 g wet weight inoculum (Soil from either PPT or PA)

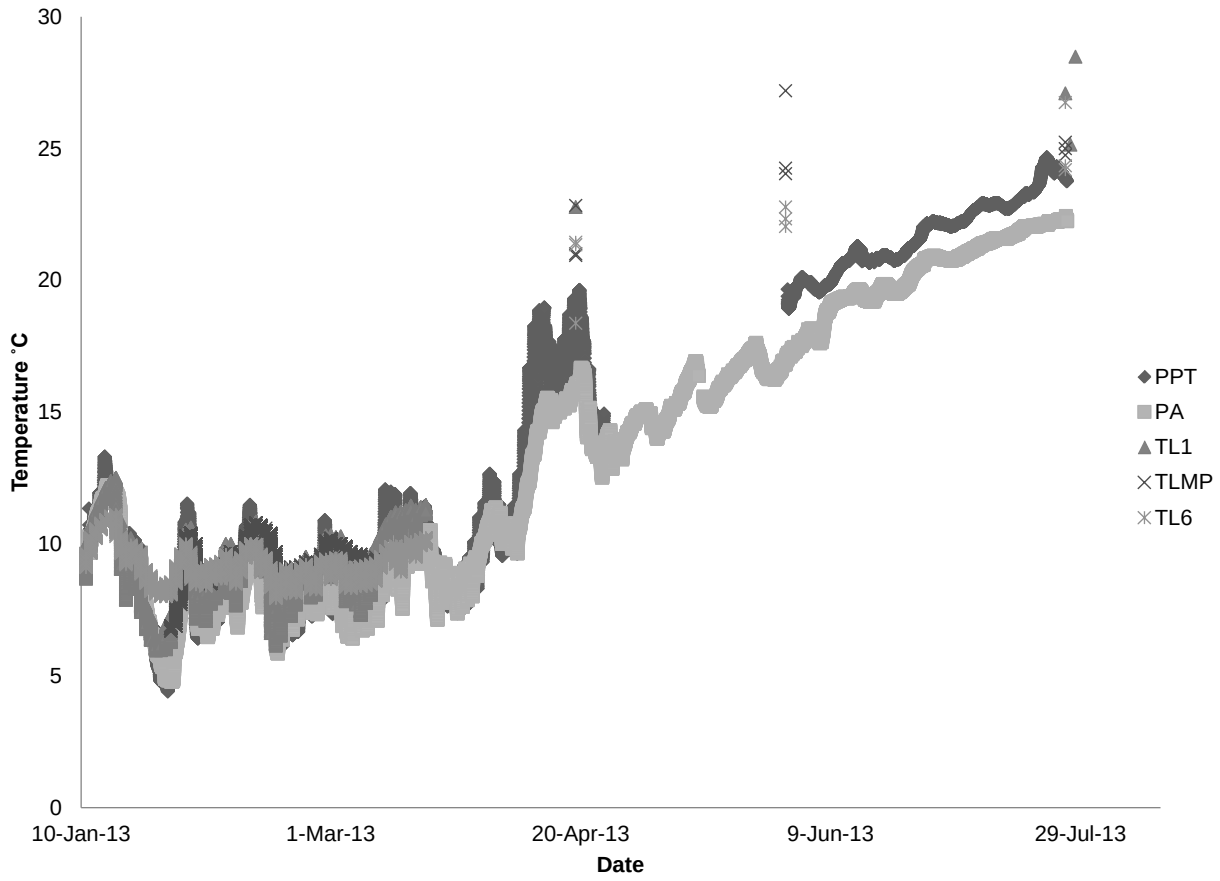
APPENDIX B

Supplemental Data

Field Experiment



Supplemental Figure 1. Measured conductivity for each field site over the duration of the field study. Measurements were taken both continuously, by data loggers, and on sampling days, by hand held monitors.



Supplemental Figure 2. Measured water temperature for each field site over the duration of the field study. Measurements were taken both continuously, by data loggers, and on sampling days, by hand held monitors.

Supplemental Table 1. Total number of macroinvertebrates found in each leaf litter bag, for bags that had macroinvertebrate presence. Also included is the site to which the bags were deployed and the taxa present in each bag. DS signifies a sample that was destroyed beyond recognition.

Bag	Site	Total Macroinvertebrates	Taxa Present
2	TL6-1	3	Amphipoda
10	TL6-2	1	Tipulidae
11	TL6-3	1	Coleoptera
14	TL6-1	1	Anisoptera
15	TL6-1	3	Anisoptera, Oligochaeta, Tipulidae
21	TL6-2	1	Coleoptera

26	TL6-2	2	amphipoda
33	TL6-3	1	Anisoptera
46	TL6-1	3	Amphipoda
48	TL6-3	1	Anisoptera
49	TL6-2	2	Coleoptera, Oligochaeta
55	TL6-1	1	Amphipoda
72	PA-1	1	Amphipoda
85	TL1-1	1	Tipulidae
98	TL1-3	1	Tipulidae
126	TL1-1	1	Tipulidae
149	PPT-1	2	Amphipoda
150	PPT-1	5	Hirudinea, Simuliidae
151	PPT-3	2	Physidae, Oligochaeta
161	PPT-2	1	Amphipoda
162	PPT-2	3	corydalidae, Tipulidae, Simuliidae
173	PPT-1	1	corydalidae
175	PA-3	1	Coleoptera
184	PPT-2	3	Zygoptera, amphipoda, Anisoptera
185	PPT-3	7	Simuliidae
198	PPT-2	1	corydalidae
199	PPT-3	1	Dytiscidae
201	PPT-2	1	Oligochaeta
206	PPT-3	1	Coleoptera
212	PPT-3	2	corydalidae, amphipoda
221	PPT-1	5	Amphipoda
227	TLMP-1	3	Amphipoda
233	TLMP-3	1	Oligochaeta
234	TLMP-3	1	Oligochaeta
236	TLMP-3	2	Amphipoda
238	TL1-2	1	Tipulidae
244	TL1-3	1	Tipulidae
247	TLMP-3	2	Amphipoda
251	TLMP-2	4	Amphipoda, Coleoptera
259	TLMP-3	1	amphipoda
267	TLMP-1	12	Anisoptera, Oligochaeta, Hirudinea
268	TLMP-1	1	DS
271	TLMP-3	1	Amphipoda
276	TLMP-3	1	Amphipoda
290	TLMP-1	3	Amphipoda
294	TLMP-1	1	Amphipoda
298	TLMP-2	4	Oligochaeta
309	TLMP-1	1	Amphipoda
315	TLMP-1	3	Oligochaeta

Supplemental Table 2a. Physiochemical water measurements taken on the 19-Apr-2013 sampling day by a hand held sampler. NA measurements represent a lack of sufficient water depth for measurement.

Site	Temperature ˚C	Conductivity mS/cm	Dissolved Oxygen %	Dissolved Oxygen mg/L	pH	Oxydation Reduction Potential mV
19-Apr-13						
PPT-1	19.37	1.889	2	0.18	6.31	-198.9
PPT-2	19.73	2.261	1.1	0.1	6.19	-269.9
PPT-3	20.92	1.824	9.6	0.84	6.35	-236.3
PA-1	20.78	0.213	24	2.12	4.58	-13.3
PA-2	21.22	0.208	32	2.84	4.32	56.9
PA-3	20.45	0.206	26.2	2.34	4.61	44.9
TL1-1	22.78	0.276	35.5	3.04	4.78	64.8
TL1-2	NA	NA	NA	NA	NA	NA
TL1-3	NA	NA	NA	NA	NA	NA
TLMP-1	20.94	0.099	15.6	1.39	4.8	-137.9
TLMP-2	21.01	0.11	6.7	0.57	5.04	-109
TLMP-3	22.84	0.099	27	2.32	5.7	-131.5
TL6-1	21.35	0.108	10.2	0.9	4.96	105.8
TL6-2	21.44	0.108	12.5	1.09	5.15	-3.9
TL6-3	18.37	0.135	3.1	0.29	5.21	26.9

Supplemental Table 2b. Physiochemical water measurements taken on the 31-May-2013 sampling day by a hand held sampler. NA measurements represent a lack of sufficient water depth for measurement.

Site	Temperature ˚C	Conductivity mS/cm	Dissolved Oxygen %	Dissolved Oxygen mg/L	pH	Oxydation Reduction Potential mV
31-May-13						
PPT-1	21.44	2.335	17.2	1.5	6.49	-250.6
PPT-2	21.02	2.079	9.5	0.83	6.34	-242.3
PPT-3	20.5	1.866	4.2	0.39	6.15	-215.9
PA-1	NA	NA	NA	NA	NA	NA
PA-2	NA	NA	NA	NA	NA	NA
PA-3	NA	NA	NA	NA	NA	NA
TL1-1	NA	NA	NA	NA	NA	NA
TL1-2	NA	NA	NA	NA	NA	NA
TL1-3	NA	NA	NA	NA	NA	NA
TLMP-1	24.04	0.12	10.5	0.85	5.43	-138.9
TLMP-2	24.25	0.111	17.7	1.42	5.36	-106.6
TLMP-3	27.19	0.111	8.5	0.72	5.42	-108.8
TL6-1	22.33	0.143	13.5	1.14	4.8	35
TL6-2	22.78	0.133	30.3	2.56	4.87	4.2
TL6-3	22.04	0.128	16	1.39	5.06	-26.1

Supplemental Table 2c. Physiochemical water measurements taken on the 26-Jul-2013 sampling day by a hand held sampler. NA measurements represent a lack of sufficient water depth for measurement.

Site	Temperature ˚C	Conductivity mS/cm	Dissolved Oxygen %	Dissolved Oxygen mg/L	pH	Oxydation Reduction Potential mV
26-Jul-13						
PPT-1	23.27	2.116	7.8	0.65	6.81	-266.3
PPT-2	23.07	2.299	9.1	0.79	6.57	-278.4
PPT-3	23.48	2.166	9	0.78	6.49	-269.7
PA-1	22.24	0.218	25.2	2.24	5	88.3
PA-2	22.14	0.235	14.2	1.2	4.3	136.3
PA-3	22.35	0.0171	12.9	1.58	4.69	96
TL1-1	27.09	0.241	28.4	2.24	4.59	134.9
TL1-2	25.14	0.372	40.3	4.32	4.29	175
TL1-3	28.48	0.355	47.6	3.71	4.29	198.8
TLMP-1	24.99	0.118	6.5	0.54	5.79	-136.1
TLMP-2	24.74	0.092	11.8	0.98	5.56	-136.1
TLMP-3	25.23	0.093	15.5	1.27	5.7	-115.3
TL6-1	24.19	0.12	20.5	1.66	5.08	-39.3
TL6-2	26.74	0.118	28.3	2.27	4.81	73.3
TL6-3	24.34	0.128	7.5	0.59	5.29	-90.9

Supplemental Table 3. Linear regression R^2 and p-values for mean field enzyme activity against temperature, dissolved oxygen, pH, and oxidation reduction potential.

Enzyme	Temperature ˚ C		Dissolved Oxygen %		pH		Oxidation Reduction Potential	
	R^2	p-value	R^2	p-value	R^2	p-value	R^2	p-value
Beta-glucosidace	0.42	0.24	0.67	0.09	0.93	0.01	0.85	0.03
Arylsulfatase	0.07	0.68	0.57	0.14	0.87	0.02	0.92	0.01
N-acetylglucoseaminidase	0.18	0.48	0.06	0.69	0.29	0.35	0.24	0.40
Lucine aminopeptidase	0.01	0.9	0.29	0.40	0.82	0.03	0.69	0.08
Acid phosphatase	0.01	0.86	0.24	0.40	0.51	0.18	0.59	0.13
Phenol Oxidase	0.30	0.34	0.88	0.02	0.83	0.03	0.85	0.03

Microcosm Experiment

Supplemental Table 4. Mean dissolved oxygen (mg/L), dissolved oxygen (%DO), and Temperature ˚C from all microcosm replicates separated into inoculum type and treatment on each sampling day. NA represents no measurement taken.

Day	Inoculum	Treatment	Mean DO (	±Std Err	Mean DO %	± Std Err	Mean T ˚ C	± Std Err
0	PA	H	69.68	0.12	6.142	0.009	NA	NA
		L	69.64	0.12	6.138	0.008	NA	NA
	PPT	H	69.54	0.24	6.128	0.020	NA	NA
		L	69.54	0.16	6.13	0.012	NA	NA
2	PA	H	2.78	0.16	0.24	0.012	NA	NA
		L	3.56	0.45	0.312	0.040	NA	NA
	PPT	H	2.62	0.44	0.23	0.038	NA	NA
		L	2.86	0.18	0.25	0.014	NA	NA
8	PA	H	4.8	1.19	0.434	0.118	NA	NA
		L	4.84	0.41	0.428	0.036	NA	NA
	PPT	H	3.58	0.77	0.312	0.069	NA	NA
		L	2.92	0.21	0.256	0.016	NA	NA
15	PA	H	3.08	0.36	0.272	0.032	21.17	0.05
		L	9.1	0.34	0.798	0.030	21.35	0.06
	PPT	H	12.96	2.66	1.14	0.235	21.34	0.12
		L	6.02	0.50	0.53	0.048	21.22	0.07
30	PA	H	17.76	1.61	1.578	0.141	21.32	0.03
		L	22.64	2.04	2.006	0.181	21.34	0.01
	PPT	H	39.98	3.49	3.542	0.308	21.35	0.04
		L	22.28	1.64	1.988	0.138	21.33	0.01
65	PA	H	12.72	1.14	1.094	0.099	22.55	0.05
		L	15.02	1.00	1.296	0.085	22.72	0.06
	PPT	H	17.96	3.30	1.546	0.296	22.65	0.05
		L	13.98	1.01	1.216	0.083	22.56	0.05
85	PA	H	8.98	1.41	0.792	0.127	21.26	0.01
		L	23.46	3.78	2.08	0.336	21.25	0.04
	PPT	H	7.04	0.70	0.604	0.064	21.39	0.01
		L	13.4	2.12	1.182	0.190	21.22	0.01
99	PA	H	5.26	0.54	0.452	0.053	21.22	0.02
		L	12.34	2.61	1.092	0.231	21.20	0.04
	PPT	H	10.82	1.64	0.952	0.150	21.20	0.02
		L	9.04	1.84	0.796	0.166	21.21	0.04

Supplemental Table 5. Mean total nitrogen (TN mgN/L) and mean dissolved organic carbon (DOC mgC/L) from all microcosm replicates separated into inoculum type and treatment on each sampling day.

Day	Inoculum	Treatment	Mean TN mgN/L	±Std Err	Mean DOC mgC/L	±Std Err
0	PA	H	2.404	1.656	68.91	8.74
		L	0.381	0.313	79.97	12.41
	PPT	H	0.680	0.315	58.17	4.43
		L	0.464	0.310	58.95	2.78
2	PA	H	1.974	0.150	1022.79	49.50
		L	1.550	0.136	1100.07	29.08
	PPT	H	1.650	0.069	814.27	171.30
		L	1.656	0.126	1034.71	80.29
8	PA	H	1.914	0.113	1190.68	48.25
		L	1.713	0.086	1235.20	26.89
	PPT	H	1.940	0.129	1176.20	19.37
		L	2.014	0.171	1290.72	36.66
15	PA	H	2.040	0.132	1165.52	14.11
		L	2.079	0.156	1234.76	22.84
	PPT	H	1.843	0.170	1161.72	13.69
		L	1.918	0.102	1259.20	15.41
30	PA	H	1.845	0.223	1141.52	23.80
		L	1.823	0.156	1155.56	20.99
	PPT	H	1.689	0.218	1153.00	23.16
		L	1.495	0.094	1245.16	32.36
65	PA	H	1.509	0.119	1061.79	46.13
		L	1.813	0.067	1046.15	47.51
	PPT	H	1.433	0.162	835.99	38.90
		L	1.804	0.133	1391.71	106.94
85	PA	H	0.976	0.082	912.47	47.70
		L	1.164	0.061	864.03	48.02
	PPT	H	1.061	0.043	628.99	64.18
		L	1.302	0.201	1014.55	57.46
99	PA	H	1.242	0.074	948.99	30.66
		L	1.239	0.129	936.59	66.81
	PPT	H	0.945	0.075	574.03	30.46
		L	1.066	0.171	930.39	66.28

APPENDIX C

Litter Bag Retrieval, Processing, and AFDM Protocol

Procedure in the field

1. Take the end bag from each of the attached lines at each sampling point.
2. Put in the appropriately labeled ziplock bag (site and collection date).
3. Bring all leaf packs back to the lab and place them in the refrigerator

Procedure in the lab

1. Using a plastic pan (or sieves) and squirt bottle remove large insects and sediments without breaking the leaf material.
2. Store macroinvertabrates in scintillation vials with 70% Ethanol and label (site, leaf pack #, collection date).
3. Punch a total of 50 leaf disks with a 13.1 mm cork borer.
4. Use ~1g from each pack for enzyme analysis. Place leaves in 1.5 mL microcentriguge tubes. If analysis cannot be completed immediately, place in -80^{o} C freezer.
5. Use 30 disks from each pack for microbial analysis (both genetic work (1.5 mL microcentrifuge tubes-20 disks) and epifluorescense microscopy (glass 2 dram vials with 2% Formalin-10 disks)). If analysis cannot be completed immediately, place disks in freezer.
6. Place the remaining 10 leaf disks from each bag in separate pieces of aluminum foil, with label, and dry them at 72° C for 24 hrs.
7. Put all litter bags in foil trays and in the oven at 72°C for 24 hours. Keep labels with leaves at all times.
8. Remove leaves from oven and weigh them (record as DM in datasheet)
9. Grind the leaf packs
10. Take approximately 1 g (and record the weight) and ash at 500 C for one hour. Once cool re-weigh the sample and record its weight.

Detailed AFDM procedures are found in the book Methods in Stream Ecology Chapter 30. Benfield E (2006) Decomposition of leaf material. Methods in Stream Ecology Edt. Hauer RF, Lamberti GA. Academic Press, San Diego, Chapter 30 pp 711-720.

Fluorometric and Oxidative Enzyme Assay Protocol

(Modified from Allison Lab Protocol, Steve Allison 06/2009. Origionally from S. Schmidt Lab/M. We Lab, January 2008; and Saiya-Cork, K. R., R. L. Sinsabaugh, and D. R. Zak. 2002. The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. Soil Biology & Biochemistry **34**:1309-1315.)

Reagents:

1.0 M NaOH
DI water
6**odium acetate buffer, pH 5.0** (can maNe a 10X stock solution)

Substrate solutions (200 µM unless otherwise noted): Enzyme	**Substrate**
β-Glucosidase (BG)	4-Methylumbelliferyl β-D-glucopyranoside 6.77 mg/100 ml
β-N-acetylglucosaminidase (NAG)	4-Methylumbelliferyl N-acetyl-β-D-glucosaminide 7.59 mg/100 ml
Leucine aminopeptidase (LAP)	L-Leucine-7-amido-4-methylcoumarin Hydrochloride 6.5 mg/100 ml (dissolve in 0.5 ml acetone first)
Acid phosphatase (AP)	4-Methylumbelliferyl phosphate 5.12 mg/100ml
Arysulfatase (AS)	4-Methylumbelliferyl Sulfate 5.89 mg/100ml
Polyphenol oxidase (PO)	25 mM L-dihydroxyphenylalanine (DOPA) **WARNING:** L-DOPA is hazardous; wear gloves. 493 mg/100 ml
MUB standard	100 µM 4-Methylumbelliferone 1.76 mg/100 ml Dissolve in 1 ml acetone first
AMC standard	100 µM 7-Amino-4-methylcoumarin 1.75 mg/100ml Dissolve in 1 ml acetone first

Make substrate and fluorescent standard solutions in 125 ml **amber glass bottles** using sterile DI water and a microbalance. Preheat water for L-DOPA solution in microwave. Store solutions in the 4°C refrigerator. Substrates are in freezer. Remake substrate solutions **every week**. Remake standard stock solutions **every 2 weeks**. Standards should be **diluted to 10 µM every day** by combining 0.5 ml stock solution with 4.5 ml sterile DI water.

Label Plates with sample numbers and the enzyme names.

For an assay with more than three substrate samples, multiple plates for each substrate will be necessary (e.g. LAP 1, LAP 2, LAP 3…). 3 samples can be run per plate, so running 12 samples would require 4 plates for each assay.

Sample Preparation
If the dry weight of the sample is unknown, obtain fresh litter samples and split each into two parts.

Weigh one part, record mass, place in foil envelope dry at about 70C to constant weight (24hrs). Record dry mass.

Weigh other part (1-2 g wet weight), record mass, and place in labeled 500 ml container. Add 125 ml acetate buffer and blend on highest speed for 1 minute to make a slurry. If the dry weight of the sample is already known, it can be added directly to the 500 ml container. **Do not let samples sit in slurries for more than 30 minutes!**

Rinse blender with DI water between samples.

Assay Set-up
Using multichannel pipettors and wide-mouth tips (cut off tip ends with scissors if necessary), pipette 200 µl of the soil slurry into the 96 well plates, keeping slurry well stirred with a stir bar/plate. 1, 2, and 3 are the different samples. Note on datasheet which sample is which. All assay plates should be pipetted as follows, leaving columns 1- 3 empty:

1	2	3	4	5	6	7	8	9	10	11	12
none	none	none	1	1	1	2	2	2	3	3	3
none	none	none	1	1	1	2	2	2	3	3	3
none	none	none	1	1	1	2	2	2	3	3	3
none	none	none	1	1	1	2	2	2	3	3	3
none	none	none	1	1	1	2	2	2	3	3	3
none	none	none	1	1	1	2	2	2	3	3	3
none	none	none	1	1	1	2	2	2	3	3	3
none	none	none	1	1	1	2	2	2	3	3	3

Next add the following amounts (µl) of sodium acetate buffer:

1	2	3	4	5	6	7	8	9	10	11	12
250	200	200	50			50			50		
250	200	200	50			50			50		
250	200	200	50			50			50		
250	200	200	50			50			50		
250	200	200	50			50			50		
250	200	200	50			50			50		
250	200	200	50			50			50		
250	200	200	50			50			50		

Next add the following amounts of MUB (use AMC instead for the LAP assay):

1	2	3	4	5	6	7	8	9	10	11	12
	50			50			50			50	
	50			50			50			50	
	50			50			50			50	
	50			50			50			50	
	50			50			50			50	
	50			50			50			50	
	50			50			50			50	
	50			50			50			50	

Finally, add the following amounts of substrate:

1	2	3	4	5	6	7	8	9	10	11	12
		50			50			50			50
		50			50			50			50
		50			50			50			50
		50			50			50			50
		50			50			50			50
		50			50			50			50
		50			50			50			50
		50			50			50			50

All wells should now contain 250 µl.

For the PO plates add the following amounts (µ0) of sodium acetate buffer:

1	2	3	4	5	6	7	8	9	10	11	12
250	200		50			50			50		
250	200		50			50			50		
250	200		50			50			50		
250	200		50			50			50		
250	200		50			50			50		
250	200		50			50			50		
250	200		50			50			50		
250	200		50			50			50		

Then add the following amounts of substrate:

1	2	3	4	5	6	7	8	9	10	11	12
	50			50	50		50	50		50	50
	50			50	50		50	50		50	50
	50			50	50		50	50		50	50
	50			50	50		50	50		50	50
	50			50	50		50	50		50	50
	50			50	50		50	50		50	50
	50			50	50		50	50		50	50
	50			50	50		50	50		50	50

All wells should now contain 250 µl, except column 3 which is empty.

Incubate fluorometric and oxidase plates at room temperature. Incubation times will depend on your sample type.

Assay Termination
Add 10 µL of 1 M NaOH to each well of all fluorescence plates noting time. Note: this is not done in advance. Add the NaOH to a set of plates for one assay only, then read those plates, then add the NaOH to another set and read those, etc.

Wipe any condensation from the bottoms of the clear plates with a paper towel before reading. The data for these plates will not be accurate unless you wipe the plate bottoms off first.

Reading Plates
Read fluorescence plates at 365 nm excitation and 450 nm emission. Read PO plates at 460 nm absorbance. Save your data.

Fluorescence Activity
Activity (µmol h^{-1} g^{-1}) = (NFU / Standard FU) × 0.3125 / (DW × hours) =
{NFU / [Standard FU / (10 µmol/L × 0.00005 L)]} / [0.0002 L × (DW g / 0.125 L) × hours]

NFU = net fluorescence units =
{(Assay – Sample) / [(Quench Control – Sample) / Standard]} – Substrate

DW = dry weight of soil sample contained in 125 ml buffer

Assay: Mean fluorescence of the 16 soil slurry + substrate wells

Negative control: Mean fluorescence of the 8 Buffer + Substrate wells

Sample Blank: Soil slurry only

Quench: Mean fluorescence of the 8 Soil slurry + Fluorescence standard wells

Reference standard: Mean fluorescence of the 8 Buffer + Fluorescence standard wells

Quench coefficient: Quench / reference standard

Net fluorescence: ((Assay – sample blank) / quench coefficient) – negative control

-.the sample blank is subtracted from the assay value before the quench coefficient is applied

– this is because the quench coefficient is applied to both the assay and sample blank values

– because they're from wells with soil in them

Emission Coefficient: Reference standard / 0.5 nmole per well

nmol h-1 g-1: (net fluorescence * 125 ml total soil slurry) / (emission coefficient * 0.2 ml soil slurry per well

* incubation time * g dry soil per g wet)

Phenol Oxidase and Peroxidase

OD = (Sample + Substrate ABS) - (Sample Blank ABS) - (OD for L-DOPA + Buffer)

Activity (µmol/h/g) = OD / (7.9 / µmol) (incubation time, h) (g sample / mL of sample homogenate)

7.9 / µmol is the micromolar extinction coefficient for DOPA under the conditions of the assay.

This is the slope of a standard curve of (oxidized) L-DOPA vs. ABS

NPOC-TN Analysis Protocol

Preparation

1. Use 1 ml sample that has been filtered through 0.7 µm GF/F glass microfiber and 19 ml reagent grade DI to dilute samples.
2. Prepare blanks and standards and quality controls (QC).

Reagents

Acid

Use special grade concentrated sulfuric acid (36 N) and pure water for dilution.

1. Pour 50 ml of sulfuric acid to 150 ml of pure H2O. This will cause an exothermic reaction in the solution so be sure to agitate the solution while carefully adding sulfuric acid.
2. Pour the prepared acid into the acid container.

Standards

NPOC standard stock solution 1000 mg C/L

1. Accurately weigh 2.125 g of reagent grade potassium hydrogen phthalate
2. Transfer into a 1 L Volumetric flask
3. Add Deionized water to the 1 L mark and stir the solution
4. Dilute stock solutions to desired concentrations.
5. Solution is stable for approximately 2 months if it is refrigerated.

NPOC QC

1. Accurately weigh .01063 g of reagent grade potassium hydrogen phthalate.
2. Transfer into a 250mL Volumetric Flask and add Deionized water to the 250mL mark.
3. This makes a 20 mg C/L solution.
4. Remake Weekly.

TN standard stock solution 1000 mg N/L

1. Weigh 7.219 g of reagent grade potassium nitrate

2. Transfer the weighed material to a 1 L volumetric flask.
3. Add zero water up to the 1 L marker and mix well.
4. Solution is stable for approximately 2 months if refrigerated.

TN QC

1. Accurately weigh .00722 g of reagent grade potassium hydrogen phthalate.
2. Transfer into a 250mL Volumetric Flask and add Deionized water to the 250mL mark.
3. This makes a 4 mg N/L solution.
4. Remake Weekly.

NPOC & TN Mixed Standard solution 100 mg C/L and 25 mg N/L TN

1. Take 100ml of the NPOC standard stock solution and 25 ml of the TN standard stock solution into a 1 L Volumetric flask
2. Add 50 ml of 1 M hydrochloric acid to the flask.
3. Add zero water up to the 1 L marker and mix well.
4. The HCl concentration will be about 0.05 M after dilution.
5. Stable for about 1 month if refrigerated.

Using the Shimadzu TOC-TN Analyzer

Before analyses:

1. Open gas tank and record gas level (PSI). (Each run takes between 200-400 PSI. Do not run large quantities of samples if under 500 PSI)
2. Check water level of dilution water- refill with DI if necessary
3. Check the level of the acid- replace if necessary (see Reagents section for preparation procedure).
4. Check the level of the Drain Vessel- make sure the level is near the position of the overflow tube. Replenish with pure water if necessary. If it needs to be replenished remove bottle from clip. Remove the black cap. Using a wash bottle pour pure water into the cooler drain vessel. Reattach the rubber cap and return the vessel to its position in the mounting clip.

5. Check the Humidifier water level (Bottle inside the instrument). Verify the water level in the humidifier is above the “Lo” mark. If the water level is below the mark, replenish using a wash bottle and the water supply port on top of the vessel.

Turning on the instrument

1. Turn on the main power switch on the left side of the instrument (Right if you are looking at it).
2. Turn on the Autosampler on the right side of the instrument (left if you are looking at it).
3. It takes about 45 minutes for the furnace to reach the correct temperature.

Opening the sample Table Editor

1. Click on TOC-Control icon on the desktop.
2. Click on Sample Table Editor
3. Hit OK when it asks for User name.
4. The TOC-L Sample Table editor will open.
5. Click on New under Sample Table
6. Choose TOC-TN System and Table Type Normal
7. Create a calibration curve, or use a calibration curve from previous runs.
8. Click Connect and Monitor.

Create a calibration curve

1. Click New in the Calibration curve tab (Bottom Left in the New Sample Table)

2. The Calibration Curve Wizard appears

3. Select the System: TOC-TN and click Next

4. Select Normal and click next, click use dilution from standard solution.
5. Select Type of Analyses: NPOC, Give the file a name and click Next.
6. Select the units: mg/L, injections 2/3, washes 2, the default values should be ok. Click Next
7. Click on Add. to add the values of the Calibration Points (add at least 3 calibration points)(Start with concentration of Standard, i.e. 100mg C/L, then enter desired concentration, i.e. 25 mg C/L. Dilution will be done automatically). Click Next

8. Click on Use Default settings
9. Click on Finish

Add unknown samples to the table

Adding a Single Sample

1. Right Click on the row you wish to add a new sample, and click insert sample. Sample wizard will appear.
2. Choose the most recent NPOC-TN.met file for the method and click next.
3. Name the Sample and identification and click next
4. Choose NPOC calibration curve and click next until you reach page 5.
5. Make sure “use default settings” is selected and click next.
6. Repeat steps 4 and 5 for TN.
7. Click none for Pharmaceutical Water Testing and click finish.

Adding Multiple Samples

8. Right Click on highlighted row and select insert multiple samples. Wizard will appear.
9. Select the newest NPOC-TN.met file and click next.
10. Choose the number of samples and name them. Choose what vial number the samples will start at and where you would like numbering to start. Make sure “insert cal curves/control samples” is checked and Click next.
11. Add Calibration curves for NPOC and TN and click next until finish.

Assigning vials for Sampling

1. Click on the birthday cake icon in the top right.
2. Have the vial you wish to assign highlighted and Double click on the location you wish to assign it to on the tray icon.
3. Repeat for every vial, making sure they match with the physical set up.

Staring the run

1. Check the Monitor. Make sure that all checks are green and that the Graphs have leveled out.
2. Press start.