ABSTRACT

Title of Dissertation: **ANTHROPOGENIC DISTURBANCE ALTERS PLANT AND MICROBIAL COMMUNITIES IN TIDAL FRESHWATER WETLANDS IN THE CHESAPEAKE BAY, USA**

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Tidal freshwater wetlands are often found near urban centers, and as a result of human development they are subject to multiple environmental stressors. Increases in nutrient runoff, sedimentation, and hydrologic alterations have had significant impacts on these systems and on the ecosystem services they provide. One of the consequences of these stressors is the expansion of invasive species that can affect native biodiversity and the many biogeochemical processes that are key to wetland ecosystem function. This research looked at how human activities affect microbial communities in tidal freshwater wetlands, and explored various aspects of an invasive plant's ecology in the Chesapeake Bay.

In our first study, we found that microbial community composition differed along a rural to urban gradient and identified microbial taxa that were indicators of either habitat. Rural sites tended to have more methanogens and these were also

indicators in these system, whereas in urban systems nitrifying bacteria were the main indicator taxa. This study suggested that urban wetlands have different microbial communities and likely different functions than those in rural areas, particularly concerning nitrogen and contaminant removal. Our second study looked at management of an invasive lineage of *Phragmites australis* which is commonly found in wetlands impacted by nitrogen enrichment. We evaluated the effects of different C:N ratios on the competitive ability of this lineage and a native North American lineage. Even though carbon addition did not improve the native's competitive ability, we identified facilitative interactions when both lineages were growing together. This suggests that native and invasive *Phragmites* might coexist if there are no additional disturbances to the system. Our last study focused on plant-fungal interactions, and found that both *Phragmites* lineages benefitted from inoculation with fungal endophytes under salt stress. These results suggest that studies of plant-fungal interactions can yield insights into mechanisms of invasion, and could be further investigated in native wetland plants susceptible to increased salt stress following sea-level rise. Our results provide insights into plant and microbial ecology in the Chesapeake Bay's tidal freshwater wetlands, and improve our understanding of the invasion process and management strategies of *Phragmites australis*.

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by

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Chapter 1: Review of nutrient pollution in the Chesapeake Bay and its effects on plant and microbial ecology

The Chesapeake Bay is the largest estuary in the United States with an area of approximately 11,601km^2 approximately and 315km of tidal zones on its main stem (1,2). Due to the Bay's shallowness relative to its length, it is one of the few estuaries that holds a semi-diurnal tide (3). These tidal currents play a key role in transport and accumulation of contaminants in the Bay through resuspension and redepositions of bottom sediments (4), and are responsible for salinity gradients across the major tributaries in the Bay.

Salinity levels can range from ~0.1% in the freshwater upper tributaries, to 25-30% near the mouth of the Bay (5). These salinity variations result in different types of wetlands that have developed across the Bay; in the upper reaches of the estuary there are tidal freshwater wetlands characterized by salinities below 0.5ppt (6), and as salinity increases these transition into brackish and salt marshes (7).

Compared to brackish or salt marshes, freshwater wetlands support the greatest diversity of plants (7,8) and are considered to be one of the most productive ecosystems on Earth (9,10). Tidal exchanges of nutrients and particulate matter contribute to the high productivity of these systems and help support a diverse food web. Tidal freshwater wetlands act as sources, sinks, and transformants of organic matter (11,12) and therefore play key roles in biogeochemical cycles of major nutrients.

The landscape position of tidal freshwater wetlands makes them intermediates between land and aquatic systems and therefore have critical ecosystem functions

(10). Some of the many services that these wetlands provide include water filtration, flood mitigation, climate regulation and wildlife habitat (12). Due to their inland location, freshwater tidal wetlands tend to be close to urban centers and therefore are prone to human impacts (13). About 18 million people live in the Chesapeake Bay watershed and population is expected to increase leading to further degradation and loss of these wetlands (13).

Sea level could also lead to increased wetland losses; and in the Chesapeake Bay coastal wetland losses to sea-level rise has already been reported (14). In this context, freshwater tidal wetlands could be at risk due to salt intrusions that can alter C cycling (15,16) and promote nutrient release from sediments (17,18) exacerbating nutrient pollution, and altering plant community structure (19,20). Due to its topographical location, freshwater wetlands would not be able to migrate further upstream and freshwater plant communities will likely be replaced by salt-tolerant species (21) increasing the loss of these valuable systems.

Current wetland cover in the Chesapeake Bay is estimated to have decreased by 60% in the last 300 years (22). Before European settlements the Bay area was predominantly covered by forests, but urban development and agricultural fields quickly replaced these systems increasing surface runoff and soil erosion, and filling natural wetlands and streams (23,24). The increase in sedimentation rates together with the overexploitation of oyster reefs that filtered particulate matter, made the Bay prone to overfertilization (4). Specifically, humans contribute to nutrient pollution in the Bay through municipal treatment plants, sewage, runoff from fertilized field, animal feedlots (5) and atmospheric depositions from fossil fuel combustions (23). As

a result, nitrogen and phosphorus loads have increased 6-8 fold and 13-24 fold respectively from pre-colonial times to the mid 1980s (25).

Nutrient pollution can affect the structure and function of freshwater tidal wetlands by altering its biogeochemistry, plant, animal and microbial communities. Nutrient enrichment of the Bay's waters has led to an increase in phytoplankton that shades and kills submerged aquatic vegetation, and subsequently depletes dissolved oxygen causing a decrease in benthic macrofauna (26) and commercially valuable species (2,27). In addition, oxygen depletion decreases redox potential leading to changes in nutrient cycling and biogeochemistry in the sediments. Among these changes, low oxygen conditions can release NH_4 and PO_4 from the sediments creating a feedback loop that further promotes the eutrophic state (26). Eutrophication has also contributed to the spread of undesirable invasive plant species that can replace native vegetation and significantly alter the ecosystem's structure and function. The next section further explores the link between nutrient enrichment and plant invasion, and focuses on the spread of a non-native lineage of *Phragmites australis*.

Effects of nutrient pollution on invasive plant ecology in tidal freshwater wetlands

Although wetlands only occupy <6% of the Earth's landmass, 24% of the world's most invasive species are wetland plants (28). The availability of invasive propagules, together with nutrient enrichment or release, can ultimately lead to successful wetland plant invasions (29). Tidal freshwater wetlands can be very susceptible to nutrient pollution and invasive propagule dispersal because of their landscape position; they are usually found downstream of the nutrient rich sources

(30) and their connection to the estuary means tidal action can also promote the spread of contaminants and invasive species (31).

Invasive plants tend to share similar traits that allow them to be successful in nutrient-rich environments. Some of these traits include high growth rates, high photosynthetic rates, efficient dispersal and efficient use of N and P (32). Increases in atmospheric nitrogen deposition, fertilization and disturbances that promote nutrient release allow fast-growing, nitrophilic species to outcompete native species that are adapted to low nutrient conditions (33–35). Shifts in competitive balance between native species and more competitive invasive plants have been reported in various nutrient-enriched ecosystems including grasslands (36), deserts (37), and wetlands (38)

In the case of the Chesapeake Bay as well as other areas across the U.S., excessive use of fertilizers has had a major impact in freshwater wetland plant communities as it facilitated the spread of invasive plants like *Phragmites australis*. This invasion was initially considered to be a results of human activities that caused disturbances like hydrological changes and nutrient pollution (39); and although these factors contributed to *Phragmites* expansion, the main determinant was the introduction of a non-native European lineage that thrived under those disturbed conditions (40).

Native and invasive lineages of *Phragmites* differ morphologically and physiologically. Morphologically, the native can be differentiated based on its caducous leaf sheathes, longer ligule and glume length (41), lighter colored leaves, and reddish smooth stems (42) that grow less dense than the invasive lineage (43).

Physiologically, native *Phragmites* has lower photosynthetic rates, nitrogen uptake, growth rate, phenotypic plasticity (44) and is less tolerant to salinity (45).

Establishment of invasive *Phragmites* can affect plant and animal communities, hydrology and biogeochemical cycles. Areas that are invaded by dense mono-specific stands of invasive *Phragmites* have less plant diversity and different plant species composition than areas dominated by native vegetation (46,47). Animal communities can also be affected by this invasion as non-native *Phragmites* modifies wetland topography and can decrease fish recruitment, which could limit the availability of prey for wading birds (48). In addition, invasive stands have been reported to support shorter food chains (49). Concerning hydrological changes, invasive *Phragmites* can promote sedimentation and over time develop stands that are elevated, have a relatively flat surface, are infrequently flooded and have little standing water (50). Although this increase in elevation and changes in the hydroperiod might have negative effects on marsh function (51), it could also increase marsh sustainability in areas threatened by sea-level rise (52). Finally, nitrogen and carbon cycles can be affected due to greater litter inputs, and rooting depth of the invasive lineage. These aspects are further summarized in the next section.

Effects of nutrient pollution on wetland microbial ecology and biogeochemistry

Microbial communities play a key role in biogeochemical cycles of tidal freshwater wetlands affecting relevant processes like greenhouse gas emissions, water quality and primary productivity. The predominantly saturated conditions create reducing conditions while the presence of vegetation results in soil oxygenation near

roots zones promoting chemical and microbially-mediated transformations of elements like carbon, phosphorus, nitrogen, iron and manganese (9). Microbial substrate availability and hydrology determine process rates and occurrences (53,54), and in tidal freshwater wetlands the tidal hydrology leads to open element cycles through exchanges of water and solutes with both terrestrial and aquatic systems.

Nutrient enrichment in freshwater wetlands can alter microbial community structure and ecosystem function. For example, eutrophic sediments have been reported to have bacterial communities with greater number of anaerobic and beta-proteobacteria (55,56), and less diversity of annamox bacteria (57). Concerning ecosystem function, greater nitrate inputs can inhibit iron (Fe) reduction as N reducers outcompete Fe reducers (58). Similarly, N and P enrichment has been seen to decrease methanogenesis likely due to increased competition for substrates of methanogens with denitrifiers (39,40). **Chapter 2** further explores the effects of urbanization, and nutrient enrichment on microbial community structure of freshwater tidal wetlands.

Biogeochemical cycling, particularly of C and N, has also been affected by the invasion of *P. australis*. Decomposition rates of *Phragmites* litter can be much slower than that of native vegetation due to the high C:N of its stems, binding nutrients to organic material and thus making them unavailable to other wetlands plants (60,61). Another of the mechanisms by which this invasive plant alters C and N cycling is through its deeper rooting profile relative to native wetland species, which "primes" microbial communities deep in the soil promoting carbon and nitrogen mineralization (62,63). The deeper rooting profiles also allow invasive *Phragmites* to access deep

nitrogen pools that are unavailable to native wetlands plants immobilizing it in its biomass. In addition to immobilization, the rooting system can also promote nitrogen removal from the system by coupling nitrification and denitrification through efficient ventilation of sediments (64). Although nutrient removal is considered a valuable ecosystem service provided by wetlands which usually act as nutrient sinks, *Phragmites* invaded sites may act as sinks or sources of nitrogen depending on the rates of the aforementioned processes as well as litter decomposition rates, and environmental or anthropogenic nitrogen inputs (65)

Wetland restoration and invasive *Phragmites* management

Recognition of the importance of tidal freshwater wetlands and the ecosystem services they provide led to the Clean Water Act and an increase in restoration and conservation efforts of these systems. Some of the key functions that motivate wetland restoration include flood abatement, biodiversity support, carbon management and water quality improvement (66). But in spite of great efforts, many times the restored wetlands don't achieve functional equivalence to reference sites (67).

Some of the challenges when restoring wetlands to support diverse vegetation include constraints in dispersal, poor conditions for species establishment, priority effects in species recruitment, unforeseen biotic interactions, and changes in the available species pool that might include invasive species (68). The aggressive spread of invasive *Phragmites australis* in many freshwater wetlands poses major difficulties to restoration due to its great dispersal ability (69,70), phenotypic plasticity (44,71), and its potential to alter wetland hydrology (72,73) and soil biogeochemistry (74). As

a result, control or eradication of *Phragmites* has become an important conservation goal for many wetland restoration projects.

Controlling the spread and eradicating stands of invasive *Phragmites* has proven to be a great challenge, and resource management agencies have used multiple techniques and approaches with varying degrees of success. The most common method is chemical control using herbicide, which usually requires multiple years of applications to be effective (75). However, the lack of data on appropriate doses, and its possible impacts on non-target vegetation (76) and on the long-term recovery of native vegetation, make it hard to assess its success (77). Other methods include mechanical control (mowing, excavating or burning) and biological control using grazers like cattle. Although they can be somewhat effective at a local scale, these methods are difficult to apply at a landscape scale which is necessary for effective eradication (77).

These commonly used methods for invasive *Phragmites* control have been costly and not very successful (78) leading to the development and research of new approaches. Given that this invasion has been linked to eutrophic conditions, nitrogen management has been proposed as a method to control the spread of *Phragmites* (77). One way to achieve this is through the addition of C rich amendments that promote N immobilization making excess nutrients unavailable to invasive plants. This has shown some promise in controlling other invasive grasses and promoting native plant restoration. For example studies by Blumenthal et al. (79) and Averett et al. (2004) (80) observed that sawdust additions can decrease plant available N and biomass of undesirable grass species biomass, and promote restoration of native grasses. In spite

of some successful results using this method, other studies in grasslands have found no effect of sawdust addition on native plant restoration (81,82). The potential use of this approach in wetland restoration or for control of invasive *Phragmites* is investigated in **Chapter 3**.

A more novel but complex approach to controlling invasive plants like *Phragmites* involves microbiome manipulations (83). This approach suggests that characterizing the microbiome of invasive plants like *Phragmites* can lead to the identification of either pathogens or relevant mutualists that could either help control or manage the fitness of the plant. Although there has been some success in identifying pathogenic oomycetes that are commonly found in invasive *Phragmites* soils and are rare in the native lineage (84), the virulence of these organisms against each lineage the and potential success of these organisms as biocontrol agents remains to be evaluated. In **Chapter 4** we characterized the root fungal endophytes of native and invasive *Phragmites* and explored the role of dark septate endophytes in salt tolerance of these lineages.

In conclusion, tidal freshwater wetlands provide many valuable ecosystem services, but are susceptible to loss and degradation due to human activities. Nutrient enrichment can be particularly detrimental to these systems as it affects microbial communities and therefore biogeochemical cycles, and promotes plant invasions that can alter the ecosystem's structure and function (28). In tidal freshwater wetlands invasion by a non-native lineage of *Phragmites australis* has been linked to nitrogen enrichment and other anthropogenic disturbances (47,85) and once established it can decrease plant biodiversity and alter hydrology and nutrient cycles. Management

efforts to eradicate this invasive plant have rarely been successful, so novel methods like N immobilization and microbiome manipulations are being investigated as alternatives (77). These methods might also aid in the restoration of native species, including native *Phragmites* genotypes, and if successful they could constitute a more comprehensive wetland management and restoration tool.

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Chapter 2: Urbanization Altered Bacterial and Archaeal Composition in Tidal Freshwater Wetlands Near Washington DC, USA, and Buenos Aires, Argentina

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Abstract

Urban expansion causes coastal wetland loss, and environmental stressors associated with development can lead to wetland degradation and loss of ecosystem services. This study investigated the effect of urbanization on prokaryotic community composition in tidal freshwater wetlands. Sites in an urban, suburban, and rural setting were located near Buenos Aires, Argentina, and Washington D.C., USA. We sampled soil associated with two pairs of functionally similar plant species, and used Illumina sequencing of the 16S rRNA gene to examine changes in prokaryotic communities. Urban stressors included raw sewage inputs, nutrient pollution, and

polycyclic aromatic hydrocarbons. Prokaryotic communities changed along the gradient (nested PerMANOVA, Buenos Aires: $p = 0.005$; Washington D.C.: $p = 0.001$), but did not differ between plant species within sites. Indicator taxa included *Methanobacteria* in rural sites, and nitrifying bacteria in urban sites, and we observed a decrease in methanogens and an increase in ammonia-oxidizers from rural to urban sites. Functional profiles in the Buenos Aires communities showed higher abundance of pathways related to nitrification and xenobiotic degradation in the urban site. These results suggest that changes in prokaryotic taxa across the gradient were due to surrounding stressors, and communities in urban and rural wetlands are likely carrying out different functions.

Introduction

Global urbanization has rapidly increased throughout the last 60 years, and by 2050, two-thirds of the world population will live in urban settlements (1). Population growth and the resulting urban expansion is a major cause of wetland loss worldwide (2,3). Coastal wetlands are particularly susceptible to loss and habitat degradation, as the most dramatic population growth has occurred in cities located near coastlines (4,5).

Urban development has a direct effect on wetland ecosystem services, and the loss and degradation of these systems can impact human health and well-being (6). Increased impervious surfaces facilitate the transport of pollutants into waterways and directly impact wetland habitat quality (7). On average, in the United States, urban storm water runoff can carry 0.26 mg/L of phosphorus, 0.2 mg/L of nitrogen, and 54.5 mg/L of suspended sediments (8), which can transport various contaminants like

heavy metals and polycyclic aromatic hydrocarbons (PAHs) (9,10). As a result, urban wetlands tend to have more non-native plant species, typically favored by nutrient inputs, and higher sediment concentrations of heavy metals and organic chemicals (11,12).

Changes in urban wetland habitat can affect the diversity and richness of biota. For example, urbanization has been seen to reduce macro-organism species diversity by creating homogeneous plant and animal assemblages (13,14). Johnson et al. (15) reported that urban wetlands had significantly less richness of aquatic insects, mollusks, amphibians, aquatic reptiles, and crayfish than wetlands in non-urban systems. A study on urban rivers, which are subject to similar stressors as urban wetlands, found microbial richness decreased in the urban areas and had higher abundance of genes related to xenobiotic metabolism (16). To our knowledge, no studies have looked at the effects of urbanization on tidal freshwater wetlands (TFW) prokaryotic communities, but it is expected that they would also be susceptible to anthropogenic disturbances (17,18).

Wetland prokaryotic communities may change directly in response to added nutrients or shifts in the length and intensity of the hydroperiod, but may also indirectly be impacted by vegetation. In order to investigate the role of different plant functional groups on wetland prokaryotic communities, we sampled the root zones of two plant species. Root morphology and plant type can influence rhizosphere prokaryotic communities through plant exudates and oxygen release (19,20). Some studies have found that prokaryotic community structure can vary between wetland plant species (21) and even between lineages of the same species (22,23). Other

studies reported site-specific effects (24) or found that soil characteristics were the main drivers of microbial community composition (18).

We examined the prokaryotic communities in TFWs located near two major capital cities: Buenos Aires (Argentina), and Washington, D.C. (United States). Tidal freshwater wetlands are located in the upper part of estuaries and are characterized by salinity levels lower than 0.5% (25), and daily tidal fluctuations due to the influence of the nearby estuary. Their location often represents the most inland point that can still be reached by ships in the estuary, supporting early settlements in these tidal areas that continued to develop over the next few centuries (26). As a result of increased economic activity and population growth, tidal wetlands are particularly vulnerable to environmental degradation and loss associated with urban development (27,28).

Our study used next-generation DNA sequencing to test three hypotheses: (1) Prokaryotic community composition and function will vary across the established urban gradient; (2) prokaryotic communities will differ between plant species at each site; and (3) specific prokaryotic taxa will be indicative of urban wetlands, regardless of geographic location of the city.

Materials and Methods

Site Description

The cities of Buenos Aires and Washington D.C. are two examples of large cities that developed near tidal freshwater wetlands. Both cities are located at similar latitudes North and South of the Equator (Buenos Aires: 34.6037◦ S; D.C.: 38.9072◦

N) and have experienced significant population growth over the last few decades. This trend is expected to continue in both areas, and current population estimates are around 3 million people in Buenos Aires with ~376,000 residents in the county where we sampled (29), and almost 700,000 in Washington D.C. (30).

Argentinian sites were located in the Lower Paraná Delta, in the upper portion of Rio de la Plata estuary. Islands formed in this area in the last ~750 years and the delta continues to expand due to sediment deposition (31). About half of this area has been affected by human activities, mainly forestry and development associated with tourism and recreation (32). To allow these activities, hydrological modifications like levees and dikes were constructed, which altered the natural environment and affected hydrologic regimes (33). Wetlands in the Lower Paraná delta have been increasingly lost to urban expansion (34). Most of the developed area uses septic systems, and as drainfields become less effective over time, wastewater is released into the waterways. Water quality in this area is also affected by inadequate disposal of solid wastes, industrial pollution, and fuel spills from heavy boat traffic (35,36).

The United States sites were located in the Chesapeake Bay estuary. These tidal marshes originated during the Holocene (~10,000 years ago), when Pleistocene valleys were gradually inundated by rising sea level (37). As a result of human intervention and sea level rise, over half of the tidal wetlands in the Bay are considered to be degraded (38). The main perturbations associated with development near these wetlands are pollutant runoff that is promoted by large impervious surfaces (27,39) and nutrient enrichment from agriculture and sewage treatment facilities (40). Washington D.C. has a combined sewer overflow system, so after heavy rainfalls a

mix of storm water and sewage is released into nearby waterways, introducing bacterial pathogens and degrading water quality (41).

The study sites are located in freshwater tidal wetlands in the Paraná River delta (Buenos Aires, Argentina), and in the Chesapeake Bay (Maryland, USA). Within each region, sites corresponding to urban, suburban, and rural environments were identified (Figure 1).

In Buenos Aires the sites were located in the Lower Paraná Delta, just north of the City of Buenos Aires. The urban gradient was established by locating sites that had varying degrees of development. The urban site is on the Sarmiento River near its confluence with the Luján River (34◦24′48.81″S, 58◦34′1.76″W), which is considered to be highly contaminated with wastewater and industrial waste (42). The selected area is influenced by tidal inputs from the Luján River, experiences heavy boat traffic, and has been modified to accommodate residential houses. The suburban site (34◦23′8.27″S, 58◦34′6.30″W) is located upriver from the urban site, and the rural site is located on the Unión River (34◦22′55.73″S, 58◦31′38.77″W) on unmanaged land with no signs of human development upstream.

In Maryland the sites are located in the Anacostia River (38◦92′41.1″N, 76◦94′58.8″W; soil series Zekiah and Issue), Patuxent River (38◦78’58″N, 76◦71′30.8″W; soil series Nanticoke and Mannington), and Choptank River (38◦48′52.67″N, 75◦53′19.82″W; soil series Nanticoke and Mannington). The Anacostia River runs along the border of Washington D.C. and is highly urbanized and affected by industrial activities and sewage inputs from the city’s combined sewer system. The Patuxent watershed is located between Washington D.C. and

Baltimore, representing a site of intermediate urban development, and the Choptank River is located across the Chesapeake Bay in Eastern Maryland, where agriculture is the predominant land use (43).

We selected two species that have different morphological features at each site. In Washington D.C. the selected species were *Phragmites australis* (Cav.) Trin. ex Steud., and *Peltandra virginica* (L.) Schott, and in Buenos Aires, *Hymenachne grumosa* and *Sagittaria montevidensis*. *Phragmites* and *Hymenachne* are clonal grasses that have thick rhizomes, tall and rigid stems, and horizontal cable-like stolons. *Peltandra* and *Sagittaria* have fleshy triangular leaves and a shallower root system with bulbous vertical corms or tubers. In addition, *Phragmites* is an invasive species that has higher nutrient requirements (44) and produces more biomass than other native species (45).

Sample Collection

Samples were collected on a summer day at each location (January in Buenos Aires and August in Maryland). In Maryland, three soil samples were collected from the rhizosphere of *Phragmites australis*, and three from Peltandra virginica at each of the sites. We used a half circle Russian peat borer (Eijelkamp, Giesbeek, Netherlands) to collect a 50-cm deep soil sample next to the stem to get plant-influenced soil. In Buenos Aires four soil samples were collected from the rhizosphere of Hymenachne grumosa and Sagittaria montevidensis. We used a spade shovel with measurement markings to collect soils from a depth of 30 cm, as these plants have a shallower root system than those sampled near D.C. Approximately 2 g of homogenized rhizosphere soil were added into sterile Falcon tubes that contained 4 mL of LifeGuard soil

preservation solution (MoBio Laboratories, Carlsbad, CA) and shipped to the University of Maryland for soil and prokaryotic community analysis.

Soil Analysis

Key soil properties known to have a significant impact on prokaryotic ecology were analyzed using the methods outlined by Yarwood et al. (22). Total C and N content were determined by combustion analysis at 950◦C and soil organic matter was calculated using loss-on-ignition (550 ◦C for 24 hr). To measure pH, five grams of soil were added to 25 mL of distilled water to make a 1:5 ratio slurry, which was then measured using a pH electrode. Finally, particle size analysis (PSA) was carried out using the hydrometer method for the Washington D.C. samples and the pipette method (46) for the Buenos Aires samples. Logistics of working in different countries prevented the use of the same core type and particle size analysis method.

DNA Extraction and Illumina Library Preparation

DNA extractions were carried out using the Qiagen DNeasy PowerLyzer PowerSoil kit (Qiagen, Hilden, Germany). The Buenos Aires samples were centrifuged, and the excess solution drained before beginning the extractions. These were carried out following the manufacturer's instructions, except for the homogenization step that was done using a FastPrep-24 (45 s at 6 m/s; MP Biomedicals, LLC, Solon, OH). Samples were quantified using a Qubit 2.0 fluorometer (Invitrogen) and diluted to 5ng/ul for PCR amplification and subsequent amplicon sequencing. The 16S rRNA region was targeted using the primers 515F+adapter (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAG

ACAGGTGCCAGCMGCCGCGGTAA-3′) and 806R+adapter (5′ -
GTCTCGTGGGCTCGGAGAT
GTGTATAAGAGACAGGGACTACVSGGGTATCTAAT -3′) (47). The PCR reaction had 3.5 uL of DNA, 17.5 uL of ThermoScientific TM PhusionTM Flash High-Fidelity PCR Mastermix (Thermo Fisher Scientific), and 7 uL of each primer (1 ng/uL). The PCR product was then processed for Illumina sequencing using the 16S Metagenomic Sequencing Library Preparation protocol (Part # 15044223 Rev. B, support.illumina.com). The cleanup was carried out using AMPure XP beads (Beckman Coulter, Pasadena, CA), and the Nextera XT 96 index kit (Illumina) was used for sample indexing. Samples were pooled, and amplicon size of the library was checked using a Bioanalyzer 2100 (Agilent Technologies). Q-PCR was used for library quantification, and the final library was diluted to 12 pM, spiked with 30% PhiX (Illumina), and run on an Illumina MiSeq using a 600-cycle v3 cartridge.

Data Analysis

R was used for statistical analysis and drawing figures (48). Illumina sequencing output was processed using the dada2 package (version 1.6) (49) for filtering, dereplication, sample inference, merging of pair end reads, and chimera checking. The algorithm used for chimera checking in this performs a Needleman-Wunsch global alignment of each sequence to compare it with more abundant sequences, and check if the “child” sequence can be obtained from exact combinations of right and left segments of “parent “sequences, which would classify them as chimeras. Taxonomic assignments were carried out by matching sequences to the SILVA database (SILVA v128, arb-silva.de) and the resulting amplicon sequence

variant table was analyzed using the phyloseq (v1.2) (50) and vegan (v2.4-4) (51) R packages. Samples were rarefied to the minimum sequence number for each data set (Buenos Aires = 11,811, Maryland = 59,939). Rarefaction curves were produced and confirmed that coverage of sampling was appropriate, as all but one sample curve leveled off at the proposed sequence cut-off (Figure S1 in supplementary materials). Non-metric multidimensional scaling (NMDS) based on a Bray-Curtis dissimilarity matrix was used to visualize differences between sites and plant species, and homogeneity of group dispersion was checked using the vegan functions betadisper and permutest. Nested PERMANOVA was used to test for statistical differences with the adonis function and a calculated p value < 0.05 was considered significant. Significant factors identified through permutational multivariate analysis of variance (PERMANOVA) were further examined with pairwise comparisons using permutation MANOVAs to assess differences between group levels. This was done using the pairwise.perm.manova function from the RVaideMemoire package (52) with 999 permutations on the Bray-Curtis distance matrix and Bonferroni corrections to adjust p-values after multiple testing. The associations between community composition and the most abundant phyla were evaluated using the envfit function, and vectors that showed significant correlations were fitted to the non-metric multidimensional scaling (NMDS) ordination. Correlations between community composition and soil variables were also evaluated with the envfit function to identify significant environmental factors. Prokaryotic amplicon sequence variants (ASVs) were classified into urban or rural habitat generalist or specialist using a multinomial species classification method (CLAMtest) in vegan. We used a coverage limit of 10,

an alpha of 0.005, and a specialization value of 0.67, which is considered conservative (53). Furthermore, indicator taxa for urban and rural habitats were identified using the multipatt function in the indispecies package (54), and significant associations between taxa and sites were evaluated using permutation tests. The t4f (Tax4Fun) function in the R package of the metagenomics (55) was used to explore functional traits and predict metabolic capabilities based on 16S rRNA sequencing data and the KEGG pathway database. Mean relative abundance of prokaryotic taxa were calculated by dividing the number of sequences of that taxa by the total number of sequences in a sample. We used either t-tests for pairwise comparisons or ANOVA (using Type III Sums of Squares for Argentina samples) to examine differences in mean relative abundance of certain taxa between sites. Log transformations of the data were carried out when assumptions of normality or homogeneity of variances were not met.

Results

Soil analysis revealed few differences between urban, suburban, and rural sites in Buenos Aires, and only soil organic matter (%SOM) increased from rural to urban (Table 1). In Washington D.C., pH increased from rural to urban sites, while %SOM decreased along the gradient. Prokaryotic communities in each area were correlated to different soil parameters. In Buenos Aires, %SOM and %clay were significantly correlated to community composition ($R2 = 0.63$, $p = 0.001$ and $R2 = 0.41$, $p = 0.014$, respectively), and those variables were correlated to each other ($r = 0.78$). The Washington D.C. soil variables associated with prokaryotic communities were pH and soil organic matter ($R2 = 0.58$, $p = 0.001$ and $R2 = 0.48$, $p = 0.009$,

respectively), and these variables were also correlated to each other ($r = -0.75$). Illumina sequencing generated approximately 3.7 million high quality sequences with a median of 76,000 sequences per sample (min=11,811, max=910,766). Nested PerMANOVA revealed that microbial communities were significantly different along the urban gradient in Buenos Aires and in Washington D.C. (Buenos Aires: $F=2.6$, $p=0.005$; Washington D.C.: $F=2.3$ $p=0.001$), and differed between plant types at the $p<0.1$ significance level (Buenos Aires: $F=1.5$ $p=0.089$; Washington D.C.: $F=1.3$, $p=0.085$) (Figure 2). Pairwise comparisons of the different sites showed that in Buenos Aires only urban and rural sites differed in community composition ($p=0.006$), while in Washington D.C. the urban site communities differed from both the suburban and rural sites ($p=0.018$ and $p=0.015$ respectively). Shannon and Simpson diversity indexes did not differ between sites at either location (results not shown). The average OTU richness in urban, suburban and rural sites in Buenos Aires was 751, 220 and 503, respectively. In Washington D.C. 2382 taxa were identified for the urban, 2347 for the suburban and 2379 for the rural site.

The most abundant bacterial phyla were *Proteobacteria* (63.9%), *Firmicutes* (7.8%), and *Chloroflexi* (7.4%) in Buenos Aires; and *Proteobacteria* (16.5%), *Chloroflexi* (13%), and *Acidobacteria* (11.5%) in Washington D.C. The most abundant archaea phylum was *Euryarchaeota* in both areas (1.8% in Buenos Aires; 9.3% in Washington D.C.). In Buenos Aires, *Chloroflexi* was strongly correlated to urban communities, *Proteobacteria* to urban and suburban, and *Euryarchaeota* to communities in rural samples (Figure 2a). In Washington D.C., *Proteobacteria* and *Acidobacteria* were correlated to urban prokaryotic communities, while *Chloroflexi*

and *Euryarchaeota* were better correlated to rural communities (Figure 2b). In both areas the most abundant class within *Euryarchaeota* was *Methanomicrobia* (56.5% in Buenos Aires; 44.9% in Washington D.C.).

Both urban sites had more unique *Proteobacteria* ASVs than suburban or rural sites (460 in Buenos Aires and 1102 in Washington D.C.) (Figure 3). Most unique ASVs in those urban sites belonged to the Class *Deltaproteobacteria*, with 201 unique ASVs in Buenos Aires and 493 in Washington D.C. This was particularly surprising in Buenos Aires, where relative abundance of *Deltaproteobacteria* did not differ between sites ($F = 3.03$, $p = 0.075$), while *Gammaproteobacteria* significantly increased from rural to urban sites ($t = -2.26$, $p = 0.047$), and comprised 93.4% of all Proteobacteria ASVs in the urban site.

Within the *Gammaproteobacteria*, *Enterobacteriaceae* were most abundant in Buenos Aires and significantly higher in the urban site compared to the rural site ($t = 3.6$, $p = 0.005$). Bacteria putatively identified in the genus *Escherichia/Shigella* were exclusively found within the urban site. There was markedly higher relative abundance of coliform bacterial families in the suburban and urban sites compared with the rural site, which was mostly driven by *Enterobacter* and *Citrobacter* (Figure S2a in supplementary materials).

In Washington D.C., *Xanthomonadales* was the most abundant taxon within the *Gammaproteobacteria* and also showed a higher relative abundance in the urban than rural site ($t = 2.58$, $p = 0.028$). Members of the *Xanthomonadales* have the ability to break down PAHs, as well as other complex substrates (57,58). Following

this observation, other bacterial groups that have that capability were further explored.

The relative abundance of members of the *Xanthomonadales* order (*Arenimonas*, *Dyella*, *Luteimonas*, *Lysobacter*, *Rhodanobacter*, and *Xanthomonas*), as well as other bacterial genera known to break down PAHs (59), were significantly higher in the urban sites relative to the suburban and rural sites ($F = 4.4$, $p = 0.013$) (Figure S2b in supplementary materials).

KEGG metabolic pathways of the prokaryotic communities, and specifically those associated to xenobiotic biodegradation and nitrification, were investigated (Table S1 in supplementary materials). For the Buenos Aires samples, most ASVs could be mapped to KEGG organisms, allowing a relatively good functional prediction based on 16S rRNA data. The average FTU, which indicates the fraction of ASVs that could not be mapped to KEGG organisms, was 55% for rural, 36% for suburban, and 31% for urban sites. The obtained KEGG profiles suggest that functional traits were more similar between urban and suburban sites, while rural communities appear to be more functionally distinct (Figure S3a in supplementary materials). Metabolic pathways associated with expected urban stressors, such as pollution and excess nitrogen, were further analyzed. There was a significant enrichment in xenobiotic degradation pathways in the urban site relative to the rural site ($t = 2.29$, $p = 0.05$) and the same pattern was observed for nitrification pathways ($t = 2.7$, $p = 0.025$) (Figure 4a). In Washington DC, sites exhibited distinct metabolic capabilities (PerMANOVA $F = 4.17$, $p = 0.007$) (Figure S3b in supplementary materials), and an enrichment of xenobiotic degradation pathways in the urban

relative to the rural site ($t = 2.4$, $p = 0.049$) was observed. Nitrification capacity was lowest in the suburban site and similar in the rural and urban sites ($F = 4.52$, $p = 0.029$) (Figure 4b). However, the average FTUs for the prokaryotic communities in Washington D.C. was much higher (around 92.5% on average), so only a small portion of the total community was represented in the KEGG profiles. Therefore, prediction of functional traits from taxonomic data in Washington D.C. would be inaccurate.

Urban and rural sites in Buenos Aires and Washington D.C. contained ASVs that were classified as specialists for each habitat based on the CLAM test (Figure 5). In Buenos Aires, 5% were classified as urban specialists and 9% as rural specialists, while 82% of ASVs were too rare to classify. In Washington D.C., 21% were urban specialists, 16% were rural specialists, and 52% were too rare to classify.

In Buenos Aires only 12 taxa were indicators of the urban site while 182 were identified in the rural site. In Washington D.C. there were 153 indicator taxa of the urban and 130 of the rural site. Among these indicator taxa, those that had the highest fidelity and specificity values as defined by Dufrêne and Legendre (60) were further examined to identify useful indicator taxa and compared between Buenos Aires. This resulted in 6 urban and 46 rural indicator taxa in Buenos Aires and 26 urban and 37 rural indicators in Washington D.C. (Table S2 in supplementary materials).

Nitrite-oxidizing bacteria of the genus *Nitrospira* and *Nitrolancea* were indicators of the urban environments in Washington D.C. and Buenos Aires, respectively. Bacteria of the family *Nitrospiraceae* were also relevant indicators of the rural environments at both sites. In Buenos Aires, ammonia-oxidizing archaea within the *Thaumarchaeota*

were indicators of the urban location and had significantly higher relative abundance in the urban site ($F = 7.4$, $p = 0.006$) (Figure S4 in supplementary materials).

In addition to being highly correlated to rural sites in Buenos Aires and Maryland (Figure 2), *Methanobacteria* within the phylum *Euryarchaeota* were identified as rural indicators in both regions. Relative abundance of this phylum was significantly lower in urban than rural sites (Buenos Aires: $t = 2.47$, $p = 0.03$; Washington D.C.: $t = 2.53$, $p = 0.03$) and was mostly driven by a decrease in *Methanobacteriales* in both areas (Buenos Aires: $t= 2.35$, $p = 0.04$; Washington D.C.: $t = 5.9$, $p < 0.001$) (Figure 6).

Discussion

In support of hypothesis one, we observed differences in the prokaryotic community composition across the urban to rural gradient (Figure 2). A common factor that shapes prokaryotic community structure is soil pH (18,61). In our study, pH did not vary significantly across the urbanization gradient in Buenos Aires, but did increase with urbanization in Washington D.C. Additionally, pH was negatively correlated to %SOM. In contrast, SOM was higher in urban than rural sites in Buenos Aires. In both cases, %SOM was significantly correlated to community composition along the gradient. A study by Arroyo et al. (62) found similar results in natural and constructed wetlands, where SOM and not pH was the main soil variable related to microbial communities.

Species richness and diversity did not change significantly across the urban gradient in either location. Our results differ from those of other studies that found urbanization was negatively related to species richness and diversity in urban rivers

(63,64), but agrees with one study on headwater streams, where alpha diversity did not change due to urbanization (65). Even though we did observe enrichment of certain bacterial groups in the urban sites (Figure S2 in supplementary materials), richness and diversity indexes did not differ across the established gradient at either area.

Consistent with previous studies in freshwater systems, *Proteobacteria* were the most abundant taxa in all sites (66,67). This phylum was correlated with urban prokaryotic communities in Buenos Aires and Washington D.C. (Figure 2), and the Class *Gammaproteobacteria* was the main driver of that relationship in both cities. Associations between *Proteobacteria* and urbanization have been reported in other systems (63,68,69), and urban sites in our study had the greatest number of unique ASV's (Figure 3). The nature of this association has been related to nutrient enrichment (63,70), which would be common in areas receiving sewage inputs or stormwater runoff.

Within the Class *Gammaproteobacteria*, the order *Enterobacteriales* was the most prevalent across sites in Buenos Aires, while the order *Xanthomonadales* was the most abundant in Washington D.C. These orders were significantly more prevalent in urban rather than rural sites, suggesting that this could be related to specific environmental stressors at each of the urban sites. For example, some members of the *Enterobacteriales* are widely used as indicators of fecal contamination (71), and the presence of *E. coli* and other gastrointestinal associated bacteria in the urban site in Buenos Aires (Figure S2a in supplementary materials) was likely related to sewage flow in that area (34). In addition to being common

residents of the human intestinal tract, *Enterobacter* and *Citrobacter* are capable of degrading various types of hydrocarbons (72,73). The increase of these genera in suburban and urban sites (Figure S2a in supplementary materials) may be attributed to heavy boat traffic and industrialization. This observation was supported by the community's KEGG functional profiles that showed an enrichment in xenobiotic biodegradation pathways in the urban site (Figure 4a).

In the Washington D.C. urban site, contamination with PAHs of industrial origin is a major environmental concern (74,75). Although our study did not specifically test for PAHs, other studies observed an increase in these compounds in our specific urban location. Studies by Pinkney et al. (76,77) found that sediment PAH concentrations were considerably higher in the Anacostia than in the Choptank River (15–39mg/kg and 1.5 mg/kg dry weight respectively), where our urban and rural samples were collected. In our study, we found that there was a marked increase in the relative abundance of bacterial genera capable of degrading PAHs at the urban location relative to the rural and suburban sites (Figure S2b in supplementary materials). Our KEGG functional profiles were very limited at these sites, but the small subset of data obtained suggested that pathways associated to xenobiotic metabolism were more prevalent in the urban site, and these sites were functionally distinct from the rural sites (Figure 4b). We speculate that specific phyla were enriched in the urban sites in Buenos Aires and Washington D.C. as a result of major environmental pollutants affecting those areas, but functional gene analyses in future studies would help to corroborate this conclusion.

Archaea of the class *Methanobacteria* were relevant indicators in rural sites of Buenos Aires and Maryland, and were significantly more abundant in soils from rural compared to urban locations (Figure 6). Nutrient additions have been related to decreases in methanogenesis (78,79), and nitrate concentrations were found to be relevant in structuring archaeal communities in an urban river (63). Our urban sites did not have significantly higher levels of total nitrogen, but inputs of mineral nitrogen associated with more impervious surfaces and sewage inputs could explain the observed reduction in *Methanobacteria*. This can be confirmed for our Washington D.C. sites, where levels of ammonia were significantly higher in our urban compared to our rural site (22,24). Even though we lack mineral nitrogen data for the Buenos Aires sites, metabolic pathways associated with nitrification were more prevalent in the urban than rural sites (Figure 4a), and ammonia oxidizing archaea that are stimulated by high organic nitrogen loads (80) were identified as relevant indicators of the urban site in Buenos Aires. In addition, we observed a significant increase in ammonia-oxidizing archaea and bacteria in the urban sites relative to the suburban or rural sites in Buenos Aires and Washington D.C. (Figure S3 in supplementary materials). These results suggest that excess nitrogen inputs are likely related to changes in prokaryotic community composition in our urban sites.

The largest number of unique ASVs corresponded to the *Deltaproteobacteria* in Buenos Aires and Washington D.C. (Figure 3). *Deltaproteobacteria* are capable of anaerobic respiration of nitrogen and sulfur compounds and degradation of organic compounds in wetlands (67). This group includes multiple families of sulfate reducing bacteria, which can utilize a variety of C sources, including lipids and

PAHs, to carry out dissimilatory sulfate reduction (81–83). Some sulfate reducing bacteria can remove toxic materials from the water (84), and increases in these bacterial groups have been related to wastewater loadings in some freshwater wetlands (85). In our urban sites, factors like stormwater runoff, hydrocarbon pollution, and sewage inputs could therefore explain the unique community of *Deltaproteobacteria*.

Chloroflexi were significant components of the prokaryotic communities in both cities (Figure 2) and were predominantly represented by bacteria of the class *Anaerolineae*. A study on low tidal flats of an estuarine wetland found that the Class *Anaerolineae* was significantly correlated to total nitrogen and soil microbial respiration (86). Members of the Class *Anaerolineae* are common components of anaerobic digesters, which would suggest that these bacteria could have an important role as part of the microbial heterotrophic community, but their ecological roles are still unknown (87). Even though the functions of *Anaerolineae* remain elusive, they appear to be relevant in soil community structure and establish interactions with other groups of bacteria (88,89).

Acidobacteria belonging to the Class Subgroup 6 were the main group associated with urban communities in Maryland (Figure 2b). Bacteria belonging to this group have a complete set of genes to carry out assimilatory nitrate reduction and contain operons for detoxification of heavy metals (90). Members of Subgroup 6 are abundant in nutrient rich soils and multiple groups of *Acidobacteria* can tolerate pollutants, such as PCBs and petroleum compounds. This has led to speculation that *Acidobacteria* might play a role in the degradation of such compounds (91).

Our results suggest that urban sites in Washington D.C. and Buenos Aires are subject to similar stressors, particularly higher concentrations of pollutants, and the shifts in prokaryotic community composition and putative functions reflected these conditions. Sewage inputs are also a concern at both urban sites and the presence of fecal bacteria in the Buenos Aires site was expected, as there is no sewage infrastructure. Our urban site in Washington D.C. also experiences frequent contamination from sewage (92), but our sampling time likely corresponded to a time when sewage concentrations were low. The closest combined sewer overflow outfall is located downstream of our sampling location and there were no heavy rainfall around our sampling date that would have resulted in overflow and subsequent upstream transport due to tides.

Our second and third hypotheses were not fully supported. Concerning hypothesis 2, which stated that prokaryotic communities would differ between plant species, we found that plant identity had a relatively small influence on community composition at each site (Figure 2). Therefore, factors associated with site differences had a greater role in structuring prokaryotic communities than plant properties. It is likely that other factors related to urbanization, such as various pollutants, override plant differences. Other studies in freshwater wetlands have found similar results, and concluded that either edaphic factors (93), site-specific factors (24), or certain landscape factors (94) have a greater effect in structuring prokaryotic communities.

Concerning our third hypothesis, we did not find a specific indicator taxa that was common for both urban locations. This is in part due to the large difference between prokaryotic communities in the two regions. When we compared the two

regions, they were more different from each other than across the gradients (data not shown). We did, however, observe that methanogens were relevant indicators of both rural locations, and identified other functionally similar indicators at the different sites. Nitrite-oxidizing bacteria were indicators of urban and rural areas in Washington D.C., and those belonging to the *Nitrospira* have been previously identified as common indicators of freshwater sediments (16). Some organisms within the genus *Nitrospira* can carry out complete nitrification (95), which would make them a relevant functional group in urban as well as in rural sites, where agriculture is the prevalent land use. In Buenos Aires, *Chloroflexi* bacteria belonging to the genus *Nitrolancea* were indicators of the urban site. These nitrite-oxidizing bacteria have only recently been described, and are considered to be better competitors at higher levels of nitrite than *Nitrospira* (96). The identification of similar functions among the different indicator taxa at each location suggests that studies of functional traits rather than specific taxa would be a better approach to characterize prokaryotic communities in these urban wetlands.

The results of this study should be interpreted in light of the following limitations: we were unable to collect contaminant data for the different sites which could have helped support our findings, and functional data had to be inferred from community composition. In addition, sample size was low, and even though it was enough to detect site differences, a larger sample size might have allowed a better resolution of community differences between plant types. Studies of the proposed patterns and processes in other urban wetlands would be of interest.

Conclusions

Prokaryotic community composition shifted along the urban gradient in TFWs in Buenos Aires and Washington D.C. Given the important roles of bacteria and archaea in biogeochemical cycles, changes in community composition in these systems could have an effect on ecosystem function. In our study, differences in prokaryotic groups between sites likely reflected variation in environmental stressors, such as nutrient and hydrocarbon pollution. A loss of methanogens and an increase in nitrifying bacteria across the rural to urban gradient at both locations could have implications for nutrient and carbon processing in these systems, and might serve as an indicator of an altered state. Future studies of prokaryotic communities in other cities that experience either similar or different stressors would help confirm our observations. Our results also suggest that prokaryotic communities in these urban wetlands could be carrying out different functions than those in rural sites, particularly concerning pollutant transformation or removal.

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Table 1: Soil characteristics for the urban, suburban and rural sites within each area. Results are arithmetic means ± standard errors, except for texture results that are shown as percent values per site.

	Buenos Aires			Washington D.C.		
Parameter	Rural	Suburban	Urban	Rural	Suburban	Urban
pH	6.4±0.2	5.4±0.08	5.8±0.1	4±0.3	4.7±0.1	5.7±0.1
SOM (%)	11.6±0.5	6.6±0.02	24.7±2.2	35.6±0.5	11.8±1.2	6.5±0.7
C (%)	2.7±0.2	1.7±0.1	3.54±0.7	1.9±0.2	5.6±0.6	3±0.4
N (%)	0.2±0.02	0.2±0.01	0.32±0.1	0.12±0.1	0.4±0.1	0.2±0.03
C/N	13	11.67	11.77	15.63	14.1	15.7
Sand (%)	0.31	0.38	0.33	0.26	0.35	0.17
Silt (%)	0.43	0.4	0.42	0.34	0.53	0.58
Clay (%)	0.25	0.22	0.25	0.4	0.12	0.26

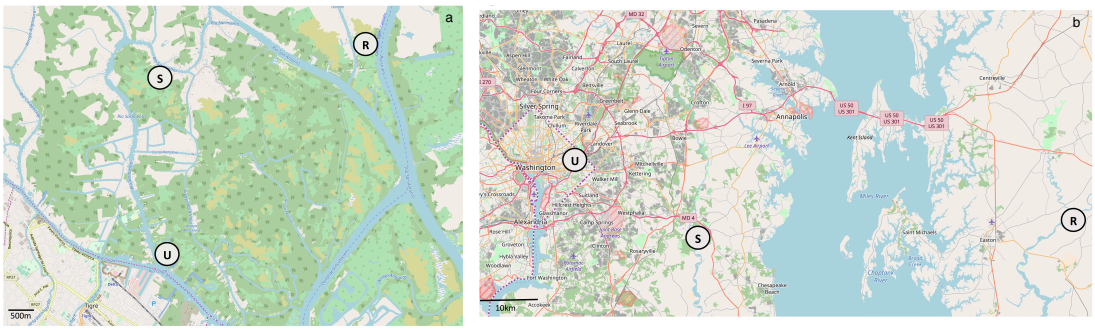

Figure 1: Location of sampling sites in Buenos Aires (a) and Washington D.C. (b). U=urban, S=suburban and R=Rural. Base map: OpenStreetMap (https://www.openstreetmap.org)

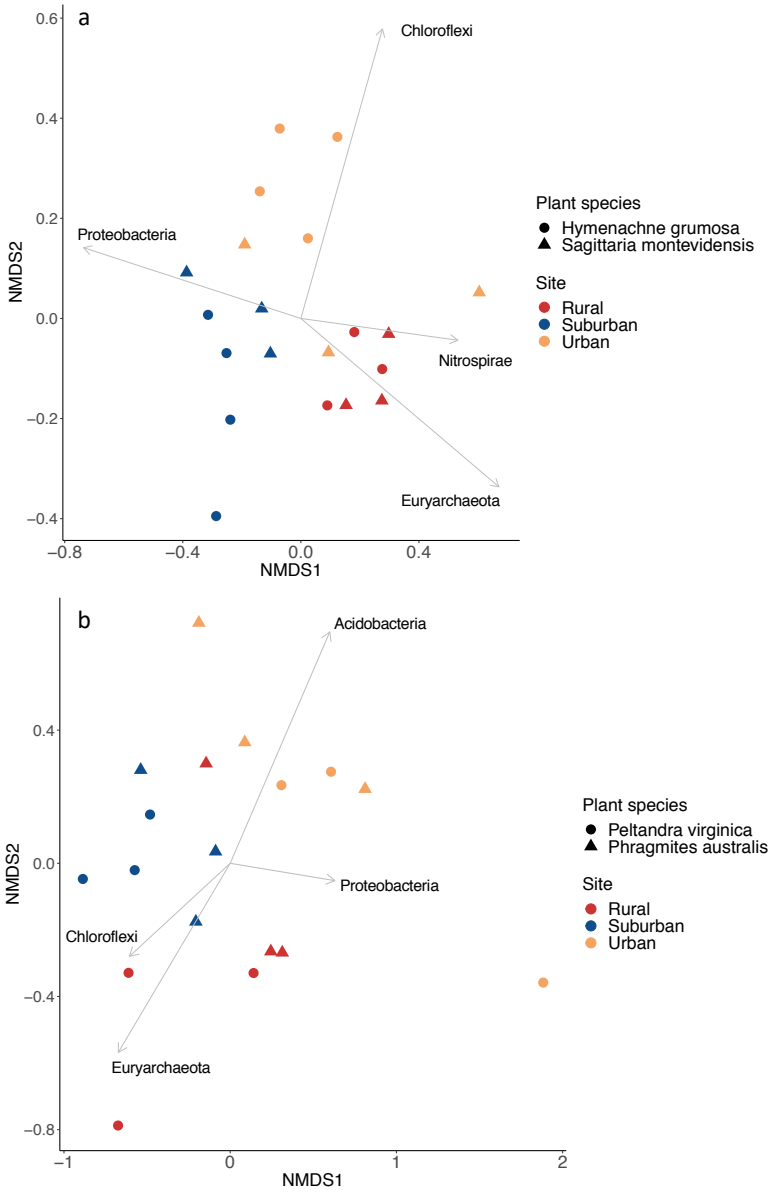

Figure 2: Non-metric multidimensional scaling ordination (NMDS) constructed using a Bray-Curtis dissimilarity matrix. Each point represents a sample with colors corresponding to sites, and shapes to plant species. The final stress values were 0.193 and 0.124 for the Buenos Aires (a) and Washington D.C. (b) ordinations respectively. Vectors show the correlation of the most abundant phyla to community composition at the different sites.

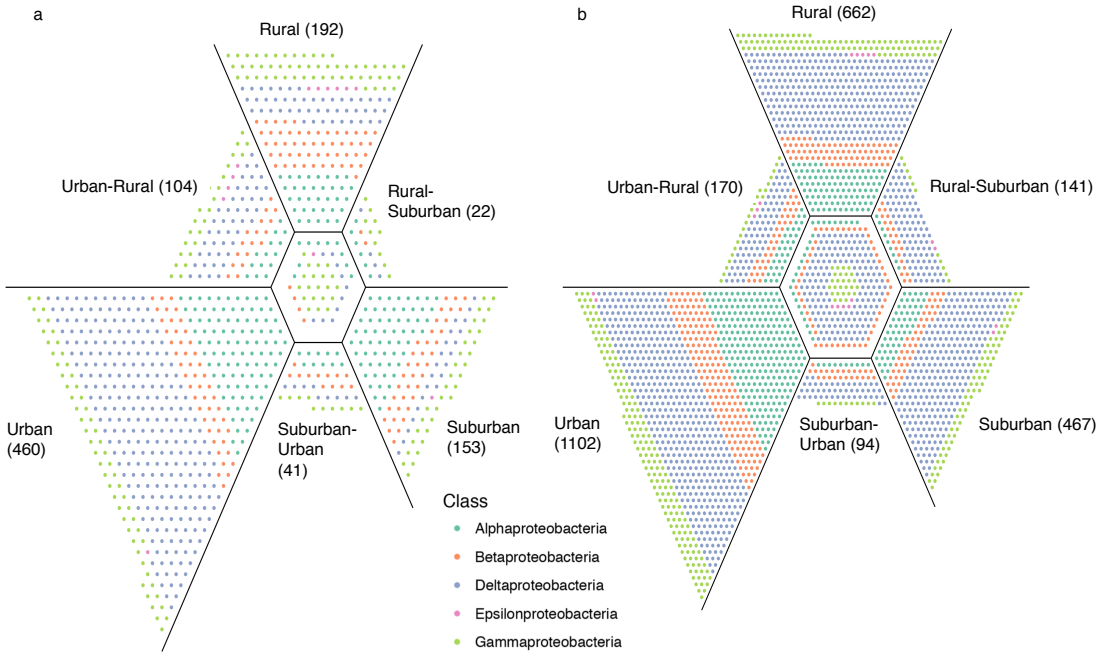

Figure 3: Venn-like representation of overlap between OTUs at urban, suburban and rural sites for the phylum Proteobacteria. Each point represents an individual OTU with colors corresponding to different classes of Proteobacteria. Numbers in parenthesis indicate the total number of unique OTUs at each site. The center hexagon contains OTUs shared by all sites, while the trapezoids contain OTUs either exclusive to each site or those shared between two of them. In Buenos Aires (a) there were 45 OTUs shared by all sites and in Washington D.C. (b) 280. The plots were created using the unionplot R package (97)

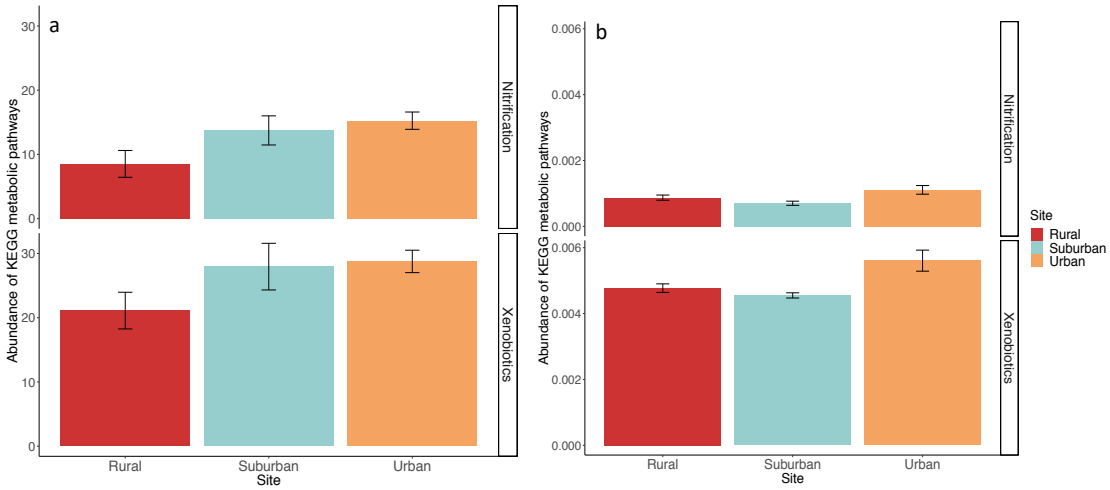

Figure 4: Bar plot showing mean abundance and standard error of metabolic pathways of nitrification and xenobiotic biodegradation across sites in Buenos Aires (a) and Washington D.C. (b)

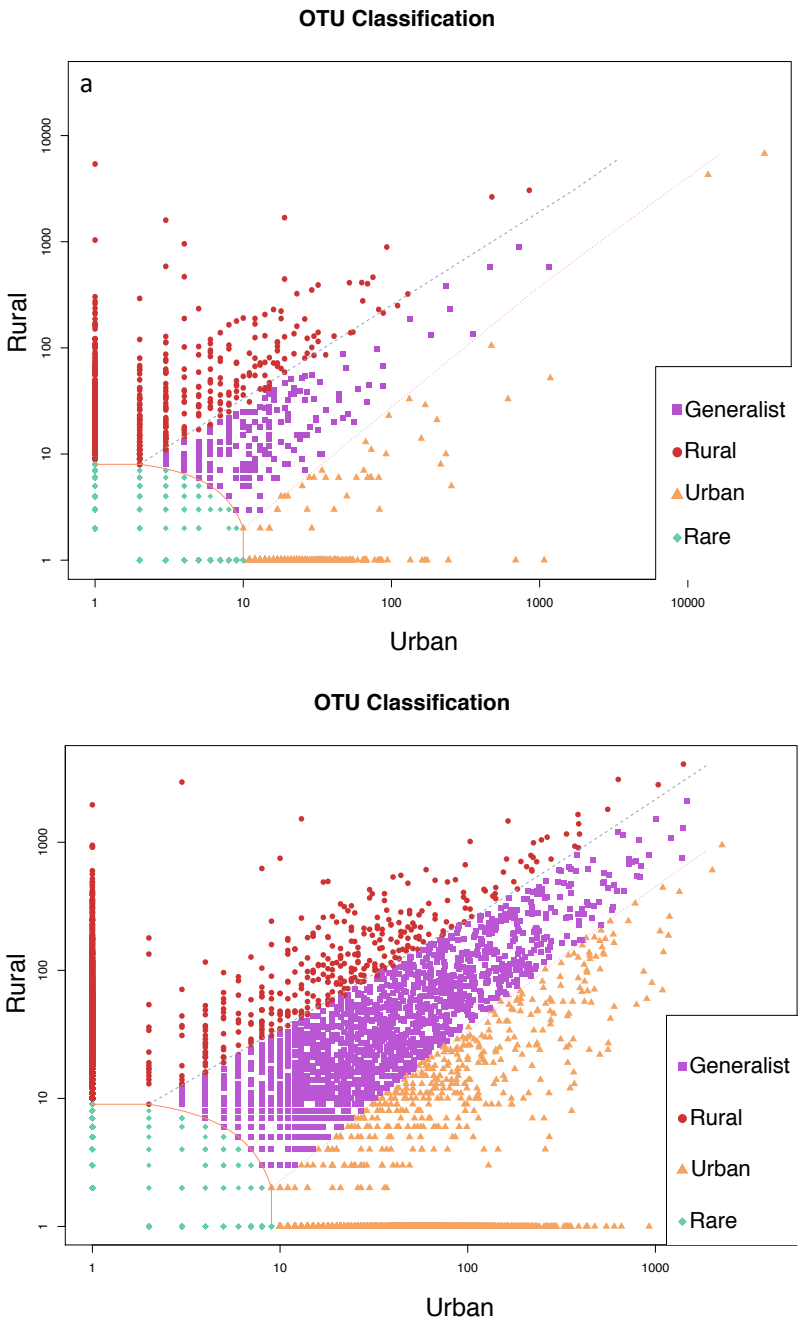

Figure 5: CLAM test results showing the classification of OTUs as habitat specialists or generalists based on relative abundance of species at each site for Buenos Aires (a) and Washington D.C. (b) OTUs were divided into four categories: urban specialists (Urban), rural specialists (Rural), generalists with no habitat preference, and too rare to classify (Rare). Note: some points are stacked so specific values for each category can be found in the text

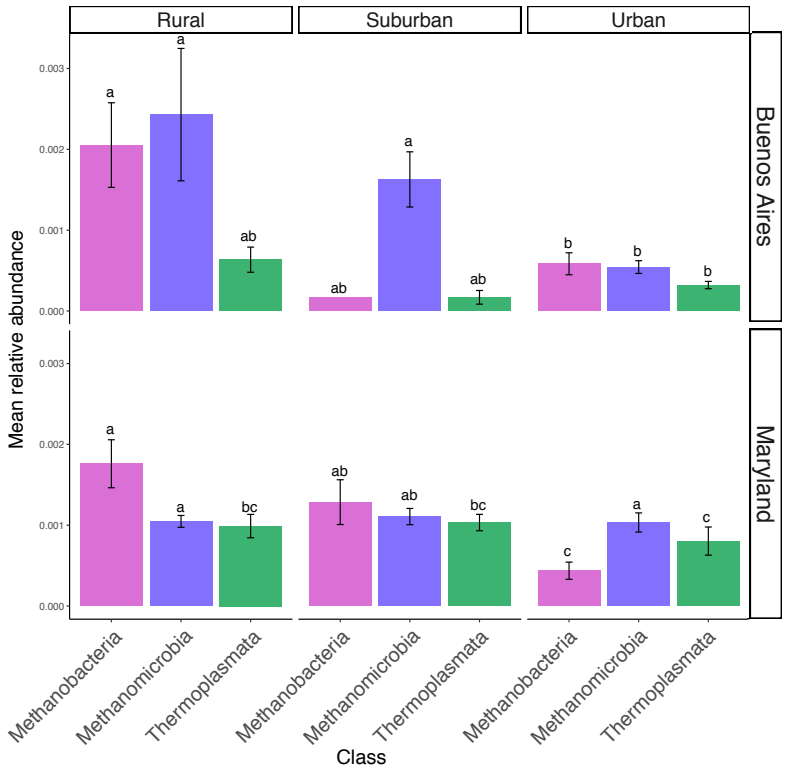

Figure 6: Mean relative abundance of Euryarchaeota classes for Buenos Aires and Washington D.C. in urban, suburban and rural sites. Bars represent standard errors and different letters indicate significant differences between means (Tukey $p<0.05$). Unclassified archaea are not included and only comprised 0.2% of total Euryarchaeota.

Table S1: Relative abundance of metabolic pathways associated to xenobiotic degradation (a) and nitrification (b).

a)

Sample	Area	Site	K00626	K00365	K00980	K00983	K00362	K00627	K00364	K00622	K00625	K00624	K00643	K00791	K00930	K00363	K00621
HI1	Buenos Aires	Suburban	4.9519	0.0138	0.0614	0.2021	9.0389	6.1517	2.7760	0.0008	0.0397	0.0076	0.1113	2.6460	2.2207	0.7844	0.0014
HI2	Buenos Aires	Suburban	7.9996	0.1140	0.1096	0.3570	6.1794	6.6772	1.3424	0.0074	0.6249	0.0457	0.4781	2.4544	2.0057	0.5912	0
HI3	Buenos Aires	Suburban	1.7479	0.0291	0.0258	0.0652	1.6829	1.7023	0.4476	0.0076	0.0890	0.0131	0.0499	0.6535	0.5764	0.1680	0.0006
HI4	Buenos Aires	Suburban	6.6535	0.0267	0.0757	0.2360	9.2943	7.1373	2.8146	0.0008	0.2226	0.0103	0.2346	2.9431	2.5171	0.8617	0
HR1	Buenos Aires	Rural	4.8293	0.0433	0.0581	0.3375	3.4757	4.0959	0.7052	0.0038	0.2704	0.0250	0.1335	1.4956	1.3692	0.3337	0
HR2	Buenos Aires	Rural	7.3143	0.0335	0.1772	0.3002	7.6717	6.4129	2.1499	0.0017	0.7158	0.0035	0.1022	2.7646	2.4774	0.7232	0
HR4	Buenos Aires	Rural	4.1083	0.0396	0.0591	0.2539	3.3312	3.6669	0.7301	0.0028	0.3192	0.0259	0.1116	1.6162	1.2562	0.3130	0
HU1	Buenos Aires	Urban	4.8010	0.0167	0.0586	0.1957	6.8711	5.9036	2.1329	0.0027	0.1564	0.0058	0.1135	2.3051	2.0152	0.6413	0.0010
HU2	Buenos Aires	Urban	4.6268	0.0051	0.0726	0.2162	7.3467	5.5135	2.3878	0.0009	0.2501	0.0022	0.0889	2.4579	2.0471	0.6801	0.0024
HU3	Buenos Aires	Urban	4.8527	0.0164	0.0533	0.1749	7.6124	5.7465	2.4284	0.0023	0.0966	0.0055	0.1049	2.4024	2.0381	0.7145	0.0005
HU4	Buenos Aires	Urban	5.2435	0.0314	0.0588	0.1963	8.0392	5.9456	2.5406	0.0034	0.1188	0.0128	0.1326	2.5176	2.1235	0.7542	0
SI1	Buenos Aires	Suburban	9.1009	0.1771	0.1429	0.2938	8.5256	7.4719	2.3162	0.0040	0.9017	0.0302	0.2772	3.2563	2.8716	0.8159	0

Table S1a: continued

SI2	Buenos Aires	Suburban	5.7071	0.0218	0.0862	0.2123	10.349	6.9815	3.2610	0.0012	0.1656	0.0016	0.1107	3.0449	2.5534	0.9190	0.001
SI3	Buenos Aires	Suburban	5.2112	0.0304	0.0735	0.2606	7.7233	5.9367	2.5797	0.0025	0.1864	0.0076	0.0644	2.6499	2.3003	0.7770	0.0026
SR1	Buenos Aires	Rural	3.4356	0.0386	0.0413	0.2109	2.6621	3.0456	0.5708	0.0059	0.2199	0.0377	0.0951	1.1397	0.9638	0.2543	0
SR2	Buenos Aires	Rural	4.7841	0.0135	0.0750	0.2469	7.5595	5.7387	2.4556	0.0013	0.1764	0.0201	0.1007	2.5689	2.1781	0.7254	0.0012
SR3	Buenos Aires	Rural	5.1805	0.0081	0.2653	0.3794	2.7522	3.9314	1.6338	0.0021	2.2837	0.0073	0.0523	3.2784	3.4048	0.2510	0.0003
SU1	Buenos Aires	Urban	6.4587	0.0155	0.2304	0.2619	10.573	7.9049	3.7607	0.0014	0.6740	0.0110	0.1171	3.6629	2.7708	0.9003	0
SU3	Buenos Aires	Urban	5.9322	0.0189	0.0630	0.2282	9.7937	7.0128	3.2151	0.0030	0.0598	0.0102	0.1029	3.0529	2.5777	0.9496	0.0011
SU4	Buenos Aires	Urban	6.3170	0.0780	0.2997	0.2319	5.1708	4.9195	1.8844	0.0006	1.6792	0.0051	0.0556	2.7141	2.2727	0.4882	8E-05
S11	Washington	Suburban	0.0019	6E-06	1E-05	0.0001	0.0004	0.0008	2E-05	1E-06	0.0001	3E-06	6E-05	0.0005	0.0007	4E-05	4E-07
S12	Washington	Rural	0.0016	1E-06	1E-05	0.0002	0.0003	0.0009	1E-05	3E-08	0.0001	8E-09	2E-05	0.0005	0.0007	3E-05	8E-07
S23	Washington	Suburban	0.0018	4E-06	5E-06	0.0003	0.0004	0.0009	3E-05	3E-07	0.0001	6E-07	3E-05	0.0005	0.0005	3E-05	0
S24	Washington	Rural	0.0018	1E-05	9E-06	0.0001	0.0005	0.0012	3E-05	2E-06	0.0002	3E-06	0.0001	0.0005	0.0006	6E-05	2E-06
S35	Washington	Suburban	0.0015	9E-06	3E-05	0.0002	0.0003	0.0009	4E-05	1E-06	0.0002	2E-06	3E-05	0.0005	0.0007	3E-05	2E-06
S36	Washington	Rural	0.0018	2E-06	2E-05	0.0002	0.0003	0.0008	3E-05	7E-08	0.0001	1E-07	2E-05	0.0005	0.0007	3E-05	1E-06
S47	Washington	Suburban	0.0013	7E-06	2E-05	0.0002	0.0003	0.0010	3E-05	2E-07	0.0002	1E-06	3E-05	0.0006	0.0006	2E-05	0

Table S1a: continued

S48	Washington	Rural	0.0018	1E-05	1E-05	0.0001	0.0005	0.0012	3E-05	2E-06	0.0001	5E-06	0.0001	0.0005	0.0006	6E-05	1E-06
S59	Washington	Urban	0.0021	3E-05	6E-06	0.0001	0.0006	0.0015	3E-05	2E-06	0.0002	2E-05	0.0002	0.0006	0.0005	7E-05	2E-06
S60	Washington	Suburban	0.0017	5E-06	6E-06	0.0002	0.0005	0.0010	2E-05	6E-07	0.0001	2E-06	5E-05	0.0006	0.0006	5E-05	7E-07
S71	Washington	Urban	0.0018	6E-06	4E-06	0.0001	0.0003	0.0010	2E-05	3E-08	0.0001	3E-06	4E-05	0.0005	0.0006	4E-05	2E-06
S72	Washington	Suburban	0.0015	4E-06	6E-06	0.0002	0.0003	0.0012	2E-05	6E-07	0.0001	5E-07	5E-05	0.0006	0.0006	3E-05	1E-06
S78	Washington	Rural	0.0017	1E-05	7E-06	0.0002	0.0005	0.0012	3E-05	7E-07	0.0001	5E-06	9E-05	0.0005	0.0006	6E-05	2E-06
S79	Washington	Urban	0.0026	5E-05	2E-05	4E-05	0.0007	0.0020	4E-05	2E-05	0.0002	2E-05	0.0003	0.0005	0.0004	8E-05	3E-06
S83	Washington	Urban	0.0020	2E-05	5E-06	0.0001	0.0007	0.0014	3E-05	2E-06	0.0002	8E-06	0.0002	0.0006	0.0005	8E-05	1E-06
S84	Washington	Urban	0.002	2E-06	3E-06	0.0002	0.0004	0.0012	7E-05	1E-08	0.0002	2E-07	4E-05	0.0007	0.0005	5E-05	6E-07
S95	Washington	Rural	0.0016	3E-06	5E-06	0.0002	0.0004	0.0010	2E-05	2E-07	0.0001	9E-07	5E-05	0.0006	0.0006	3E-05	7E-07
S96	Washington	Urban	0.0016	7E-06	4E-06	0.0002	0.0005	0.0013	2E-05	3E-07	0.0001	4E-06	4E-05	0.0007	0.0006	6E-05	8E-07

Table S1: continued

b)

Sample	Area	Site	K10944	K10945	K10946	K10535	K00370	K00371
HI1	Buenos Aires	Suburban	0.0007	0.0007	0.0002	0.0015	11.5115	4.6876
HI2	Buenos Aires	Suburban	0.0123	0.0181	0.0189	0.0257	5.3729	2.1869
HI3	Buenos Aires	Suburban	0.0013	0.0019	0.0019	0.00345	2.1507	0.8780
HI4	Buenos Aires	Suburban	0.0011	0.0013	0.0014	0.00461	12.3990	5.0466
HR1	Buenos Aires	Rural	0.0119	0.0128	0.0120	0.0481	3.0017	1.2459
HR2	Buenos Aires	Rural	0.0051	0.0046	0.0045	0.0327	9.1910	3.7513
HR4	Buenos Aires	Rural	0.0047	0.0063	0.0059	0.0144	2.8606	1.1810
HU1	Buenos Aires	Urban	0.0035	0.0044	0.0040	0.0079	9.4267	3.8293
HU2	Buenos Aires	Urban	0.0019	0.0021	0.0005	0.0044	10.1779	4.1331
HU3	Buenos Aires	Urban	0.0042	0.0063	0.0064	0.0098	10.6640	4.3477
HU4	Buenos Aires	Urban	0.0037	0.0055	0.0035	0.0138	11.2445	4.5699
SI1	Buenos Aires	Suburban	0.0022	0.0033	0.0035	0.0068	11.2144	4.5408
SI2	Buenos Aires	Suburban	0.0002	0.0002	0.0002	0.0007	13.7507	5.5784
SI3	Buenos Aires	Suburban	0.0032	0.0025	0	0.0119	11.8775	4.8224
SR1	Buenos Aires	Rural	0.0107	0.0135	0.0124	0.0329	2.3554	0.9712
SR2	Buenos Aires	Rural	0.0008	0.0011	0.0010	0.0019	10.3566	4.2192
SR3	Buenos Aires	Rural	0.0121	0.0169	0.0165	0.0319	8.4174	3.2734
SU1	Buenos Aires	Urban	0.0017	0.0020	0.0021	0.0064	13.2144	5.3899
SU3	Buenos Aires	Urban	0.0009	0.0013	0.0014	0.0018	14.4210	5.8645
SU4	Buenos Aires	Urban	0.0027	0.0037	0.0031	0.0085	6.6694	2.7008
S11	Washington	Suburban	1.5E-05	1.9E-05	1.4E-05	3.8E-05	0.0004	0.0002
S12	Washington	Rural	2.4E-05	3.3E-05	3.1E-05	6.3E-05	0.0004	0.0002

Table S1b: continued

S23	Washington	Suburban	1.9E-05	2.4E-05	1.7E-05	4.1E-05	0.0004	0.0002
S24	Washington	Rural	3.4E-05	4.9E-05	4.9E-05	7.9E-05	0.0005	0.0002
S35	Washington	Suburban	6.5E-06	7.2E-06	2.4E-06	1.4E-05	0.0004	0.0002
S36	Washington	Rural	1.6E-05	2E-05	1.4E-05	3.4E-05	0.0005	0.0002
S47	Washington	Suburban	9E-06	1.2E-05	8.3E-06	2.1E-05	0.0004	0.0002
S48	Washington	Rural	3E-05	4.2E-05	3.9E-05	6.6E-05	0.0006	0.0002
S59	Washington	Urban	2.9E-05	4E-05	3.5E-05	6.6E-05	0.0009	0.0004
S60	Washington	Suburban	6.1E-05	8.6E-05	8.2E-05	0.00014	0.0005	0.0002
S71	Washington	Urban	2.4E-05	3.3E-05	3.2E-05	5.2E-05	0.0004	0.0002
S72	Washington	Suburban	1.5E-05	2E-05	1.5E-05	3.1E-05	0.0004	0.0001
S78	Washington	Rural	4.9E-05	7E-05	6.5E-05	0.00011	0.0006	0.0002
S79	Washington	Urban	5.2E-06	7.7E-06	8E-06	1.1E-05	0.0007	0.0003
S83	Washington	Urban	3.9E-05	5.4E-05	4.8E-05	8.7E-05	0.0009	0.0003
S84	Washington	Urban	3.2E-05	4.3E-05	3.7E-05	6.4E-05	0.0005	0.0002
S95	Washington	Rural	2.6E-05	3.5E-05	3.2E-05	5.6E-05	0.0004	0.0002
S96	Washington	Urban	3.9E-05	5.3E-05	4.8E-05	8.8E-05	0.0007	0.0003

Table S2: Indicator taxa identified for the Urban and Rural sites in Buenos Aires and Washington D.C.

Buenos Aires Urban				
Phylum	Class	Order	Family	Genus
Verrucomicrobia	OPB35_soil_group	NA	NA	NA
Thaumarchaeota	Soil_Crenarchaeotic_Group(SCG)	NA	NA	NA
Acidobacteria	Subgroup_6	NA	NA	NA
Chloroflexi	Ktedonobacteria	Ktedonobacterales	NA	NA
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acidicaldus
Chloroflexi	Thermomicrobia	Sphaerobacterales	Sphaerobacteraceae	Nitrolancea
Buenos Aires Rural				
Phylum	Class	Order	Family	Genus
Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosaetaceae	Methanosaeta
Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium
Bacteroidetes	Bacteroidetes_vadinHA17	NA	NA	NA
Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium
Bacteroidetes	Bacteroidetes_vadinHA17	NA	NA	NA
Firmicutes	Clostridia	Clostridiales	Clostridiaceae_1	Clostridium_sensu_stricto_1
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	Leptolinea
Thaumarchaeota	Group_C3	NA	NA	NA
Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dechloromonas
Omnitrophica	Omnitrophica_Incertae_Sedis	Unknown_Order	Unknown_Family	Candidatus_Omnitrophus
Bacteroidetes	Bacteroidetes_vadinHA17	NA	NA	NA
Proteobacteria	Deltaproteobacteria	Deltaproteobacteria_Incertae_Sedis	Syntrophorhabdaceae	Syntrophorhabdus
Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	Methylocystis
Acidobacteria	Subgroup_6	NA	NA	NA

Table S2: continued

Proteobacteria	Gammaproteobacteria	Methylococcales	Crenotrichaceae	Crenothrix
Chloroflexi	Caldilineae	Caldilineales	Caldilineaceae	NA
Bacteroidetes	SB-5	NA	NA	NA
Chloroflexi	KD4-96	NA	NA	NA
Bathyarchaeota	NA	NA	NA	NA
Acidobacteria	Subgroup_17	NA	NA	NA
Actinobacteria	Actinobacteria	PeM15	NA	NA
Bacteroidetes	Bacteroidetes_vadinHA17	NA	NA	NA
Actinobacteria	Actinobacteria	Corynebacteriales	Mycobacteriaceae	Mycobacterium
Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	NA
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	Anaerolinea
Bacteroidetes	Bacteroidia	Bacteroidia_Incertae_Sedis	Draconibacteriaceae	NA
Nitrospinae	Belgica2005-10-ZG-3	NA	NA	NA
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Lentimicrobiaceae	NA
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	NA
Bacteroidetes	Bacteroidetes_vadinHA17	NA	NA	NA
Ignavibacteriae	Ignavibacteria	Ignavibacteriales	PHOS-HE36	NA
Acidobacteria	Subgroup_6	Unknown_Order	Unknown_Family	Vicinamibacter
Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobacteraceae	Desulfatirhabdium
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	NA
Saccharibacteria	NA	NA	NA	NA
Chloroflexi	Caldilineae	Caldilineales	Caldilineaceae	NA
Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	Desulforhabdus
Proteobacteria	Deltaproteobacteria	Desulfuromonadales	Geobacteraceae	Geobacter
Acidobacteria	Subgroup_6	NA	NA	NA
Verrucomicrobia	Spartobacteria	Chthoniobacterales	Chthoniobacteraceae	Chthoniobacter
Nitrospirae	Nitrospira	Nitrospirales	Sh765B-TzT-35	NA
Ignavibacteriae	Ignavibacteria	Ignavibacteriales	BSV26	NA

Table S2: continued

Bacteroidetes	Bacteroidetes_vadinHA17	NA	NA	NA
Spirochaetae	Spirochaetes	Spirochaetales	Spirochaetaceae	Spirochaeta_2
Proteobacteria	Gammaproteobacteria	Chromatiales	Chromatiaceae	NA
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	Anaerolinea
Washington D.C. Urban				
Phylum	Class	Order	Family	Genus
Nitrospirae	Nitrospira	Nitrospirales	0319-6A21	NA
Proteobacteria	Deltaproteobacteria	43F-1404R	NA	NA
Nitrospirae	Nitrospira	Nitrospirales	0319-6A21	NA
Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium
Proteobacteria	Betaproteobacteria	Nitrosomonadales	Nitrosomonadaceae	NA
Proteobacteria	Betaproteobacteria	Nitrosomonadales	Nitrosomonadaceae	NA
Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	NA
Verrucomicrobia	Spartobacteria	Chthoniobacterales	DA101_soil_group	NA
Proteobacteria	Betaproteobacteria	Nitrosomonadales	Nitrosomonadaceae	NA
Acidobacteria	Blastocatellia	Blastocatellales	Blastocatellaceae_(Subgroup_4)	NA
Proteobacteria	Deltaproteobacteria	Desulfurellales	Desulfurellaceae	H16
Acidobacteria	NA	NA	NA	NA
Proteobacteria	Deltaproteobacteria	Desulfurellales	Desulfurellaceae	H16
Chloroflexi	KD4-96	NA	NA	NA
Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium
Acidobacteria	Subgroup_6	NA	NA	NA
Nitrospirae	Nitrospira	Nitrospirales	0319-6A21	NA
Acidobacteria	Holophagae	Subgroup_7	NA	NA
Acidobacteria	Holophagae	Subgroup_7	NA	NA
Acidobacteria	Subgroup_11	NA	NA	NA
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	Nitrospira
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	Nitrospira
Latescibacteria	NA	NA	NA	NA

Table S2: continued

Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	NA
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	NA
Proteobacteria	Gammaproteobacteria	Acidiferrobacterales	Acidiferrobacteraceae	Sulfurifustis
Washington D.C. Rural				
Phylum	Class	Order	Family	Genus
Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium
Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosaetaceae	Methanosaeta
Proteobacteria	Deltaproteobacteria	Desulfarculales	Desulfarculaceae	Desulfatiglans
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	NA
Acidobacteria	Subgroup_6	NA	NA	NA
Ignavibacteriae	Ignavibacteria	Ignavibacteriales	Ignavibacteriaceae	Ignavibacterium
Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	Desulfobacca
Proteobacteria	Betaproteobacteria	Nitrosomonadales	Gallionellaceae	Gallionella
Bathyarchaeota	NA	NA	NA	NA
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	NA
Verrucomicrobia	S-BQ2-57_soil_group	NA	NA	NA
Proteobacteria	Deltaproteobacteria	43F-1404R	NA	NA
Euryarchaeota	Thermoplasmata	Thermoplasmatales	Marine_Benthic_Group_D_and_DHVEG-1	NA
Bathyarchaeota	NA	NA	NA	NA
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	NA
Euryarchaeota	Thermoplasmata	Thermoplasmatales	Marine_Benthic_Group_D_and_DHVEG-1	NA
Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	NA
Acidobacteria	Subgroup_18	NA	NA	NA
Euryarchaeota	Thermoplasmata	Thermoplasmatales	Marine_Benthic_Group_D_and_DHVEG-1	NA
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	NA
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	NA

Table S2: continued

Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	Desulfobacca
Verrucomicrobia	Spartobacteria	Chthoniobacterales	DA101_soil_group	NA
Hadesarchaea	NA	NA	NA	NA
Actinobacteria	Actinobacteria	Frankiales	Sporichthyaceae	NA
Verrucomicrobia	S-BQ2-57_soil_group	NA	NA	NA
Nitrospinae	MD2898-B26	NA	NA	NA
KSB3_(Modulibacteria)	NA	NA	NA	NA
Aminicenantes	NA	NA	NA	NA
Proteobacteria	Gammaproteobacteria	Acidiferrobacterales	Acidiferrobacteraceae	Sulfurifustis
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	NA
Candidate_division_YNPFFA	NA	NA	NA	NA
Latescibacteria	NA	NA	NA	NA
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	NA
NA	NA	NA	NA	NA
Acidobacteria	Subgroup_13	NA	NA	NA
Chloroflexi	NA	NA	NA	NA

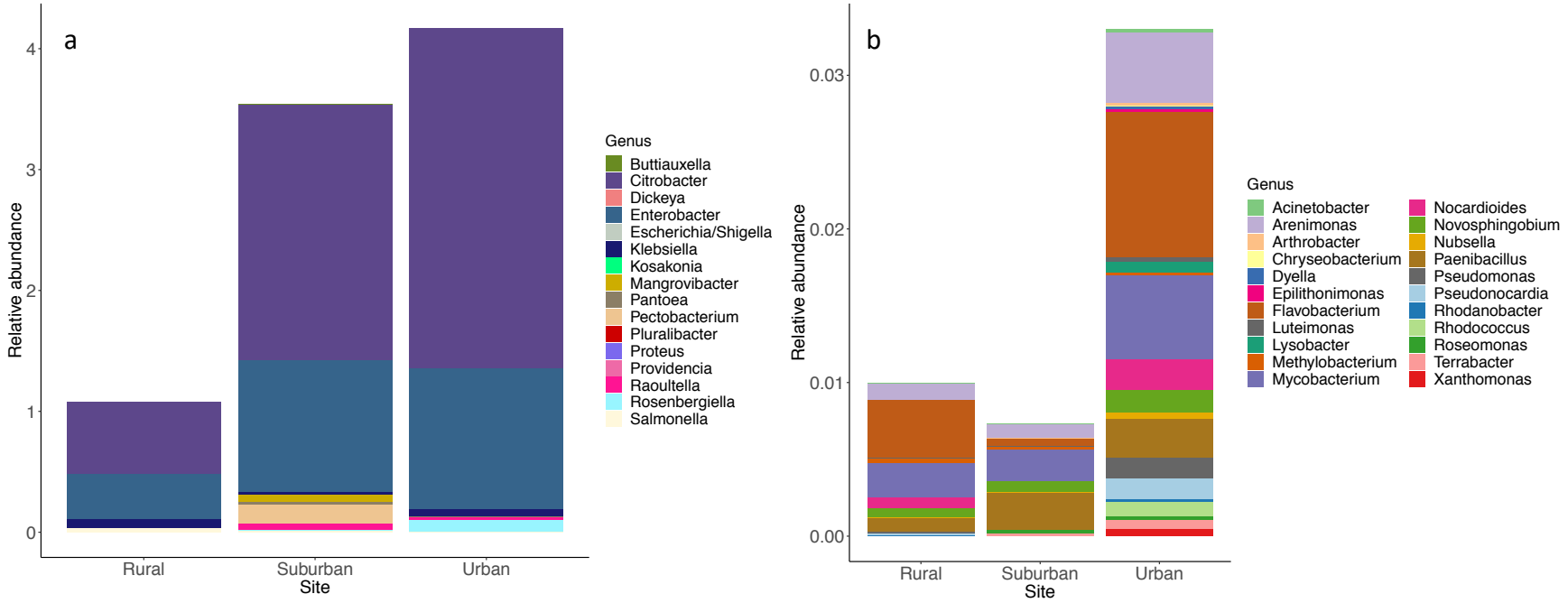

Figure S1: Relative abundance of the family Enterobacteriaceae in Buenos Aires (a) and bacterial genus capable of PAH degradation in Washington D.C. (b)

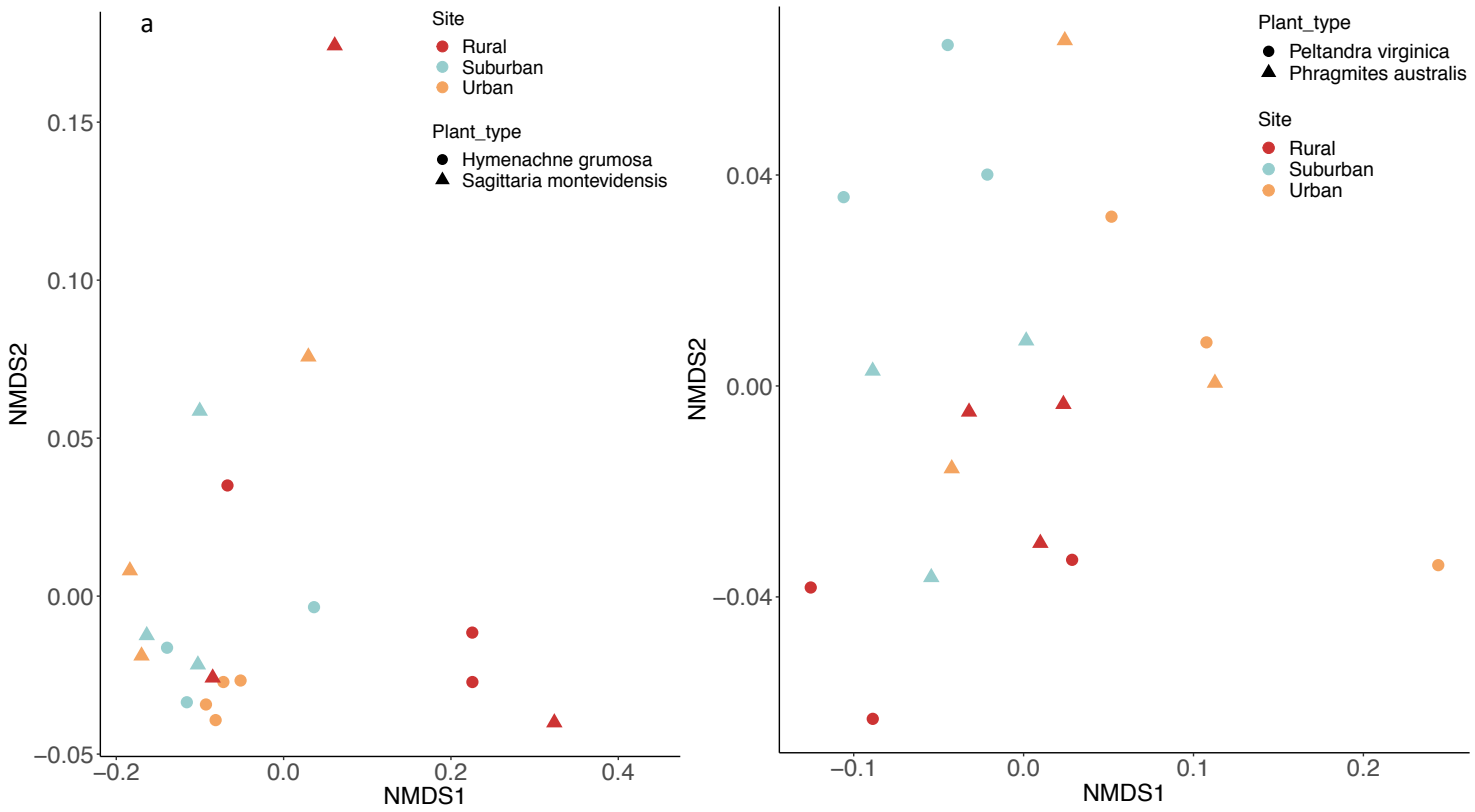

Figure S2: Non-metric multidimensional scaling ordination (NMDS) of KEGG metabolic profiles of the microbial communities. Each point represents a sample with colors corresponding to sites, and shapes to plant species. Buenos Aires (a), Washington D.C (b)

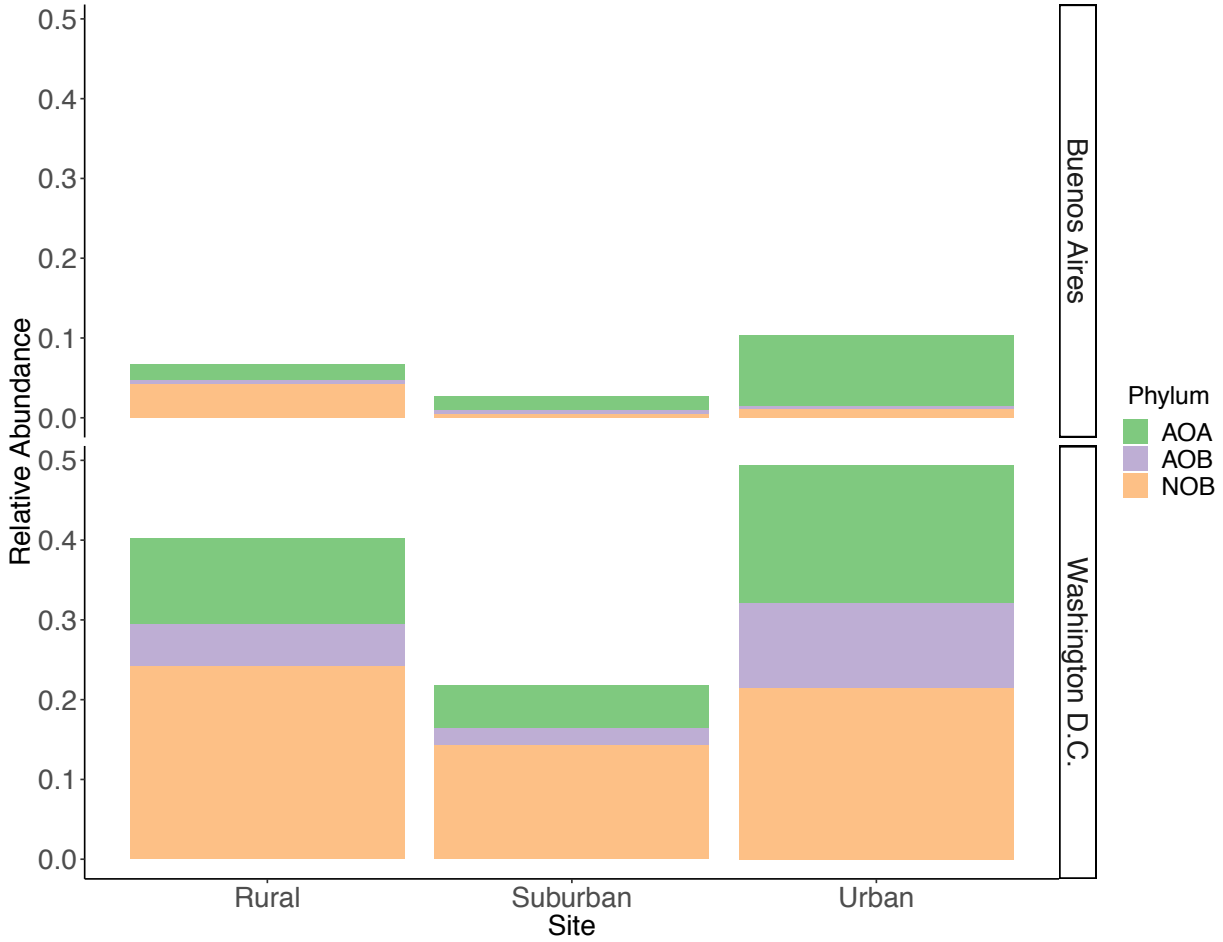

Figure S3: Relative abundance of ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) along the urban gradient in Buenos Aires and Washington D.C.

Chapter 3: Intraspecific facilitation of native and invasive *Phragmites australis* across varying C:N ratios

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Abstract

Land use changes and greater nitrogen input into waterways have facilitated the spread of an invasive lineage of *Phragmites australis*. Its establishment has led to a decrease in native wetland plant diversity, including the displacement of a native American lineage of *Phragmites* that is considered to be a low-nutrient specialist. Management efforts to eradicate invasive *Phragmites* have been extensive but not very effective in the long-term. Limiting nutrients could help control the spread of this invasive lineage, and would be expected to benefit the native lineage. Therefore, this study evaluated the use of a carbon-rich amendment as a management tool for *Phragmites*. We set up a greenhouse experiment to assess the competitive interactions of native and invasive *Phragmites* under varying C:N ratios, using either urea or sawdust additions. Our results show that there was an overyielding effect in intergenotypic treatments that was independent of C:N ratios. When grown together native and invasive *Phragmites* had greater above and belowground biomass relative to monoculture treatments, and primarily allocated carbon to rhizomes and stems. Since invasive *Phragmites* did not suppress the growth of the native but rather

enhanced it, we suggest that intergenotypic facilitation could promote coexistence of the two lineages. Concerning our C:N treatments, we found that carbon and nitrogen additions had varying effects on several parameters depending on whether the plants were growing in monoculture or mixed treatments. However, overall sawdust addition did not improve the competitive ability of native *Phragmites*, and urea additions increased aboveground biomass in both plant types. At the levels evaluated in this study, carbon additions would not constitute an effective management tool to control invasive *Phragmites* or restore the native lineage.

Introduction

The introduction of a non-native lineage of the wetland grass *Phragmites australis* to the United States has led to a dramatic increase in its relative abundance at the expense of native plants. (Farnsworth and Meyerson 1999, Saltonstall 2002, Findlay et al. 2003, Silliman and Bertness 2004). Once established, invasive *Phragmites* forms large mono-specific stands that can reduce animal and plant diversity (Minchinton 2003, Able et al. 2003), and alter biogeochemical cycles (Mozdzer and Megonigal 2013, Bernal et al. 2017).

The aggressive expansion of this introduced lineage was likely facilitated by changes in land use (Silliman and Bertness 2004, Lelong et al. 2007, Meadows and Saltonstall 2007), increased nutrient pollution (Packett and Chambers 2006, Saltonstall and Court Stevenson 2007), and the plant's own ecophysiological traits that contribute to its invasiveness (Mozdzer Thomas J. and Zieman Joseph C. 2010,

Mozdzer et al. 2013). Compared to a native American lineage of this *Phragmites australis*, the invasive European lineage has greater phenotypic plasticity (Mozdzer et al. 2013), nitrogen uptake capacity (Packett and Chambers 2006, Mozdzer Thomas J. and Zieman Joseph C. 2010), ventilation efficiency (Tulbure et al. 2012) and salt tolerance (Vasquez et al. 2005). These traits have allowed invasive *Phragmites* to become dominant in many wetland habitats, particularly in areas susceptible to disturbance and nutrient enrichment (King et al. 2007, Kettenring et al. 2015, Sciance et al. 2016) where it can gain a competitive advantage over native vegetation (Bertness et al. 2002, Holdredge et al. 2010).

Management of invasive *Phragmites* is costly and not always effective (Martin and Blossey 2013). Current eradication strategies primarily rely on herbicide application, mowing and burning which are used at local scales, and have uncertain results in the long-term (Hazelton et al. 2014). In order to achieve an effective control it is necessary to implement a watershed-scale approach and it needs to specifically target nutrient management (Rickey and Anderson 2004, Packett and Chambers 2006, Hazelton et al. 2014, Kettenring 2011).

Carbon rich soil amendments have been widely evaluated as a tool to control invasive species across different landscapes (Blumenthal et al., 2003; Eschen et al., 2006; Mitchell and Bakker, 2011; Rashid and Reshi, 2010). Increasing the C:N ratio of the soil stimulates microbial uptake of the newly added carbon together with soil available nitrogen. As nitrogen gets immobilized into the microbial biomass, it becomes temporarily unavailable to plants and other organisms (Blumenthal et al 2003; Perry et al. 2004; Rashid & Reshi, 2010) resulting in a reduction in net nitrogen

mineralization (Averett et al. 2004). Many plant invasions, including that of *Phragmites*, are facilitated by excess nitrogen (Perry et al. 2010), therefore limiting available nitrogen through carbon amendments could serve as an additional management strategy (Hazelton et al. 2014).

The goal of our study was to evaluate the potential of C:N manipulations as a tool for control of the invasive lineage and for native *Phragmites* restoration. We established a greenhouse competition experiment of native and invasive *Phragmites* and added either sawdust or urea to assess competition outcomes. We predicted that sawdust addition would increase C:N, promote nitrogen immobilization, and favor native *Phragmites* that is considered to be a low-nutrient specialist (Holdredge et al. 2010). Urea addition would instead decrease C:N, promote nitrogen mineralization, and have a greater effect on aboveground biomass production of the invasive lineage than the native making it a more effective competitor.

Methods

Greenhouse set-up

Phragmites rhizomes were collected in a freshwater tidal wetland in the Patuxent River on March 2015 from an invasive (38°40'1.16"N, 76°41'57.48"W) and a native (38°42'17.32"N, 76°42'9.11"W) stand. Rhizomes were cut to a length of two nodes, and planted in 2:1 potting soil and sand mixture where they grew for 8 weeks. Four plantlets of similar sizes were transplanted into 6.52-liter circular pots with a 2:1 mixture of potting soil and sand, and slow release fertilizer (Osmocote® Scotts Sierra

Co, Maryville, OH) was added to result in a nitrogen load equivalent of 25g N/m^2/year that would prevent nutrient limitation. Greenhouse temperature was kept at 32°C during the day and dropped to 7°C at night. To maintain moisture, pots were placed into 19-liter buckets filled with water up to 10cm below the edge of the pots. Every week, pots were removed from the bucket, leftover water was poured through them to flush accumulated solutes, and they were allowed to drain before returning them to the bucket and re-filling the water. To prevent plant chlorosis, 1ml a freshly prepared iron sulfate ($FeSO_4$) was added every week on the surface of the pot (Willson et al. 2017) at a rate of 0.1462 g/pot/week (Eller et al. 2013). After a month of growth, the individual buckets were surrounded with shade cloth (Easy Gardener Sun Screen Fabric-Saddle Tan color) to reduce lateral light penetration and keep the stems vertical as occurs in vegetation stands.

Experimental design and treatment applications

Competition experiments were established under four C:N ratios using a substitutive design (De Wit 1960) , where plant density was kept constant and relative abundance of each species was varied. This experimental design is particularly useful to evaluate if intraspecific competition differs in intensity from interspecific competition (Morin, 1999). To assess the effects of competition between native and invasive Phragmites under different C:N ratios pots were set up either as monocultures of 4 plantlets of each type, or as mixtures that combined two native and two invasive plantlets on each side of a single pot. For the C:N treatments, a high C:N was established by incorporating 1.25kg/m^2 of sawdust into the potting mixture

(Kaytee Pine pet bedding, Chilton, WI, USA) prior to planting, and a low C:N by adding 46-0-0 urea fertilizer on the surface of the pots weekly at a rate of 50g N/m^2/year; this rate represents an estimate of the annual nitrogen loading for freshwater marshes in the Chesapeake Bay area (Boynton et al. 2008, Bukaveckas and Isenberg 2013). We applied a 2x2 factorial arrangement of sawdust and urea treatments, with two levels of sawdust (none and 1.25 kg/m^2) and two levels of urea (none and $50g/m^2$/yr). This design allowed us to examine interactions between sawdust and urea in addition to main effects.

We set up the experiment as a complete randomized block design (RCBD) with four blocks following a perceived light gradient in the greenhouse. Each block contained one experimental unit for each of the four treatment combinations, resulting in a full factorial arrangement of native and invasive types (monocultures and mixture) and under the different C:N treatments (urea and sawdust).

Plant measurements and harvesting

The experiment was carried out over 13 weeks, and weekly measurements included stem height, diameter and total stem count. Twice during the experiment, after 4 and 11 weeks of growth, we used a PAM-2100 Chlorophyll Fluorometer (Waltz, Effeltrich, Germany) to record chlorophyll fluorescence as quantum yield (Y) during the day and maximum quantum yield (Fv/Fm) at night. Quantum yield is strongly related to carbon fixation, while maximum quantum yield can be used as an indicator of photosynthetic performance (Maxwell and Johnson 2000). We took two fluorescence measurements per pot of the second highest collared leaf of each plant.

At the end of the experiment we harvested the above and belowground biomass, separating leaves with leaf blades from the stems, and lateral roots from rhizomes after thoroughly washing these over a 5mm mesh sieve. The leaves for each pot were counted and their specific area was measured using a LI-3100C Area Meter (LI-COR,USA). Harvested biomass was dried at 70°C until it reached a constant mass. Using the dry biomass we calculated specific leaf area (SLA=leaf area/leaf biomass), which has been correlated with leaf nitrogen content, and relative growth rate (Garnier et al., 1997, Evans and Poorter, 2001). We also assessed how the different treatments affected biomass allocation by calculating the following ratios: leaf mass ratio (LMR=leaf biomass/total biomass), stem mass ratio (SMR=stem biomass/total biomass), rhizome mass ratio (RHMR=rhizome biomass/total plant biomass), root mass ratio (RMR=lateral root biomass/total plant biomass), rhizome:root ratio (rhizome biomass/lateral root biomass), and shoot-to-root ratio (total aboveground/total belowground biomass).

Data analysis

All data analysis was completed in R studio (v.1.0.153). We used a three-way analysis of variance (ANOVA) to evaluate the effects of lineage, urea and sawdust additions on response variables. We then grouped response variables across C:N treatments used two-way ANOVAs to assess the effects of lineage and planting treatment (monoculture versus mixture) on each response. We checked the data for normality and homogeneity of variance and performed transformations when necessary to meet ANOVA assumptions. Mean comparisons of significant factors

were evaluated using the Tukey HSD.test function in the R package “agricolae” (de Mendiburu, 2019).

To further investigate the intensity of genotypic competition we used the Relative Interaction Index (RII) (Armas 2004). This index is symmetrical around 0 and bound between -1 and 1; negative values indicate competition while positive values indicate that facilitation is the prevalent interaction. We calculated the RII index as RII = $(Bw - Bo)/(Bw + Bo)$, where Bw is the biomass of the plant growing in mixture and Bo is half of the biomass of the plant growing in monoculture. We used three-way ANOVAs to assess differences in this index based on plant type and additions of urea or sawdust, and t-tests to see if the overall RII means differed from 0.

Results

Although the original goals of this study were to evaluate the effects of changes in C:N ratio on competition of native and invasive *Phragmites*, we also found that there were surprising intergenotypic facilitation outcomes independent of treatment effects and investigated those further.

When native and invasive *Phragmites* were grown together, there was an overall increase in rhizome:root ratio (Figure 1), and biomass allocation to stems (Table 1) resulting in higher stem:leaf ratio in both lineages relative to monocultures (Figure 2). On the other hand, when growing together both lineages had lower LMR, SLA, RMR, F_v/F_m and final stem height and diameter than they did in monoculture

(Table 1, Supplementary Table 5). In addition, native *Phragmites* had greater shoot:root ratio in mixture than in monoculture (Table 1).

Overall, regardless of monoculture or mixture treatment, we found that native *Phragmites* had greater rhizome:root ratio than the invasive lineage (Figure 1); but lower stem count, leaf area, leaf count, lateral root biomass, aboveground biomass, rhizome biomass and RMR (Supplementary Table 6).

We observed predominantly positive interactions (i.e., facilitation) when native and invasive *Phragmites* were grown together in the same pots (Figure 3). The calculated Relative Interaction Index values across urea and sawdust treatments were significantly greater than 0 for aboveground (t_{30}=15.4, p<0.001) and belowground biomass (t_{27}=32.47 p<0.001). This was also observed for both components of aboveground biomass (leaves and shoots) and belowground biomass (rhizomes and lateral roots), which showed positive interactions in the mixture treatments (Supplementary figures 1-4). There was a significant difference between lineages for belowground RII, as native *Phragmites* showed higher RII values (i.e., greater facilitation of growth by the other lineage) than the invasive type ($F_{1,17}$=15.9, p=0.001); no differences were observed between native and invasive for aboveground RII ($F_{1,19}$=0.76, p=0.395). Urea and sawdust additions had no effect on the outcome of the interaction values estimated for above or belowground biomass (p>0.05).

The C:N treatment effects were sometimes dependent on lineage, and the response to these treatments sometimes differed when the plants were growing in monoculture or in mixture. (Supplementary Tables 1-4). Specifically, in mixture treatments urea addition led to greater rhizome biomass ($F_{1,21}$=6.41, p=0.0194), leaf

biomass ($F_{1,20}$=30.822, p<0.001), stem biomass ($F_{1,21}$=7.219, p=0.014), aboveground biomass ($F_{1,19}$=17.48 , p<0.001; Figure 4c), SLA ($F_{1,18}$=24.05, p<0.001; Figure 4d), higher leaf count ($F_{1,21}$=17.02, p<0.001), and higher stem count ($F_{1,21}$=4.48 , p=0.046) (Supplementary tables 2, 4 and 7). There was also a significant Lineage x Urea interaction for LMR, as urea addition increased the LMR of invasive (2-way interaction: $F_{1,20}$=4.89, p=0.039) relative to native *Phragmites* without urea.

Similarly, in monoculture treatments, urea addition increased rhizome biomass ($F_{1,20}$=8.69, p=0.008), SLA ($F_{1,18}$=68.5, p<0.001; Figure 4), aboveground biomass except when combined with sawdust in native *Phragmites* (3-way interaction: $F_{1,18}$=5.07, p=0.042; Figure 4a); and increased leaf counts for both lineages ($F_{1,21}$=11.88, p=0.002). Urea addition in monoculture also increased Fv:Fm when no sawdust was incorporated ($F_{1,21}$=4.62, p=0.042) (Supplementary tables 1 and 7).

In monoculture, sawdust addition resulted in lower SMR ($F_{1,18}$=9.36, p=0.007) and stem diameter ($F_{1,21}$=4.38, p=0.048) for both genotypes, and increased RHMR in native *Phragmites* ($F_{1,19}$=6.15, p=0.016) (Supplementary tables 1,3 and 8). The sawdust treatment also had lower stem count relative to urea or urea+sawdust ($F_{1,21}$=13.51, p=0.001). In mixture plantings, sawdust addition showed no significant main effects on any of the assessed plant parameters (p>0.05; Supplementary Tables 2 and 4).

Discussion

To our considerable surprise, we found facilitation to be the predominant interaction between native and invasive *Phragmites*, as reflected by greater above and belowground biomass of both lineages in mixture treatments than in monoculture (Figure 3). Interactions between native and invasive *Phragmites* have been considered to be overall negative, and several studies have attributed the displacement of native plants to competitive exclusion by the introduced *Phragmites* lineage (Chambers et al. 1999, Saltonstall 2002, Wilcox et al. 2003, Meadows and Saltonstall 2007). However, competitive interactions were not experimentally measured in these studies so the reported replacement of native vegetation by invasive *Phragmites* cannot be definitively considered a result of direct resource competition (Mozdzer 2013). Our finding of facilitative interactions (i.e., overyielding) between native and invasive *Phragmites*, would suggest that other mechanisms and not competitive exclusion favor the establishment of the introduced lineage in detriment of the native.

Intergenotypic interactions can result in overyielding (Rao and Prasad 1984, Crutsinger et al. 2006, Kotowska et al. 2010, Cook-Patton et al. 2011) through complementarity mechanisms which include niche partitioning and facilitation (Loreau and Hector 2001). Niche partitioning could constitute a relevant mechanism for the observed interactions given the marked morphological and physiological differences between native and invasive *Phragmites* (League et al. 2006, Mozdzer Thomas J. and Zieman Joseph C. 2010, Mozdzer et al. 2013); but it is difficult to evaluate in our greenhouse setting. Field studies have found that invasive *Phragmites*

has deeper rooting profiles than native plants and can access resources that are unavailable to natives (Moore et al. 2012, Bernal et al. 2017); this could make niche partitioning a possible mechanism for coexistence in the field. In our greenhouse on the other hand, depth separation of roots seems unlikely due to the limited space provided, but roots might still separate out in space based on kin recognition as seen in Fang et al. (2013). In that study, the authors looked at three genotypes of rice growing in cylinders with kin or nonkin and found that plants of the same genotype had significantly more overlapping roots, than plant roots in intergenotypic treatments which grew away from each other. In our study the observed decrease in RMR in intergenotypic arrangements (Table 1) could imply a more efficient use of space and belowground resources relative to monocultures. Facilitative interactions could also explain overyielding in mixture treatments. For example, it is possible that native *Phragmites* benefited from better soil aeration provided by greater belowground biomass of the invasive lineage. And it is likely that the increase in biomass of the invasive in mixture treatments resulted from a greater availability of nutrients as the native lineage has lower nitrogen demands (Mozdzer Thomas J. and Zieman Joseph C. 2010).

When grown together, native and invasive *Phragmites* allocated more biomass to rhizomes than lateral roots (Figure 1, Table 1) and to stems rather than leaves (Figure 2; Table 1). Concerning rhizome allocation, Cheplick and Gutierrez (Cheplick 2000) also found a greater allocation to storage organs in a clonal grass under competition, and suggested it might help the plant's persistence within an occupied area. The increase in biomass allocation to stems without an associated increase in

their overall height or diameter could be due to an investment in mechanical stability to support more leaf biomass, and could further promote persistence in the environment through greater translocation of nutrients and carbohydrates to rhizomes in the winter (Boar 1996).

Holdredge et al. (2010) performed a similar competition experiment, and although their two-year experiment found no significant intergenotypic suppression of native *Phragmites* by the invasive lineage, they suggested that the greater biomass and higher expansion rate of the invasive eventually leads to displacement of the native. They also found that native *Phragmites* can be a good competitor under low nutrient conditions, but based on field observations they suggest it is outcompeted under eutrophic conditions. As opposed to competitive exclusion of native *Phragmites*, our greenhouse study suggests that there can be facilitative interactions between native and invasive plants when grown together. We also found that native and invasive *Phragmites* appear to be equally capable of harnessing nutrients and had a comparable relative increase in aboveground biomass after urea additions (Figure 4, Supplementary Table 7). This is unlike the disproportionate increase in this parameter for the invasive that was seen in Holdredge et al (2010) and in line with the results reported for *Phragmites* seedlings in Saltonstall and Stevenson (2007). It is possible that the differences in our results lie in the source or our *Phragmites* populations that can result in morphological and physiological differences (Clevering et al. 2001, Saltonstall and Court Stevenson 2007). Rhizomes used in the Holdredge et al. (2010) study were excavated from a brackish marsh and re-planted at oligohaline sites, whereas our rhizomes were collected in a freshwater and nutrient

rich wetland. Native *Phragmites* is more sensitive to salinity than the invasive lineage (Vasquez et al. 2005), therefore native populations might have been affected by salinity in the Holdredge et al. (2010) study limiting their nutrient uptake (Brown et al. 2006), while invasive populations were capable of quickly responding to nutrient additions based on their greater salinity tolerance (Vasquez et al. 2005) and nitrogen uptake capacity (Mozdzer Thomas J. and Zieman Joseph C. 2010).

In addition to our greenhouse results, our field observations and others in the literature (Saltonstall and Court Stevenson 2007, Kettenring and Mock 2012) support that native and invasive lineages can co-exist even in eutrophic systems. Kettenring et al. (2012) further suggest that there is little evidence that native *Phragmites* stands are being replaced by the invasive lineage, and some of our study sites in the nutrient-rich Patuxent and Choptank Rivers in Maryland have neighboring stands of native and invasive *Phragmites* that have remained relatively unchanged for at least 10 years. Furthermore, we planted native Phragmites in an area dominated by the invasive lineage in Jug Bay Wetlands Sanctuary (Patuxent river, MD, USA) and three years later the native is still present and has expanded beyond our original plots (Appendix Figure 6). This expansion started from rhizomes after the first year because we harvested the aboveground biomass and it likely continued to expand belowground as we found stems growing around our original plots. We were unable to confirm if there was native growth from seed dispersion in the general area due to limited access. These observations support the importance of belowground spread for the native lineage (Kettenring and Mock 2012) and its ability to persist in an area dominated by the invasive lineage.

Although the C:N treatment effects were sometimes variable between monoculture and mixture treatments (Supplementary Tables 6-8), we did find an overall effect of urea addition on aboveground biomass, leaf count, SLA and rhizome biomass which matches those seen in previous studies (Saltonstall and Court Stevenson 2007, Holdredge et al. 2010, Mozdzer and Megonigal 2012). Both lineages benefitted from urea additions, but invasive *Phragmites* had greater values for these parameters supporting the idea that nitrogen enrichment can make this lineage an effective competitor and promote its invasiveness particularly following disturbance (Silliman and Bertness 2004, Rickey and Anderson 2004, Holdredge et al. 2010). Rhizome biomass showed a greater relative increase in native than invasive *Phragmites* after urea addition (Supplementary Tables 3, 4 and 7), and the native overall allocated more biomass to rhizomes than the invasive did (Figure 1) supporting the importance of vegetative reproduction in this lineage (Kettenring and Mock 2012).

Sawdust addition did not have significantly differential effects on native and invasive *Phragmites*, and did not benefit the native as we originally hypothesized. It overall did not have a significant effect on most parameters, and the increase in rhizome biomass and decrease in SMR observed with sawdust addition in monoculture might not have been detected in the mixture treatments because intergenotypic interactions likely had a stronger effect than low C:N on those parameters.

In conclusion, our greenhouse study suggests that C:N modifications at the proposed level would be unlikely to be a useful strategy for management or

restoration of *Phragmites*. Many of the plant response parameters were variable depending on whether the plants were in monoculture or mixture treatment, and sawdust addition did not improve the native's competitive ability. This study found that facilitation was the predominant interaction when the genotypes were grown together regardless of C:N treatment, and therefore supports the idea that disturbance rather than competitive exclusion is likely responsible for the extirpation of native *Phragmites* (Lelong et al., 2007; Mozdzer et al., 2013; Saltonstall and Court Stevenson, 2007).

Declarations

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Author Contributions: SAY and AHB designed the project and secured funding. MGM, SAY and AHB planned and set up the experiment. MGM collected the data,

analyzed it with assistance from AHB, and wrote the manuscript. All authors reviewed and improved the manuscript.

Permits: Jug Bay Wetlands Sanctuary allowed us to set up our experiment on their land.

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Table 1: Mean values and standard error for plant parameters that were significantly different between monoculture and mixture treatments. Within each parameter, different letters indicate significant differences between lineages and/or planting treatments ($p<0.05$). SMR=Stem Mass Ratio, SLA= Specific Leaf Area, LMR= Leaf Mass Ratio, RMR= Root Mass Ratio.

Parameter	Lineage	Monoculture	Mixture
SMR	Native	0.205 ± 0.02 b	0.304 ± 0.01 a
	Invasive	0.190 ± 0.02 b	0.386 ± 0.01 a
Rhizome:Root	Native	2.792 ± 0.08 b	3.320 ± 0.09 a
	Invasive	2.010 ± 0.08 c	2.602 ± 0.08 b
Shoot:Root	Native	0.819 ± 0.14 b	1.015 ± 0.04 a
	Invasive	0.866 ± 0.11 ab	0.844 ± 0.04 ab
Stem height	Native	89.643 ± 0.32 a	83.608 ± 0.53 ab
	Invasive	84.460 ± 0.25 a	72.54 ± 0.34 b
SLA	Native	42.742 ±0.57 ab	16.031 ± 0.76 c
	Invasive	49.798 ± 0.61 a	33.362 ± 0.70 b
LMR	Native	0.186 ± 0.02 a	0.139 ± 0.00 b
	Invasive	0.183 ± 0.02 a	0.144 ± 0.01 b
RMR	Native	0.239 ± 0.07 ab	0.191 ± 0.04 b
	Invasive	0.344 ± 0.08 a	0.238 ± 0.05 ab
Stem diameter	Native	3.265 ± 0.07 a	2.865 ± 0.08 ab
	Invasive	2.973 ± 0.03 a	2.506 ± 0.09 b
Fv/Fm	Native	0.824 ± 0.001 a	0.822 ± 0.003 b
	Invasive	0.826 ± 0.001 a	0.823 ± 0.002 b

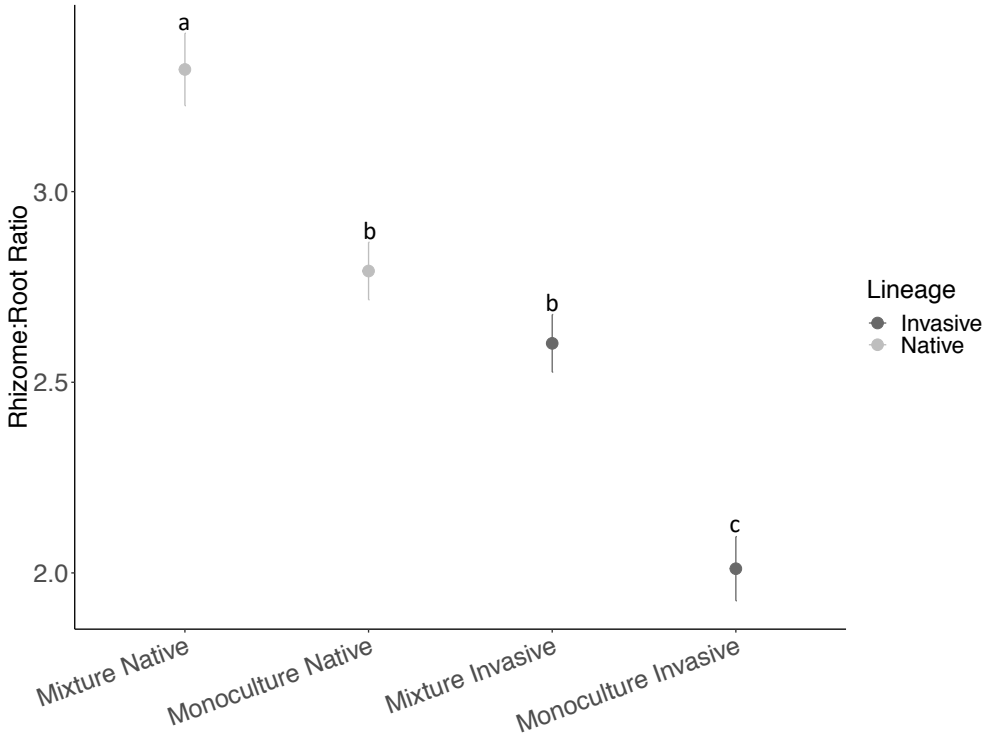


Figure 1: Mean rhizome:root ratio and standard error of native and invasive *Phragmites australis* growing together in mixtures or in monoculture. Different letters indicate significant differences between planting treatments ($p<0.05$).

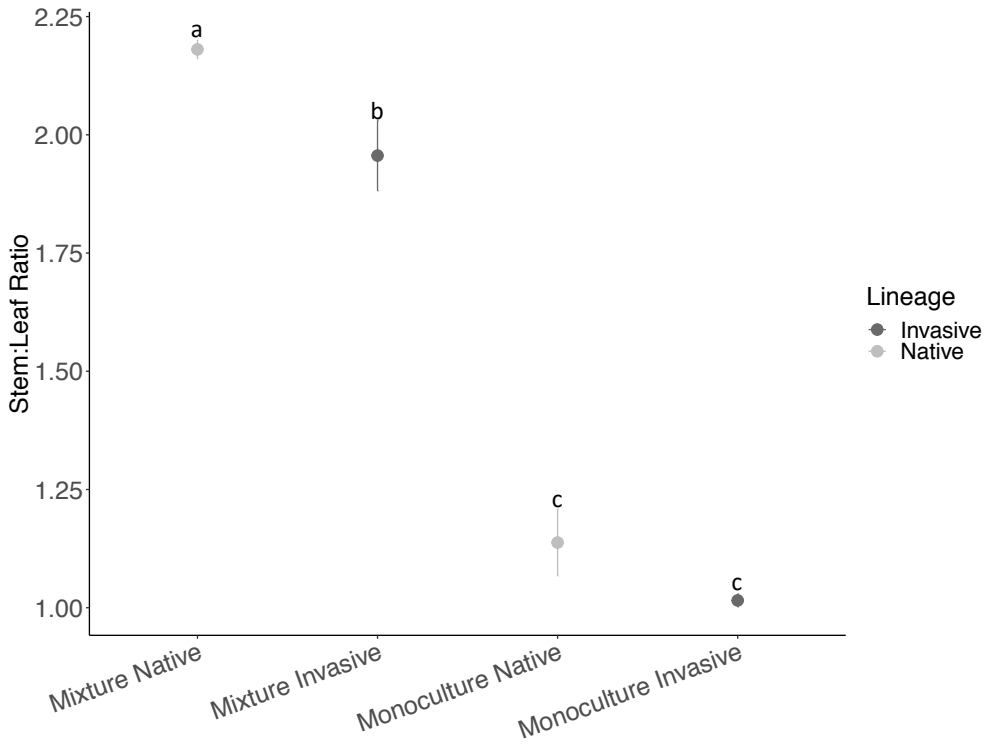


Figure 2: Mean stem:leaf ratio and standard error of native and invasive *Phragmites australis* growing together in mixture or in monoculture. Different letters indicate significant differences between planting treatments ($p<0.05$).

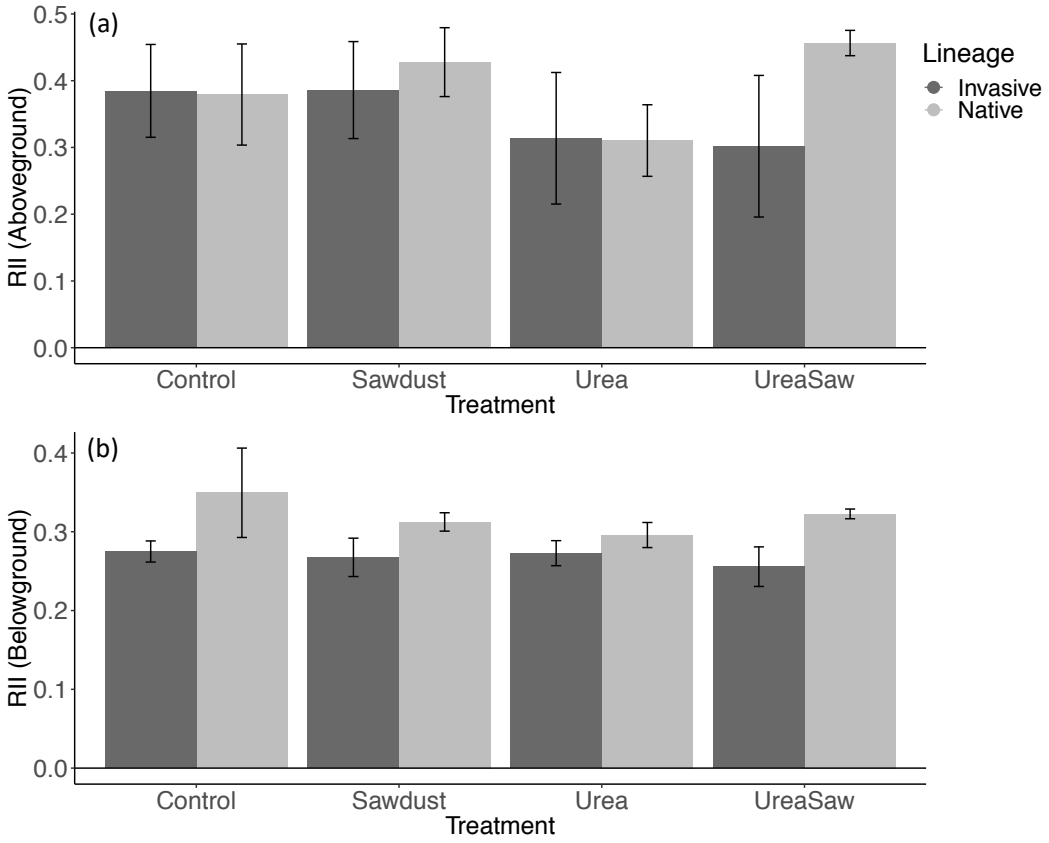


Figure 3: Relative Interaction Index (RII) for aboveground (a) and belowground (b) biomass of native and invasive *Phragmites australis* across C:N treatments. Control=No Urea/Sawdust addition, Sawdust=1.25kg/m^2 , Urea=50g/m^2/year

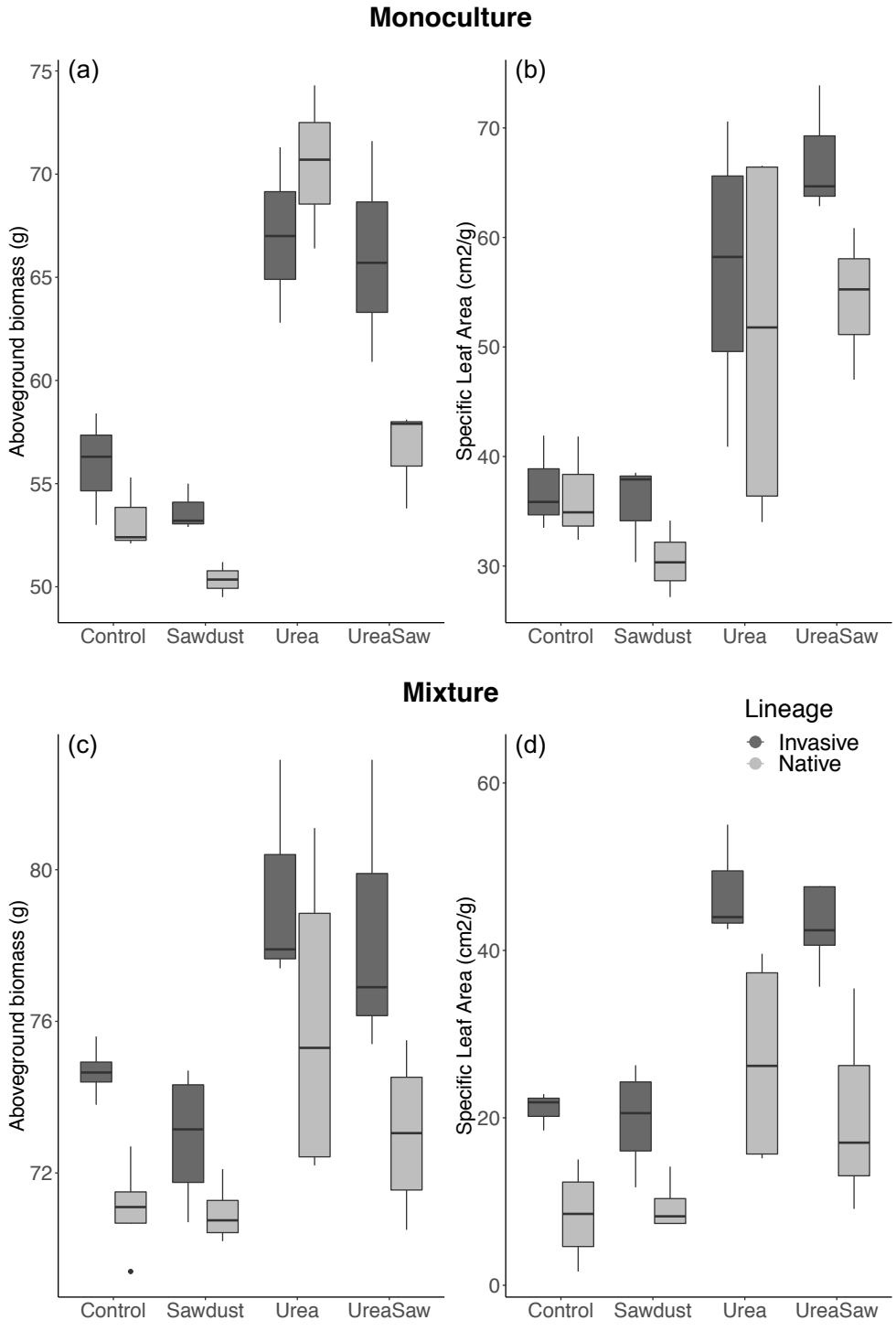


Figure 4: Boxplot of aboveground biomass and specific leaf area (SLA) in monoculture (a,b respectively) and mixture (c,d respectively) and across C:N treatments. Control=No Urea/Sawdust addition, Sawdust=1.25kg/m^2 , Urea=50g/m^2/year

Supplementary Table 1: ANOVA results of treatment effects on aboveground parameters for *Phragmites* growing in monoculture

	Aboveground biomass		Stem height		Stem diameter		Stem count		Stem biomass		SMR		Leaf count	
	F	p	F	p	F	p	F	p	F	p	F	p	F	p
Urea (S)	**97.22**	**<0.001**	0.01	0.949	0.33	0.571	**136.34**	**<0.001**	3.39	0.0799	1.56	0.228	**53.28**	**<0.001**
Sawdust (S)	**18.06**	**<0.001**	2.67	0.117	**4.38**	**0.049**	**13.511**	**0.001**	0.74	0.4	**9.36**	**0.006**	2.47	0.131
Lineage (L)	**8.57**	**0.0118**	2.94	0.101	**5.70**	**0.026**	**125.662**	**<0.001**	0.02	0.891	2.86	0.108	**148.49**	**<0.001**
UxS	2.89	0.113	1.87	0.185	0.46	0.506	2.164	0.156	0.89	0.354	0.23	0.639	0.05	0.827
UxL	0.04	0.841	0.60	0.445	1.90	0.182	**60.48**	**<0.001**	0.10	0.752	0.23	0.639	**11.88**	**0.002**
SxL	**9.52**	**0.008**	3.63	0.07	1.38	0.253	2.286	0.145	1.22	0.282	3.21	0.09	0.47	0.501
UxSxL	**5.06**	**0.042**	0.08	0.783	0.0	0.972	0.013	0.91	0.69	0.416	0.03	0.857	0.38	0.544

Supplementary Table 1: Continued

	Leaf area		SLA		Stem:Leaf		LMR		Leaf biomass		Quantum yield (Y)		Fv/Fm	
	F	p	F	p	F	p	F	p	F	p	F	p	F	p
Urea (S)	**72.23**	**<0.001**	**68.504**	**<0.001**	0.239	0.633	0.59	0.453	4.238	0.052	1.74	0.202	3.696	0.069
Sawdust (S)	2.607	0.121	0.123	0.73	0.323	0.579	0.65	0.431	1.641	0.214	0.136	0.716	0.114	0.789
Lineage (L)	**9.41**	**0.006**	**19.085**	**<0.001**	2.748	0.119	0.001	0.975	2.389	0.137	1.418	0.248	2.939	0.102
UxS	0.627	0.437	0.668	0.425	0.183	0.675	0.189	0.669	0.844	0.369	2.174	0.156	**4.617**	**0.044**
UxL	2.995	0.098	0.241	0.629	0.036	0.852	0.702	0.414	0.012	0.914	0.031	0.862	0.038	0.848
SxL	1.913	0.181	0.047	0.832	0.379	0.548	3.37	0.084	4.017	0.058	0.047	0.831	0.08	0.779
UxSxL	0.485	0.493	1.086	0.311	2.642	0.126	0.921	0.351	0.135	0.717	1.852	0.189	0.055	0.817

Supplementary Table 2: ANOVA results of treatment effects on aboveground parameters for *Phragmites* growing in mixture

	Aboveground biomass		Stem height		Stem diameter		Stem count		Stem biomass		SMR		Leaf count	
	F	p	F	p	F	p	F	p	F	p	F	p	F	p
Urea (S)	**17.48**	**<0.001**	0.365	0.552	3.392	0.079	4.48	0.046	**7.219**	**0.0138**	0.133	0.719	**17.019**	**<0.001**
Sawdust (S)	2.144	0.159	0.48	0.496	0.098	0.757	0.59	0.451	3.621	0.071	2.008	0.173	0.009	0.923
Lineage (L)	**14.174**	**0.001**	0.153	0.7	3.589	0.072	**24.6**	**<0.001**	**19.95**	**<0.001**	**13.246**	**0.002**	**100.66**	**<0.001**
UxS	0.223	0.642	1.153	0.295	2.169	0.156	0.11	0.749	2.873	0.105	2.8	0.111	0.413	0.527
UxL	0.568	0.46	0.002	0.969	0.288	0.597	0.04	0.837	0.014	0.908	0.376	0.547	0.067	0.798
SxL	0.017	0.897	0.086	0.773	0.785	0.386	0	0.988	2.265	0.147	0.53	0.475	0.196	0.662
UxSxL	1.244	0.279	0.545	0.469	0.124	0.728	0.54	0.471	1.052	0.317	0.27	0.609	0.215	0.647

Supplementary Table 3: ANOVA results of treatment effects on belowground parameters for *Phragmites* growing in monoculture

	Belowground biomass		Rhizome biomass		Lateral root biomass		RHMR		RMR		Rhizome:Root	
	F	p	F	p	F	p	F	p	F	p	F	p
Urea (S)	1.349	0.259	**8.69**	**0.008**	0.549	0.467	0.09	0.768	**66.994**	**<0.001**	0.753	0.396
Sawdust (S)	1.647	0.213	0.011	0.917	0.708	0.401	**5.334**	**0.032**	**23.113**	**<0.001**	0.592	0.451
Lineage (L)	1.459	0.24	**17**	**<0.001**	**94.87**	**<0.001**	0.256	0.619	**17.409**	**<0.001**	**20.095**	**<0.001**
UxS	2.171	0.155	2.142	0.159	0.374	0.548	0.087	0.7708	**31.181**	**<0.001**	0.152	0.701
UxL	0.006	0.939	0.139	0.724	0	0.994	1.986	0.175	0.811	0.378	0.459	0.506
SxL	2.09	0.163	0.896	0.355	2.463	0.131	**6.115**	**0.023**	0.137	0.716	3.001	0.099
UxSxL	**4.398**	**0.048**	3.11	0.093	0.974	0.335	0.023	0.88	0.046	0.831	0.042	0.839

Supplementary Table 4: ANOVA results of treatment effects on belowground parameters for *Phragmites* growing in mixture

	Belowground biomass		Rhizome biomass		Lateral root biomass		RHMR		RMR		Rhizome:Root	
	F	p	F	p	F	p	F	p	F	p	F	p
Urea (S)	0.185	0.672	**6.413**	**0.019**	1.33	0.262	0.426	0.522	**22.136**	**<0.001**	0	0.851
Sawdust (S)	0.141	0.711	0.427	0.521	0.022	0.884	0.295	0.593	**9.149**	**0.007**	0.036	0.987
Lineage (L)	**12.669**	**0.002**	**6.499**	**0.018**	**15.259**	**0.0008**	5.178	0.035	4.762	0.041	**30.042**	**<0.001**
UxS	2.23	0.15	1.807	0.193	1.047	0.318	0.622	0.44	**7.382**	**0.0133**	0.536	0.472
UxL	0.74	0.399	0.192	0.666	0.149	0.703	0.065	0.8	0.111	0.742	0.004	0.534
SxL	0.266	0.612	1.738	0.202	0.795	0.383	1.603	0.221	0.409	0.529	0.421	0.953
UxSxL	0.464	0.503	1.489	0.235	0.209	0.652	0.377	0.546	0.346	0.563	0	0.989

Supplementary Table 5: ANOVA results showing effect of lineage and planting (monoculture vs mixture) on response variables

	SMR		Rhizome:Root		Shoot:Root		Stem height	
Planting	F	p	F	p	F	p	F	p
Mono_Mix	**197.142**	**<0.001**	**22.644**	**<0.001**	**6.265**	**0.0015**	**14.437**	**<0.001**
Lineage	**4.384**	**0.041**	**38.549**	**<0.001**	0.411	0.524	0.751	0.389
Lineage x Mono_Mix	0.004	0.949	1.036	0.313	3.346	0.0681	0.157	0.694

Supplementary Table 5: continued

	SLA		LMR		RMR		Stem diameter	
Planting	F	p	F	p	F	p	F	p
Mono_Mix	**35.673**	**<0.001**	**71.317**	**<0.001**	**4.184**	**0.0456**	**14.287**	**<0.001**
Lineage	**12.73**	**<0.001**	0.076	0.784	**5.692**	**0.021**	**8.106**	**0.006**
Lineage x Mono_Mix	4.007	0.0508	0.525	0.472	0.199	0.657	0.084	0.774

Supplementary Table 6: Mean and standard error of various response variables of native and invasive *Phragmites* under different C:N treatments and across planting treatments

Lineage	Treatment	Rhizome: Root	Stem count	Leaf Area	Leaf count	Lateral root biomass	Aboveground biomass	Rhizome biomass	RMR
Invasive	Control	2.35±0.17	9.8±1.1	883.3±4.9	98.8±15	32.46±2.77	66.63±3.84	72.89±0.79	0.49±0.10
Invasive	Sawdust	2.31±0.18	8.1±1.0	710.8±3.8	94.0±12	33.33±3.81	64.69±3.92	72.13±0.67	0.25±0.06
Invasive	Urea	2.21±0.24	15.0±1.4	1721.6±5.2	175.8±20	34.80±3.91	73.22±3.08	74.75±0.99	0.21±0.04
Invasive	UreaSaw	2.36±0.25	13.5±1.6	1686.3±4.8	162.3±24	34.78±4.08	72.23±3.25	74.13±1.12	0.21±0.05
Native	Control	3.11±0.44	5.9±1.1	545.3±6.1	47.0±13	22.03±1.89	63.44±3.64	63.31±4.14	0.34±0.08
Native	Sawdust	3.10±0.06	5.4±0.9	489.7±4.9	42.1±9	23.13±0.50	64.08±4.36	71.29±0.61	0.18±0.05
Native	Urea	2.93±0.16	7.7±1.4	1269.4±7.9	64.1±14	25.60±2.07	73.61±1.83	72.63±0.76	0.15±0.02
Native	UreaSaw	3.13±0.06	7.7±1.2	1044.9±6.4	64. 7±14	22.85±0.53	64.62±3.69	71.23±0.35	0.18±0.04

Supplementary Table 7: Mean and standard error of variables affected by urea addition in monoculture and mixture treatments

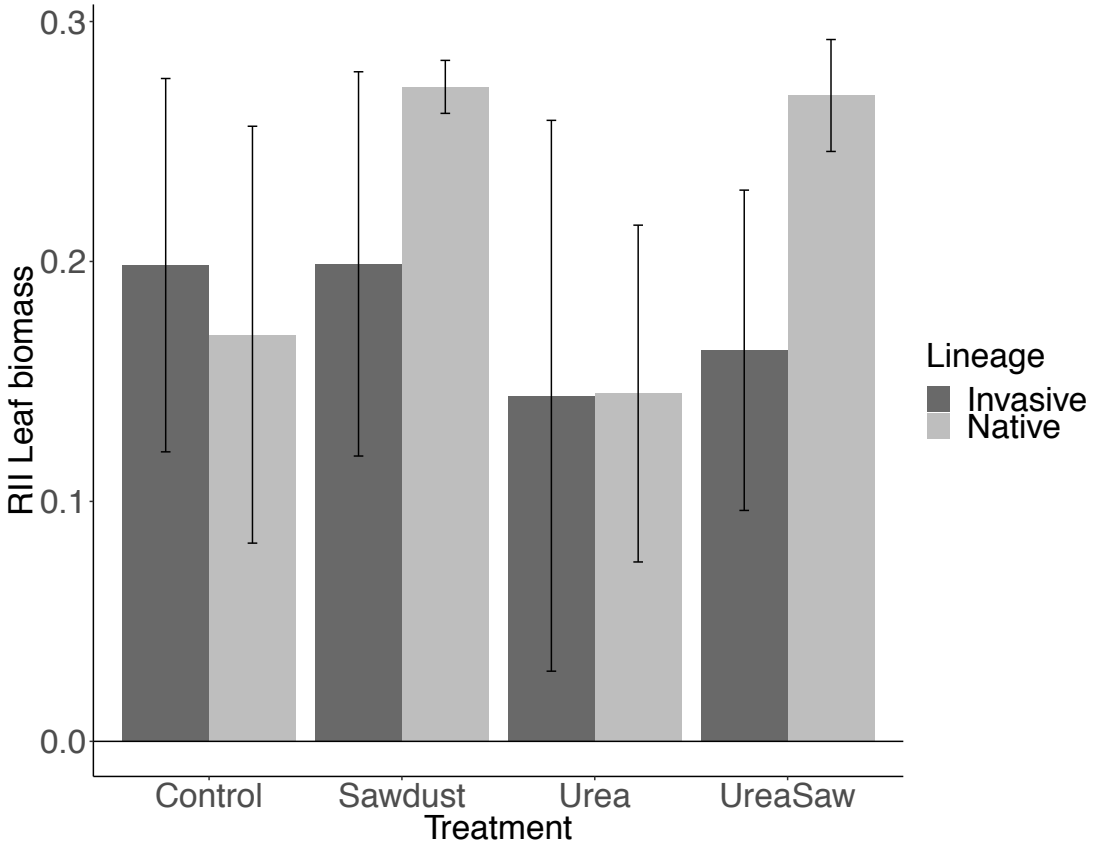
Lineage	Treatment	Mono_Mix	Aboveground biomass	Stem Count	Leaf Count	SLA	Stem biomass
Invasive	No urea	Mixture	73.80±0.59	7.4±0.9	71.71±7.1	20.33±1.83	49.91±0.37
Invasive	No urea	Monoculture	54.80±0.90	10.7±0.8	124.83±6.9	36.34±1.66	28.23±0.59
Invasive	Urea	Mixture	78.90±1.31	11.0±0.9	130.33±15.9	47.09±4.04	51.78±0.61
Invasive	Urea	Monoculture	66.55±1.77	17.5±0.2	207.83±12.9	64.74±3.01	33.30±1.22
Native	No urea	Mixture	71.01±0.36	3.6±0.5	22.63±2.9	8.96±1.58	49.08±0.25
Native	No urea	Monoculture	52.10±0.89	8.3±0.3	73.83±4.7	32.83±2.10	26.32±0.63
Native	Urea	Mixture	74.54±1.46	5.4±0.7	37.71±5.9	24.11±4.75	50.63±0.84
Native	Urea	Monoculture	63.53±3.32	10.3±0.9	95.50±7.9	55.54±4.75	33.85±1.4
Invasive	UreaSaw	Mixture	78.4±3.97	10.0±0.0	111.67±12.6	46.99±8.13	51.87±1.01
Invasive	UreaSaw	Monoculture	66.07±5.36	17.0±1.2	213.0±14.6	67.14±3.41	32.80±2.27
Native	UreaSaw	Mixture	72.63±2.58	5.3±1.3	37.67±24.7	20.54±7.80	49.40±0.66
Native	UreaSaw	Monoculture	56.6±2.42	10.0±0.0	91.67±11.6	54.38±4.02	28.77±0.68

Supplementary Table 7: continued

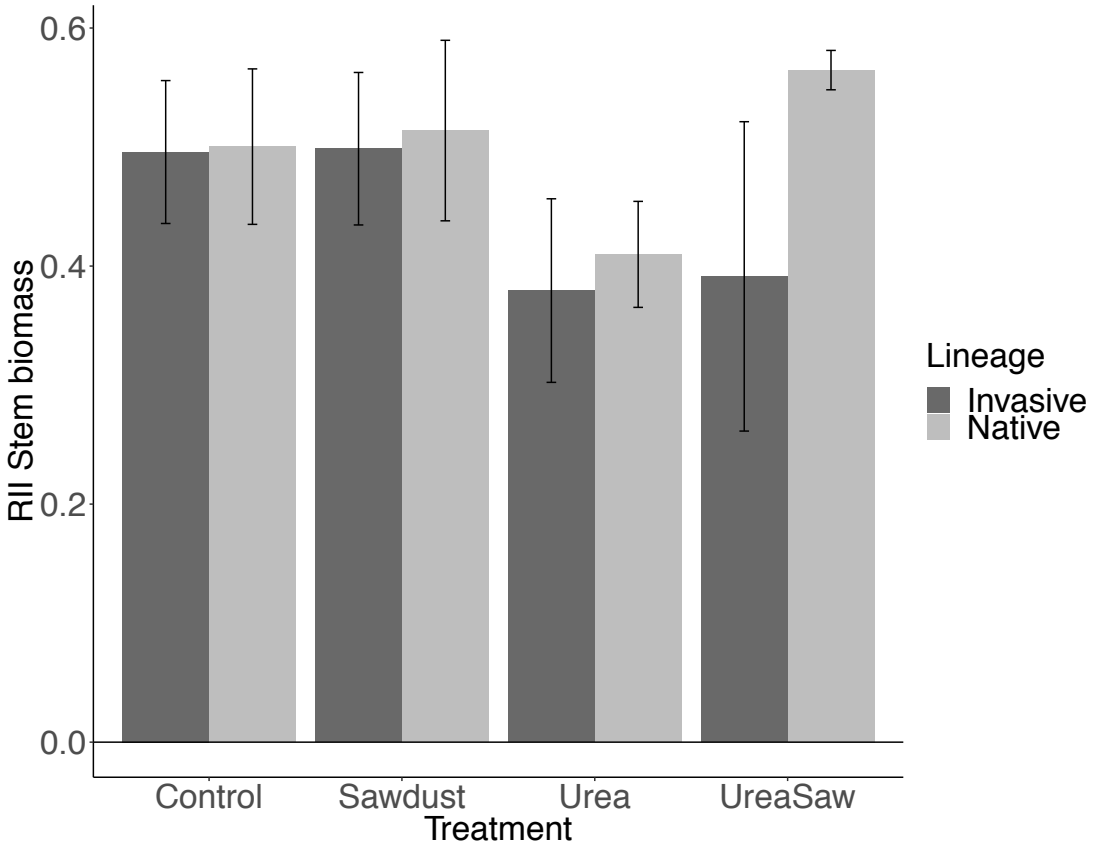
Lineage	Treatment	Mono_Mix	Leaf biomass	Rhizome biomass	LMR	Fv:Fm
Invasive	No urea	Mixture	23.76±0.28	70.89±0.33	0.13±0.002	0.822±0.003
Invasive	No urea	Monoculture	26.57±0.32	74.13±0.51	0.16±0.008	0.825±0.002
Invasive	Urea	Mixture	27.12±0.74	72.13±0.51	0.15±0.002	0.827±0.005
Invasive	Urea	Monoculture	33.25±0.61	76.75±0.69	0.18±0.002	0.827±0.001
Native	No urea	Mixture	21.94±0.14	68.51±1.52	0.14±0.002	0.829±0.002
Native	No urea	Monoculture	25.54±0.29	65.34±4.99	0.18±0.007	0.823±0.001
Native	Urea	Mixture	23.91±0.65	70.89±0.23	0.14±0.002	0.822±0.003
Native	Urea	Monoculture	29.68±1.58	72.96±0.69	0.18±0.005	0.825±0.001
Invasive	UreaSaw	Mixture	26.53±1.29	71.88±0.91	0.15±0.002	0.822±0.006
Invasive	UreaSaw	Monoculture	33.27±0.91	76.38±1.28	0.18±0.004	0.825±0.001
Native	UreaSaw	Mixture	23.23±0.85	70.70±0.37	0.14±0.003	0.821±0.003
Native	UreaSaw	Monoculture	27.83±0.72	71.75±0.49	0.18±0.001	0.824±0.001

Supplementary Table 8: Mean and standard error for variables affected by sawdust addition in monoculture and mixture treatments

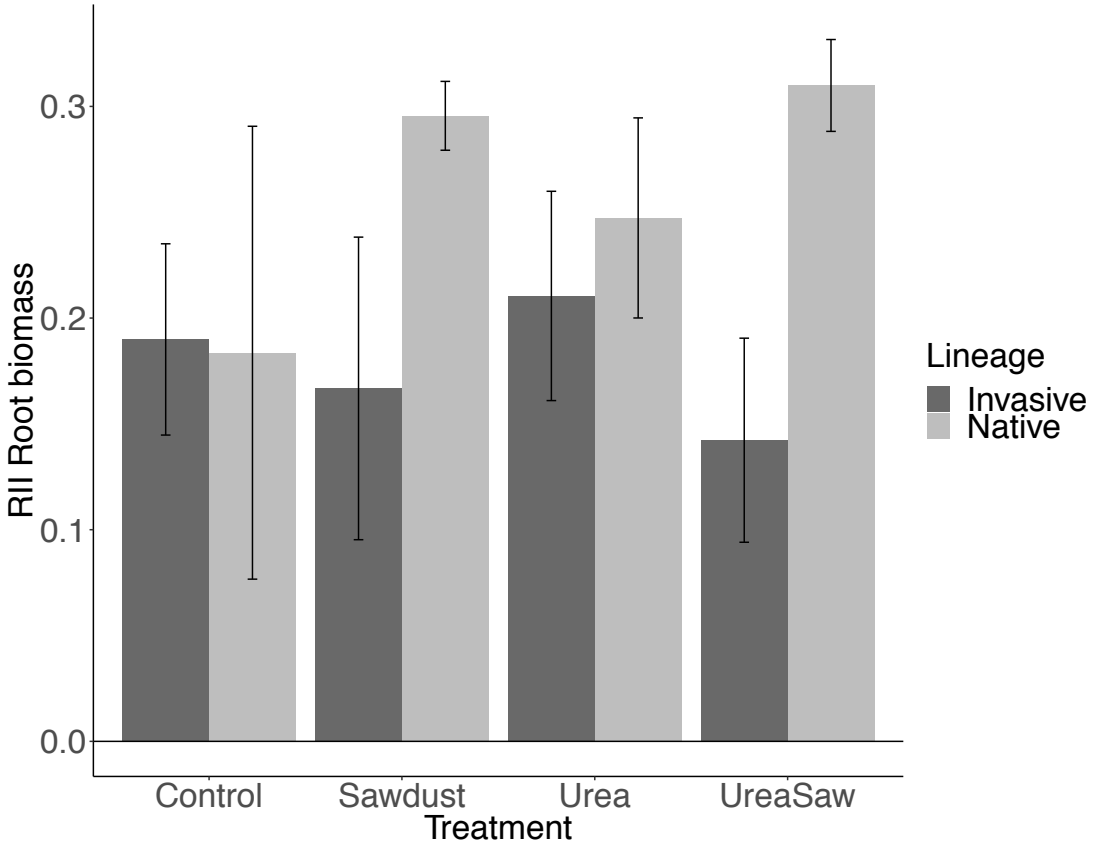
Lineage	Treatment	Mono_Mix	SMR	Stem diameter (mm)	RHMR	Stem count
Invasive	No sawdust	Mixture	0.29±0.00	2.62±0.25	0.45±0.02	10.0±1.1
Invasive	No sawdust	Monoculture	0.18±0.00	3.03±0.09	0.48±0.04	14.8±1.5
Invasive	Sawdust	Mixture	0.28±0.01	2.39±0.12	0.44±0.02	8.3± 1.1
Invasive	Sawdust	Monoculture	0.17±0.02	2.92±0.08	0.47±0.03	13.3±1.7
Native	No sawdust	Mixture	0.31±0.01	2.81±0.26	0.46±0.01	4.6±0.7
Native	No sawdust	Monoculture	0.21±0.00	3.47±0.14	0.40±0.06	9.7±1.1
Native	Sawdust	Mixture	0.30±0.00	2.92±0.10	0.49±0.02	4.3±0.7
Native	Sawdust	Monoculture	0.18±0.00	3.07±0.17	0.56±0.06	9.0±0.4



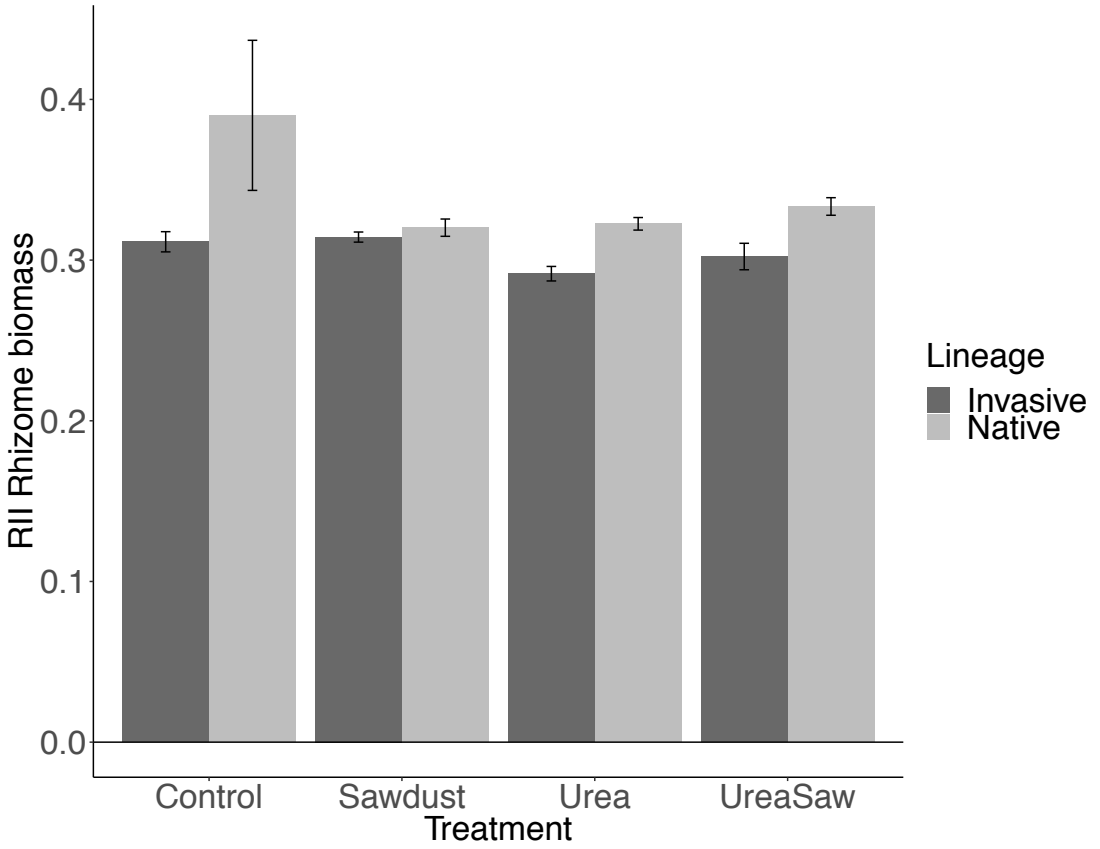
Supplementary Figure 1: Relative Interaction Index (RII) for leaf biomass of native and invasive *Phragmites australis* across C:N treatments. Control=No Urea/Sawdust addition, Sawdust=1.25kg/m^2 , Urea=50g/m^2/year. Bars show standard error. RII values were significantly different from 0 ($t=10.09$, $p<0.001$) and showed no significant differences between lineages or treatments.



Supplementary Figure 2: Relative Interaction Index (RII) for stem biomass of native and invasive *Phragmites australis* across C:N treatments. Control=No Urea/Sawdust addition, Sawdust=1.25kg/m^2 , Urea=50g/m^2/year. Bars show standard error. RII values were significantly different from 0 ($t=10.13$, $p<0.001$) and showed no differences between lineages or treatments.



Supplementary Figure 3: Relative Interaction Index (RII) for root biomass of native and invasive *Phragmites australis* across C:N treatments. Control=No Urea/Sawdust addition, Sawdust=1.25kg/m^2 , Urea=50g/m^2/year. Bars show standard error. RII values were significantly different from 0 ($t=10.13$, $p<0.001$). There were no differences between treatments, but native *Phragmites* had overall greater RII than the invasive ($F_{1,25}=12.82$, $p=0.0014$)



Supplementary Figure 4: Relative Interaction Index (RII) for rhizome biomass of native and invasive *Phragmites australis* across C:N treatments. Control=No Urea/Sawdust addition, Sawdust=1.25kg/m^2 , Urea=50g/m^2/year. Bars show standard error. Means for RII were significantly different from 0 ($t=42.6$, $p<0.001$). The native lineage had greater RII than the invasive ($F_{1,26}=12.45$, $p=0.002$), and there were no differences in RII based on C:N treatments.

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Chapter 4: Dark septate endophyte improves salt-tolerance of native and invasive lineages of *Phragmites australis*

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Abstract

Fungal endophytes can improve plant tolerance to abiotic stress. However, the role of these plant-fungal interactions in invasive species ecology and their management implications remain unclear. This study characterized the fungal endophyte communities of native and invasive lineages of *Phragmites australis* and assessed the role of dark septate endophytes (DSE) in salt tolerance of this species. We used Illumina sequencing to characterize root fungal endophytes of contiguous stands of native and invasive *P. australis* along a salinity gradient. DSE colonization was assessed throughout the growing season in the field, and their role in salinity tolerance was investigated using laboratory and greenhouse studies. Native and invasive lineages had distinct fungal endophyte communities that shifted across the salinity gradient. DSE colonization was greater in the invasive lineage and increased

with salinity. Laboratory studies showed that DSE inoculation increased *P.australis* seedling survival under salt stress; and a greenhouse assay revealed that the invasive lineage had greater aboveground biomass under mesohaline conditions when inoculated with a DSE. We observed that *P.australis* can establish mutualistic associations with DSE when subjected to salt stress. This type of plant-fungal association merits further investigation in integrated management strategies of invasive species and restoration of native *Phragmites*.

Introduction

Fungal endophytes establish mutualistic associations with most plant species, and can play a major role in plant ecology and community structure (86). These endophytes can improve host nutrient uptake (87,88), improve host defense against pathogens (89), modify trophic interactions (90–92), and improve host tolerance to abiotic stress (93,94). At the plant community level, they can affect plant diversity (95,96) and can be important factors in plant invasion ecology (97,98). A better understanding of plant-microbe interactions can help improve various aspects of invasive species management (99). Kowalski et al. (83) recently proposed a framework for a microbial-based control strategy of invasive species; the basis of this strategy is that greater understanding of key microbial association of invasive and native species can lead to new insights of invasive species success and improve management practices.

The aggressive expansion of the invasive European lineage of *P. australis* is an issue in several regions of the United States. Management of this lineage has been costly. Despite agencies spending over $4.6 million/year (78), most eradication

efforts are unsuccessful and focused on short-term results (77,83). Once established, invasive *P. australis* forms dense monotypic stands affecting native plant diversity (47,61,100), hydrology (101), and biogeochemistry (62,102) in invaded areas. Expansion of this lineage has been common in brackish marshes (103,104) and salt marshes, where it can significantly alter ecological functions (50,61).

The native North American haplotype F of *P. australis* (40) is less salt-tolerant than the invasive European haplotype M (45), and is therefore predominantly found in low-salinity habitats (43). Both lineages share the same physiological mechanisms of salt-tolerance, which are K^+ accumulation in plant tissues and Na+ exclusion (105,106); but the growth potential of the invasive lineage has been considered key to its invasiveness at higher salinities (45). Expansion of the invasive lineage into salt marshes has also been related to clonal integration (107) and temporary decreases in soil salinity (47,108). Benefits of microbial associations for salinity tolerance of *P. australis* have been theorized (109) but have not been assessed until this study.

In wetlands, one of the most common groups of root endophytes are dark septate endophytes (DSE). In these systems they are commonly found to coexist with mycorrhizal fungi and are more prevalent in monocotyledonous than dicotyledonous plant species (110,111). Dark septate endophytes are considered generalist root fungi and have been found to associate with over 600 plants, including some that are non-mycorrhizal in various ecosystems (112,113). Based on the classification by Rodriguez et al. (2009) (86), these Class IV endophytes can be characterized as sterile or conidial, they have dark melanized hyphae and microsclerotia, and are likely to

play an important role in plant ecophysiology. Several studies have found DSE colonization is common in plants exposed to abiotic stress (114–116), and experimental inoculation of plants with DSE has been reported to improve host tolerance to heavy metal contamination (117) and drought (118). Some of the possible mechanisms by which DSE can affect host fitness include the production of bioactive compounds (117,119), and increasing nutrient uptake by colonized hosts (87,118). Considering the ubiquitous nature of DSE in wetland grass species and their ability to promote stress-tolerance in various hosts, their associations with wetland plants and potential functional roles merit further investigation. Specifically, their interactions with native and invasive plants like *P. australis* could be of interest to improve management of the invasive lineage as proposed by Kowalski et al. (2015) (83).

In this study we characterized the fungal endophyte communities of contiguous stands of native and invasive *P. australis* across a salinity gradient. We used next generation sequencing and microscopy to address the role of lineage and salinity in structuring root fungal communities over a growing season. In pursuing this objective, we identified salinity-driven DSE colonization patterns that led to a follow up question: Can fungal endophytes improve salt-tolerance of *P. australis*? We hypothesized that DSE mutualists played a role in stress tolerance of the invasive *P. australis* lineage, and used laboratory and greenhouse assays to test this prediction.

Materials and methods

Study sites and sampling

We selected three sites with contiguous stands of native and invasive *P. australis* along a salinity gradient in the Choptank River in eastern Maryland, U.S.A (**Figure 1**). The salinity regimes at these tidal wetland sites range from freshwater (<0.5%) to oligohaline (0.5-5%) (6). During the summer of 2016, we collected rhizomes from native and invasive *P. australis* by excavating the plant and clipping rhizomes that had multiple lateral and fine roots. Four rhizomes were sampled from each stand from plants that were at least 5 m away from each other. Sampling was carried out approximately every two weeks between June and October, resulting in a total of 84 rhizomes of each lineage that were collected for analysis. To monitor water level, we installed loggers (HOBO U20L-04, Bourne MA, USA) in stands of native and invasive *P. australis* at sites A and C, and water level was recorded every 5 minutes from July to October. We calculated the level of inundation for each stand based on the percent time that the water was above the soil surface over the two-week period before each rhizome sampling date. Salinity was recorded at each site using a portable salinity meter (YSI, Yellow Springs OH,USA). We collected soil samples from each site in July and analyzed them for percent nitrogen (%N) and carbon (%C) by combustion at 950°C, pH using a 1:5 soil:DI water slurry and soil organic matter (SOM) using loss-on-ignition (550°C for 24 h). We characterized root morphology of native and invasive *P. australis* based on three samples from each stand and measured lateral root density and length, and root hair density (34).

Root processing and endophyte sequencing

Lateral and fine roots were clipped and separated for different uses. Some were stored in 50% ethanol for staining and microscopy, and the rest were surfaced sterilized and either stored at -80˚C for Illumina sequencing, or used to isolate fungal endophytes. Surface sterilization was carried out as described in Ban et al (2012) (120), with 99% ethanol for 1 m, 35% hydrogen peroxide for 5 m, 99% ethanol for 30 s and washed three times in sterile DI water. We confirmed the success of the root sterilization by imprinting the roots on potato dextrose agar (PDA) and confirming no signs of growth after incubation.

To isolate root endophytes we clipped the ends of the roots and placed them on PDA with ampicillin and streptomycin. Plates were incubated in the dark at 23˚C and fungi that emerged from the roots were transferred to new PDA plates. We characterized the isolated endophytes using Sanger sequencing. Endophyte recovery from the Choptank sites was low, so additional isolates were obtained using the same methods from roots of invasive *Phragmites* that was located at a mesohaline site (8 ppt) on the Patuxent River (N38°32'20", W76°40'3"). We extracted fungal DNA using a Zymo Quick DNA Fungal/Bacterial kit according to manufacturer's instructions. BigDye® Terminator v3.1 (ThermoFisher) was used for PCR amplification using the ITS1F/ITS4R and the EF1-728F/EF1-986R primer sets to amplify the internal transcribed spacer (ITS) region and alpha elongation factor (EF), respectively. EdgeBio cleanup plates were used to recover the cleaned sample, which was then vacuum concentrated using a speedvac, re-suspended in 20 µl of HiDi formamide, and denatured for 2 m at 95°C. We processed the resulting sequences

using SeqScanner v.1.0 (ABI) to check quality, DNAStar to assemble contigs at 97% similarity, and BLAST (NCBI) to assign taxonomy.

Sterile roots from two of our sampling dates (June 30th and August 24th) were used for DNA extractions and subsequent Illumina sequencing of the ITS1 region. We used a PowerPlant Pro DNA isolation kit (MoBio, Carlsbad CA, USA) for DNA extractions and followed the manufacturer's instructions, except for the lysing step, which was carried out using a FastPrep®-24 (two 60 s cycles at 6 m s^{-1}; MP Biomedicals, LLC, Solon OH, USA). We quantified the extracted DNA using a Qubit 2.0 fluorometer and diluted it to 5 ng μl^{-1} for PCR and amplicon sequencing. The ITS region was targeted using the primer+adapter for ITS1F (5'-TCG TCGGCAGCGTCAGATGTGTATAAGAGACAGCTTGGTCATTTAGAGGAAGT AA-3') and ITS2 (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTGCGTTCTTCAT CGATGC-3') with Illumina adapters. The PCR reaction had 3.5 µl of DNA, 17.5 µl of ThermoScientific ™ Phusion™ Flash High-Fidelity PCR Mastermix (Thermo Fisher Scientific), and 7 µl of each primer. PCR products were purified using AMPure XP beads (Beckman Coulter, Pasadena CA, USA) following the Illumina protocol (Part # 15044223 Rev. B, support.illumina.com), and indexed using the Illumina Nextera XT 96 index kit. Samples were pooled, and amplicon size of the library was checked using a Bioanalyzer 2100 (Agilent Technologies). We quantified the library using Q-PCR, and the final library was diluted to 12 pM, spiked with 30% PhiX (Illumina), and ran on an Illumina MiSeq using a 600-cycle v3 cartridge.

Roots that were stored in 50% ethanol were used for fungal colonization assessment using microscopy. They were cleared by autoclaving in 10% KOH for 15 m, acidified in 1%HCl for 20 m, and stained with 0.05% trypan blue for 2 h (121). Roots were de-stained overnight in 50% glycerol and stored in lactoglycerol. Percent colonization of DSE was assessed using the grid intercept method (122), with 100 intersections per slide.

Laboratory and greenhouse

We assessed the salt-resistance of the isolated endophytes from the mesohaline site by growing them in PDA with 200 mM, 400 mM and 600 mM NaCl. We then used all salt-tolerant endophytes in a laboratory experiment to evaluate their effect on survival of *P. australis* under salt-stress. We began by surface sterilizing seeds using 70% ethanol for 2 m, 10% bleach for 5 m and three sterile DI water rinses.

For the laboratory experiment, we germinated sterile seeds of invasive *P. australis* in 1% agar. After germination, four seedlings were transferred to Magenta boxes containing solid MS media with 100 mM NaCl. We then added either a disc of actively growing fungi, or a disc of sterile PDA media as a control next to each seedling. For the less salt-resistant native *P. australis* lineage, we inoculated the seeds prior to adding them to the Magenta Box because 100 mM of NaCl can be very stressful for this lineage (45) and we speculated the endophytes might improve its chances of surviving the transplant. We added the sterile seeds either to PDA plates that had different endophytes actively growing, or to sterile PDA media for control. After 24 h, we transferred the seeds to 1% agar for germination, and then added the

seedlings to Magenta boxes containing solid MS media with 100 mM NaCl and ampicillin. We recorded the number of surviving seedlings of native and invasive *P. australis* after two months. Based on these results, we selected one of the endophytes for a greenhouse experiment to further evaluate its effect on salinity tolerance of invasive *Phragmites*. In addition, we stained a subset of the seedling roots (as described in the previous section), to confirm fungal colonization and DSE classification of the endophytes.

For the greenhouse experiment, sterile seeds of invasive *P. australis* were germinated in 1% agar at 14 h of light and a 30˚/18˚C diurnal temperature shift. We transferred seedlings into Magenta boxes with half-strength Murashige and Skoog (MS) basal salt solid media and ampicillin. After 3 weeks, we planted 23 seedlings into 2 l pots containing a sterile mix of 2:1 Sungro potting soil and sand. Plant height was recorded at the beginning of the experiment and used as a covariate for analysis. One week after planting, we began the endophyte treatment by adding a disc of the selected fungal endophyte that was actively growing on PDA near the base of each plant, or a disc of sterile PDA media for control plants. A week later, we began the salt treatments by adding 100 mM of NaCl to irrigation water and gradually increasing additions by 100 mM weekly until the final treatment levels (Mesohaline=200 mM and Polyhaline=400 mM) were reached. This gave us a factorial design with 3 levels of salinity (Freshwater, Mesohaline, and Polyhaline) and 2 levels of endophyte (Endophyte and No Endophyte). We placed the pots into aluminum pans to collect drainage water, and plants were watered twice weekly with 1 l of tap water. We added NaCl weekly to the irrigation water to maintain salt

treatments, and fertilizer (Jack's All Purpose 20-20-20) was added bi-weekly in the amounts recommended by the manufacturer. After 2 months, plants were re-potted into 4 l pots and we increased watering frequency to 3 times a week. Bi-weekly measurements included plant height, number of shoots, and salinity of the drainage and reservoir water using a portable salinity meter (YSI, Yellow Springs, Ohio). At the end of the experiment, we measured chlorophyll fluorescence as an indicator of stress using a PAM-2100 Chlorophyll Fluorometer (Walz, Effeltrich Germany) on the second collared leaf of two stems per pot. We recorded the quantum yield (Y) during the day, and the maximum quantum yield (F_v/F_m) at night. We harvested the plants after 4 months and recorded leaf number, leaf area (LI-COR LI-3100), total above and belowground dry biomass, number of shoots, lateral root length and density, and rhizome diameter. Total nitrogen and total carbon of leaf tissue was analyzed by combustion using a LECO CN628 analyzer (LECO, St. Joseph, MI, USA).

Data analysis

We used R v.1.0.153 (123) for all data analysis and figure drawings. Paired end sequences from Illumina were processed using the dada2 package (124) and taxonomy assigned using the UNITE database (125). The phyloseq (126) and vegan (127) packages were then used for data analysis and ggplot2 (128) for plotting figures. Samples were rarefied to 14,705 sequences which provided overall good coverage based on rarefaction curves (**Supplementary figure 1**). Samples were then filtered based on prevalence, and taxa with a prevalence of at least 7% was kept. Non-metric multidimensional scaling (NMDS) based on a Bray-Curtis dissimilarity matrix was used to visualize endophyte community composition across sites and between

lineages. Permutational multivariate ANOVA (PerMANOVA) was used to assess differences between communities, and homogeneity of group dispersion was checked using the vegan functions betadisper and permutest. When PerMANOVA was significant, we used pairwise comparisons to contrast the specific factors using the package RVAideMemoire (129). Alpha diversity based on log-transformed observed and Fisher's alpha index was evaluated using ANOVA (type III SS). We used the FunGUILD database (130) to assess differences in functional profiles of fungal communities associated to native and invasive *P. australis*. We used the trophic modes output to evaluate the prevalence of Symbiotrophs, Saprotrophs, Pathotrops and combinations of these in each lineage.

Differences in DSE root colonization across dates and between sites for each lineages were assessed using two-way ANOVA (type III SS) and Tukey's post-hoc means comparisons test. Pearson correlation coefficients were calculated to determine the relationship of percent colonization with sampling date and salinity.

Greenhouse results were first analyzed using ANOVA (type III SS) to evaluate if initial height was a significant explanatory variable for each parameter. When it was, the data were analyzed as an ANCOVA using covariate-adjusted means with the package emmeans (131). Planned pairwise contrasts with a Tukey adjustment were used to assess differences between endophyte treatments at each salinity level. When the initial height was not significant, it was removed from the model and data were analyzed as an ANOVA (type III SS). Variables were log-transformed to meet ANOVA assumptions when necessary.

Results

Site characteristics

Percent carbon (%C), and nitrogen (%N) were higher in Site A, and pH tended to be lower at that site. There were also a few site-specific differences between native and invasive stands, specifically the native stand soils had higher %C in sites B and C and overall higher %N at all sites; and invasive stands had higher %SOM in sites B and C. Root morphological characteristics did not differ significantly overall between lineages or across sites (**Table 1**).

Fungal root endophyte community analysis

After rarefaction and filtering of Illumina sequences there was a total of 165 amplicon sequence variant (ASVs). Most fungal ASVs were present in both lineages (71%) and half of them were found at the three sites. The most abundant Orders were Lulworthiales, Agaricales, Pezizales and Pleosporales (**Supplementary figure 2**). Fungal endophytes communities did not differ between June and August (PerMANOVA, F_{41}=0.99, p=0.46), and were therefore combined for the rest of the analyses. When evaluating beta diversity between the sites and lineages, we found a significant interaction between factors (PerMANOVA, F_{37}=2.2, p=0.001). We then ran separate PerMANOVAs for each lineage and site to evaluate changes in community composition across the salinity gradient. Fungal endophytes associated with native *P. australis* in Site A (~0.7 ppt), differed from those at sites B (~1.2 ppt) and C (~3 ppt) (PerMANOVA, F_{20}=2.49, p=0.002). For invasive *P. australis,* fungal communities only differed between Site A and Site C (PerMANOVA, F_{17}=2.79, p=0.002). Contiguous stands of native and invasive *P. australis* had distinct

endophyte communities in every site (Site A: F_{12}=2.49, p=0.01; Site B: F_{14}=2.69, p=0.007; Site C: F_{11}=2.69, p=0.02) (**Figure 2 NMDS**). Alpha diversity did not differ between lineages or across sites (p>0.05). **(Supplementary figure 3).**

Only 83 (50%) of the ASVs could be assigned to the Genus level, and 55 (33.5%) to Species. This resulted in limited functional profiles from FunGUILD that uses these taxonomic levels to assign putative trophic modes to the fungal species. Based on those assignments, there were no significant differences between functional groups of endophytes of native or invasive *P. australis*. The only exception was the presence of a symbiont in one sample of the native lineage at Site A that had the arbuscular mycorrhizae (AM) *Rhizophagus irregularis* (**Supplementary figure 4**).

DSE colonization

Percent DSE colonization was consistent overall throughout the growing season, and always higher in the invasive lineage (**Figure 3a**). There was a significant correlation between DSE colonization and salinity in the invasive lineage (r=0.47, n=82, p<0.01), but no correlation in the native lineage (r=-0.037, n=79, p=0.75) (**Figure 3b**). Inundation level had no apparent relationship to the observed percent DSE colonization (**Figure 4).**

Endophyte isolation and Sanger sequencing

We isolated 15 fungal endophytes from invasive *P. australis* roots, and were categorized as DSE based on microscopic observation of inoculated plant roots. Sequencing output based on the ITS region resulted in 12 contigs that were predominantly matched to uncultured fungi (**Supplementary table 1**). Sequencing of

the alpha-elongation factor gene did not provide enough resolution to differentiate between any of the isolates.

Endophyte salinity tolerance

Fourteen of the isolated endophytes showed growth in PDA with up to 600 mM NaCl; therefore, all of these were tested in Magenta box laboratory assays to evaluate their effect on seedling survival of both lineages. Seedlings of invasive and native *P. australis* inoculated with endophyte GG2D showed the highest survival relative to the control treatment and other endophytes tested (**Table 2**). Based on these results, endophyte GG2D was selected to further evaluate its effect on salt tolerance of *P. australis* in a greenhouse experiment.

Greenhouse assay

Endophyte inoculation increased aboveground biomass of invasive *P. australis* only at the mesohaline salinity treatment (ANCOVA, contrast t_{15}=2.42, p=0.029) (**Figure 5 a**). This was mainly driven by a significant increase in average stem height (ANOVA, F_{11}=6.77, p=0.039) (**Figure 5 b**) and leaf biomass (ANCOVA, contrast t_{15}=2.58, p=0.021) (**Figure 5 c**), and an increase in stem biomass (ANCOVA, contrast t_{15}=2.1, p=0.053) at that salinity level in inoculated plants. Other aboveground parameters, including number of stems, stem biomass, leaf count, and leaf area, did not differ significantly between inoculated and non-inoculated plants (data not shown).

Belowground parameters including rhizome and lateral root biomass, rhizome diameter, and lateral root length and number, did not differ between control and

endophyte inoculated plants ($p>0.05$, data not shown). Root-to-shoot ratio tended to be lower in endophyte treatments across all salinity levels ($p<0.1$) (**Figure 5 d**). Photosynthetic efficiency based on quantum yield (Y) and maximum quantum efficiency (F_v/F_m) did not differ between salinity treatments (ANOVA, Y: $F_{16}=1.3$, $p=0.29$; F_v/F_m: $F_{16}=0.37$, $p=0.69$), or between inoculated and not inoculated plants (ANOVA, Y: $F_{16}=0.1$, $p=0.75$; F_v/F_m: $F_{16}=1.02$,$p=0.33$).

Total carbon in leaf tissue did not differ between salinity or fungal treatments (ANOVA, $F_{15}=0.28$, $p=0.75$; $F_{15}=0.08$, $p=0.78$; respectively). Total nitrogen differed between salinity treatments (ANOVA, $F_{15}=8.73$, $p=0.003$), and was highest in mesohaline conditions and lowest in freshwater. Total leaf nitrogen did not differ between inoculated and non-inoculated plants ($F_{15}=0.64$, $p=0.44$).

Discussion

Native and invasive lineages of the common reed *P. australis* are colonized by distinct fungal endophytes that can improve the salt-tolerance of these grasses. We observed an increase in DSE colonization with salinity in the invasive lineage, and greater overall colonization of this lineage relative to the native (**Figure 3a**); we speculated this was due to a mutualistic association between DSE and the invasive lineage, likely related to salt tolerance. This warranted further investigation on the potential role of DSE in salinity tolerance of invasive *P. australis*, which has not been considered a relevant factor to explain *P. australis* expansion into saline areas so far.

The 'habitat adapted hypothesis' (93) suggests that plants may associate with endophytes to improve their tolerance to environmental stress, and these endophytes can confer similar stress-tolerance to genetically distant plants. This has been

commonly reported for Class II endophytes that can benefit a host under a specific stress, and induce a similar response in closely related hosts (86,93,132). Similarly, in our study Class IV DSE isolated from roots of invasive *P. australis* improved salt-tolerance of both the invasive and native lineage (**Table 2**). This suggests that DSE mutualisms may be an additional mechanisms of salt-tolerance for *P. australis* that might enhance the invasion of the European lineage. On the other hand, these mutualisms could also be useful in restoration of the native lineage if inoculation improves its survival in areas susceptible to salt-water intrusions (45).

DSE associations can range from parasitic to mutualistic, but are predicted to be primarily the latter in plants under abiotic stress (133). Accordingly, our greenhouse study showed that invasive *P. australis* did not appear to benefit from inoculation under freshwater conditions; but had higher aboveground biomass at mesohaline salinity (**Figure 5**). These results highlight the importance of environmental conditions in the outcome of host-fungal interactions, as has been seen with mycorrhizae and other endophytic fungi (86,134); and can help explain why there are such mixed results in the literature concerning the effects of DSE in its hosts (135,136).

Photosynthetic efficiency is one of the mechanism by which DSE could enhance plant tolerance to abiotic stress (137,138). In our study DSE inoculation had no effect on quantum yield (Y) or maximum quantum yield (F_v/F_m), and did not affect C or N content in leaves; so growth promotion was likely related to other factors. These could include a reduction of reactive oxygen species (ROS) by fungal melanin,

increased osmolyte production, or greater water uptake; as water can be a limiting resource at high salinities.

Fungal inoculation led to an overall decrease in root:shoot ratio relative to non-inoculated controls (**Figure 5**), as inoculated plants generally favored above over belowground growth. This is often observed in mycorrhizal plants where extensive hyphal networks promote water and nutrient uptake (139,140), but a meta-analysis on DSE found no influence of inoculation on plant root:shoot ratio (135). In our study DSE inoculation tended to decrease root:shoot ratio across all treatments, suggesting that preferential allocation of C aboveground was not a result of an imposed abiotic stress, but rather a response of the host to inoculation.

In eutrophic wetlands, like our study system, greater aboveground biomass could translate to a significant competitive advantage. Given the abundance of nitrogen (141) and phosphorus in these wetlands (142), belowground competition for these resources is relaxed, and aboveground competition has a greater role in plant community structure (143,144)(70,71). Based on our greenhouse findings, we propose DSE could play a role in expansion and establishment of invasive *P. australis* into brackish marshes by increasing its competitive ability.

Characterizing microbial communities of the native and invasive lineages, and identifying relevant microbial associations can help improve management of *P. australis* (83,99). Our study characterized fungal endophyte communities of contiguous stands of native and invasive *P. australis* and showed that community composition was lineage and site-specific (**Figure 2**), even though most taxa were present in both lineages and half of them were found at all sites. These results differ

from those reported by Bickford et al. (2018) (145) that found no differences in root fungal endophytes between *P. australis* lineages in the Great Lakes, USA. However, soil saturation was a relevant environmental factor in that study, whereas water level did not appear to play a role in endophyte colonization or community structure in our study sites (**Figure 4**).

Dark septate endophyte colonization has been reported to vary seasonally, showing a decrease at the end of the growing season in alpine plant communities and in a tall grass prairie (146,147). In our study DSE colonization was prevalent in both lineages throughout the growing season (**Figure 3b**), and likely underestimated because our staining method did not allow detection of hyaline hyphae (115). The high prevalence of DSE and lack of evident disease symptoms in colonized plants, suggest a relevant, and yet unexplored, role of these endophytes in our study system even at low salinities.

Our study focused on the effects of DSE on salt tolerance, and specifically looked at NaCl as a stress factor; future research could further address the ecological role of DSE in wetland plants by looking at their effects on sulfide tolerance for example. This would be particularly relevant in freshwater wetlands where salt water intrusion is already affecting coastal biogeochemistry and plant community composition (18,148). *P. australis* growth is inhibited by high sulfide (149), so it would be interesting to know if DSE can also improve this plant's tolerance to sulfide toxicity. Sulfide could also have a negative effect on seedlings that colonize sites after invasive *P. australis* removal (150), so beneficial effects for plant restoration using DSE should also be evaluated.

Identification of our fungal endophyte isolates through Sanger sequencing was not possible primarily due to a lack of representation of these organisms in the database searched. We used two different primer sets for sequencing but were unable to get enough resolution to differentiate among some of the endophytes, even when they showed clear morphological differences. Similarly, only 50% of the most prevalent sequences obtained using high throughput sequencing could be matched at the Genus level, highlighting the lack of database coverage for these types of endophytes.

To conclude, our results indicate that *P. australis* can benefit from DSE colonization when exposed to salt-stress. Therefore, the role of fungal mutualists, particularly in a context of sea-level rise, is worth considering in future studies of invasion ecology, species management, and restoration of native plants.

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Competing interest

The authors declare no competing of interests.

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Table 1: Site and root morphology characteristics of native and invasive *Phragmites* stands. Different letters indicate significant mean differences ($p<0.05$).

Site and *P. australis* lineage	Site A		Site B		Site C	
	Invasive	Native	Invasive	Native	Invasive	Native
Average salinity (ppt)	0.7±0.2	0.7±0.2	1.2±0.2	1.2±0.2	3±0.6	3±0.6
pH	6bc	5.7c	6.5a	6.3ab	6.5a	6.7a
%SOM	32.9a	31.1a	20.8b	15.9d	25.7c	21.0b
%C	16.7a	16.5a	7.9b	10.6c	10.9c	12.9d
%N	1.2a	1.3b	0.6c	0.8d	0.8e	0.9f
Lateral root density	29.7a	13.7a	29.3a	21a	18a	27.5a
Lateral root length (cm)	5.4ab	5.9ab	4.3b	6.1a	4.1b	5.2ab
Root hair density	45.8a	9.1b	24.3b	25.3b	24.3b	13.2b

Table 2: Percent surviving seedlings in Control (non-inoculated) and DSE inoculated treatments for each lineage of *Phragmites australis* growing in MS media with 100mM NaCl. Numbers in parenthesis indicate the number of surviving seedlings over the total seedlings tested. For the invasive assay seedlings were added to 4 Magenta boxes, but some of the boxes were excluded due to contamination. For the native assay total numbers vary based on germination success, and NA indicates no seeds germinated after inoculation with that specific endophyte. Endophyte GG2D (bolded) was the selected endophyte for the greenhouse assay.

Endophyte	Invasive	Native
Control	50% (2/4)	13% (1/8)
GG2D	**100% (4/4)**	**86% (6/7)**
GG1E	67% (2/3)	25% (2/8)
GN	33% (1/3)	0% (0/3)
GG4B	67% (2/3)	67% (2/3)
GGI9	0% (0/4)	40% (2/5)
GG7A	33% (1/3)	50% (2/4)
GGID	67% (2/3)	50% (3/6)
GG3	25% (1/4)	NA
GG8	33% (1/3)	NA
BN3	0% (0/3)	NA
GG2C	33% (1/3)	NA
GG9	33% (1/3)	NA
GG2	0% (0/3)	NA

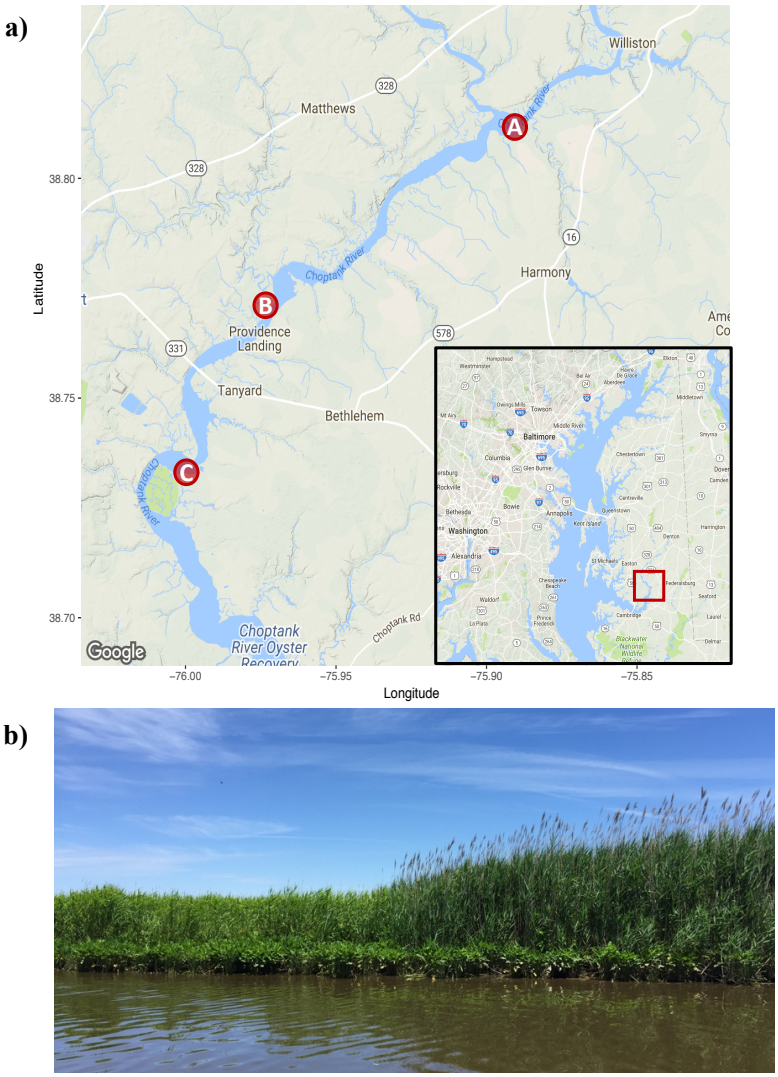


Figure 1: a) Sites located along the Choptank River in the Chesapeake Bay following a salinity gradient over 12.5 km (Site A=~0.7 ppt, Site B=~1.2 ppt and Site C=~ 3 ppt). **b**) Example of contiguous stand of native (left, shorter) and invasive (right, taller) *P. australis* in Site B.

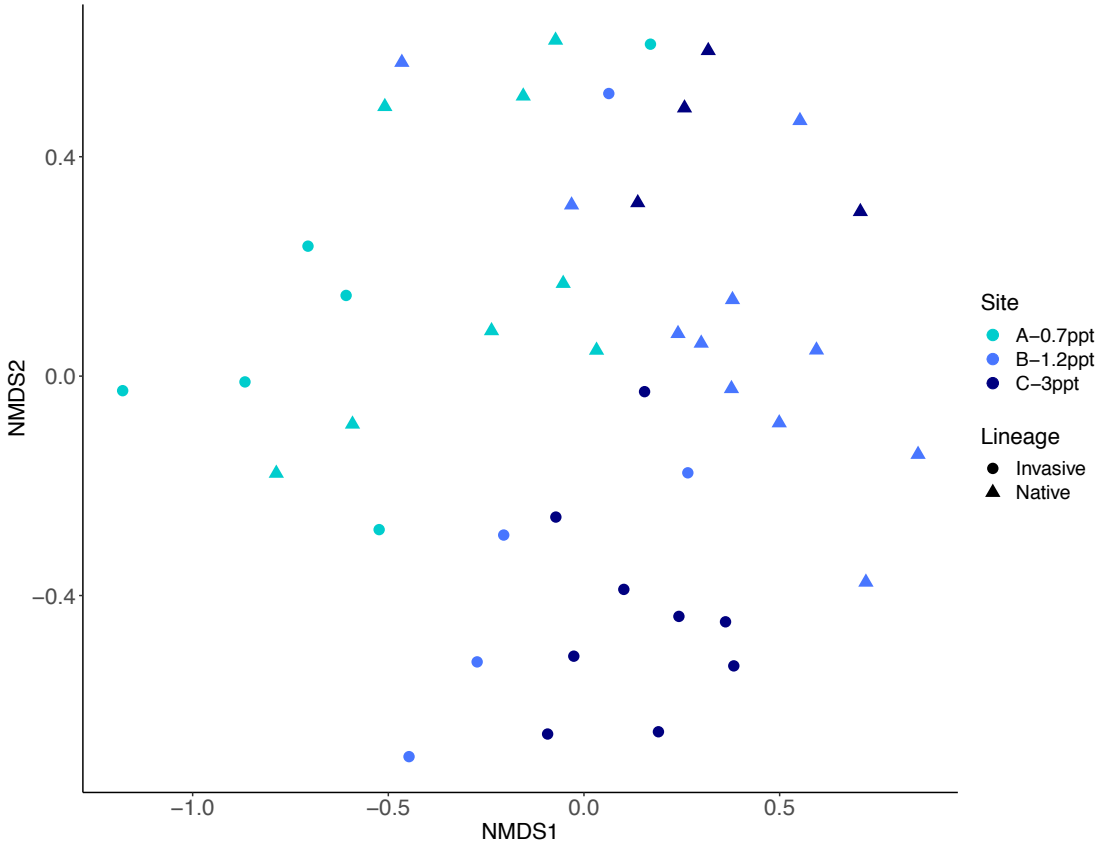


Figure 2: Non-metric multidimensional scaling plot of fungal root endophyte communities associated with native and invasive lineages of *Phragmites australis* across a salinity gradient.

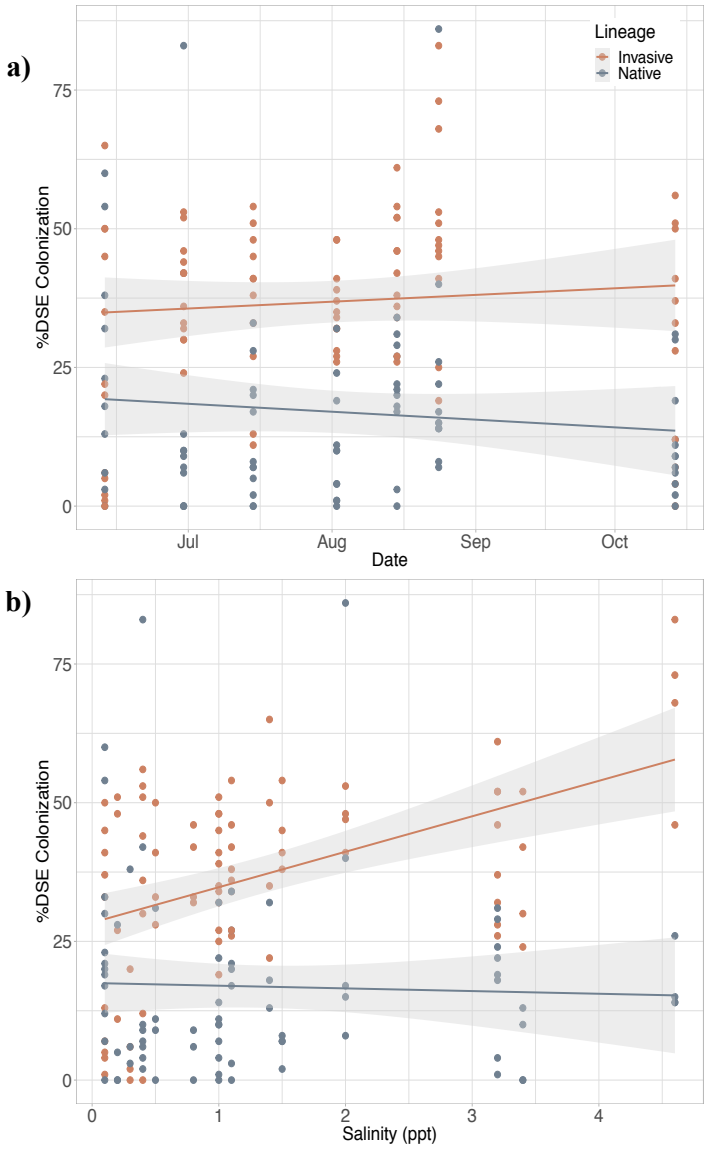


Figure 3: Percent dark septate endophye (DSE) colonization of native and invasive lineages of *Phragmites australis* **a**) across the growing season and **b**) at increasing levels of salinity.

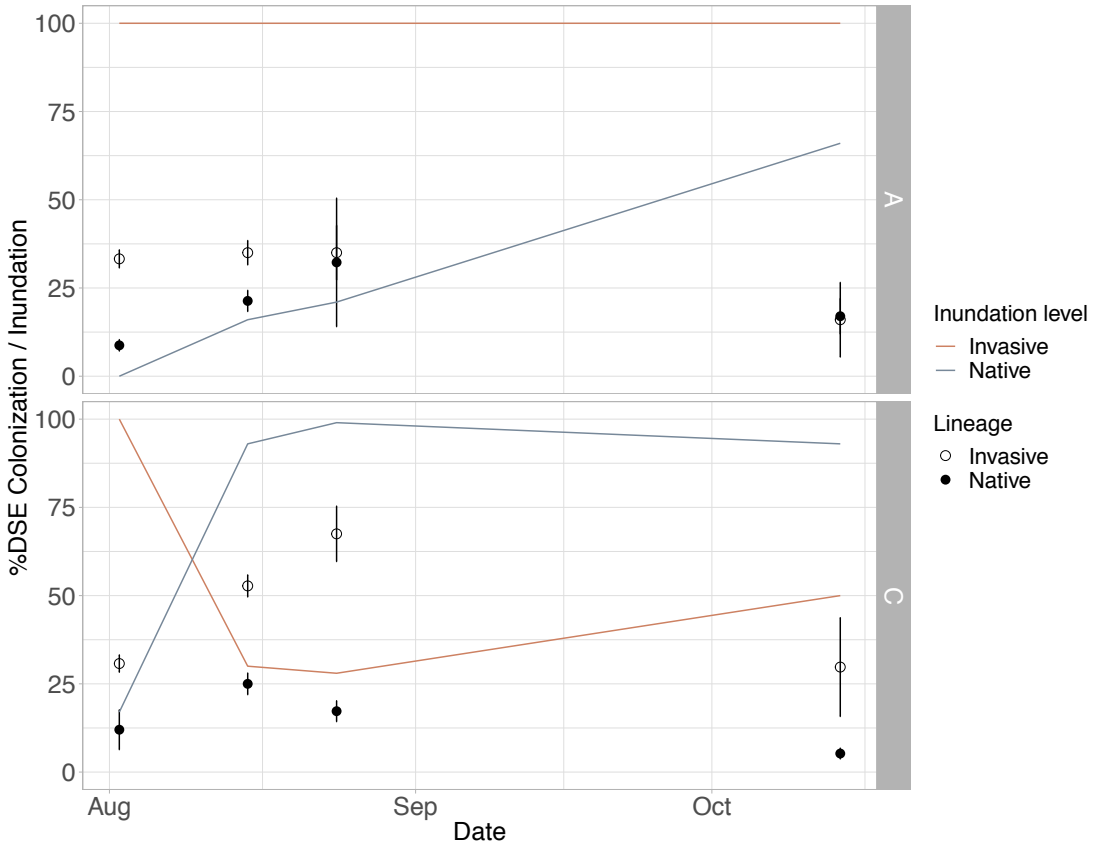


Figure 4: Percent dark septate endophyte (DSE) colonization and percent inundation over time at Site A (~0.7 ppt) and Site C (~3 ppt). The y-axis shows either the %DSE Colonization or the % Inundation (percentage of time when the water was above the soil surface in the 14 days prior to sampling). The lines indicate the %Inundation over time in loggers placed in Sites A (top) and C (bottom) in native and invasive *Phragmites* stands. The circles show the %DSE Colonization for each lineage at the different sampling times, and the error bars shows the standard error.

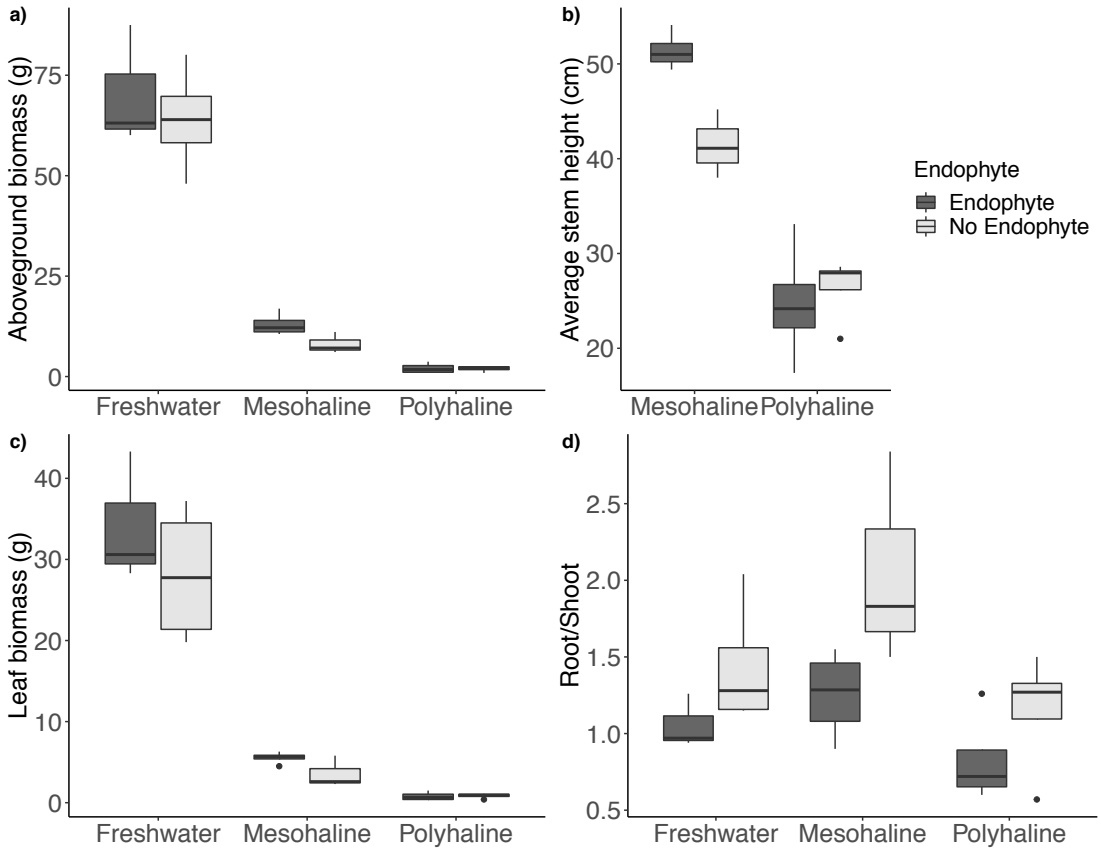


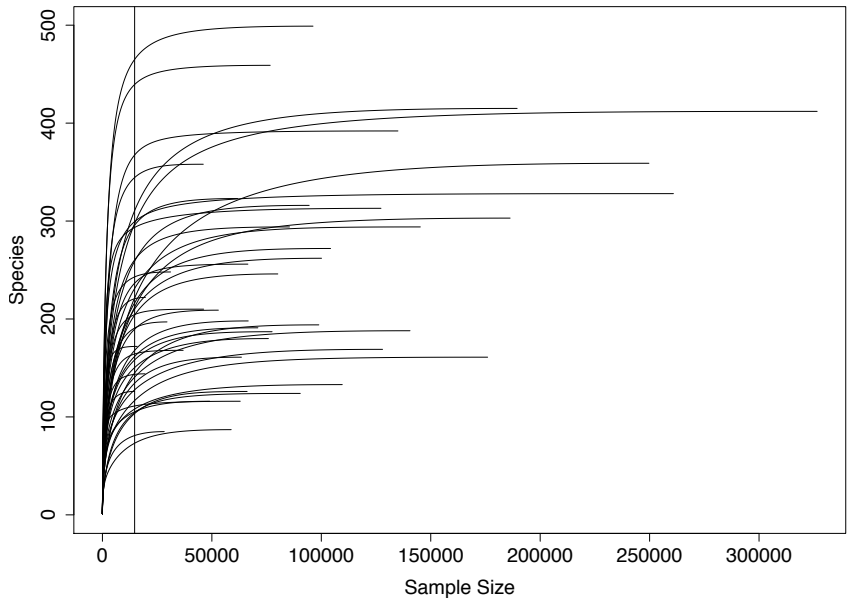
Figure 5: Boxplots showing the effects of dark septate endophyte inoculation of invasive *P. australis* at different salinity levels (Freshwater: No added NaCl, Mesohaline: 200 mM NaCl and Polyhaline: 400 mM NaCl) on a) aboveground biomass, b) average stem height, c) leaf biomass and d) root:shoot ratio.

Supplementary Table 1 Results of BLAST search of Sanger sequences from the ITS region of fungal endophyte isolates of invasive *Phragmites australis*.

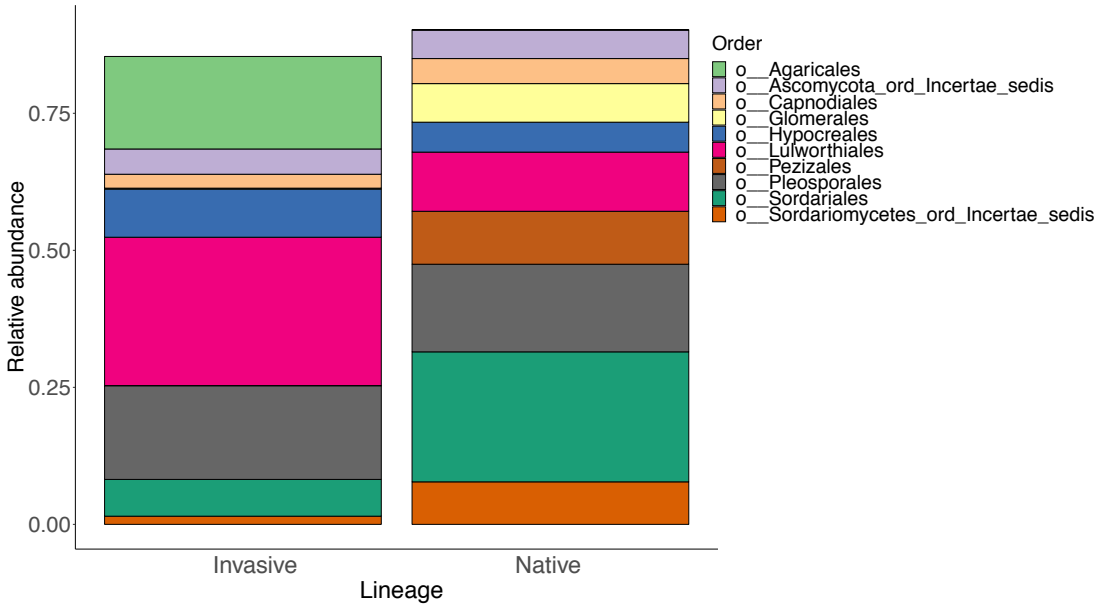
Endophyte	Similarity and Closest BLAST match	Characterization	Reference
GG2D	97%: Fungal endophyte voucher ARIZ:DM0192 18S ribosomal RNA gene and internal transcribed spacer 1, partial sequence	Fungal endophyte isolated from healthy, mature submerged root of *Persicaria amphibia* in Willow Creek Reservoir, Arizona Taxonomy: NA	Sandberg DC, Battista LJ, Arnold AE. Fungal endophytes of aquatic macrophytes: diverse host-generalists characterized by tissue preferences and geographic structure. Microb Ecol. 2014;67(4):735–747. Accession number: KF673730.1
GGIE, GG4B, GGI9, GGID, GG8, BN3, GG2C, GG4A, GG10	874%-93.1%: Fungal sp. strain S184S internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	Cultured fungus isolated from *Ammophila breviligulata* Taxonomy: NA	David, AS, Seabloom, EW, May, G. Disentangling environmental and host sources of fungal endophyte communities in an experimental beachgrass study. Mol Ecol. 2017; 26: 6157– 6169 Accession number: KU839097.1
GG7A	96%: Fungal sp. 51 SAB-2015 strain SV664 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.	Isolated from *Spartina alterniflora* in Barataria Bay, USA Taxonomy: NA	Kandalepas D, Blum MJ, Van Bael SA (2015) Shifts in Symbiotic Endophyte Communities of a Foundational Salt Marsh Grass following Oil Exposure from the Deepwater Horizon Oil Spill. PLoS ONE 10(4): e0122378. Accession number: KP757570.1

Supplementary Table 1 (cont.)

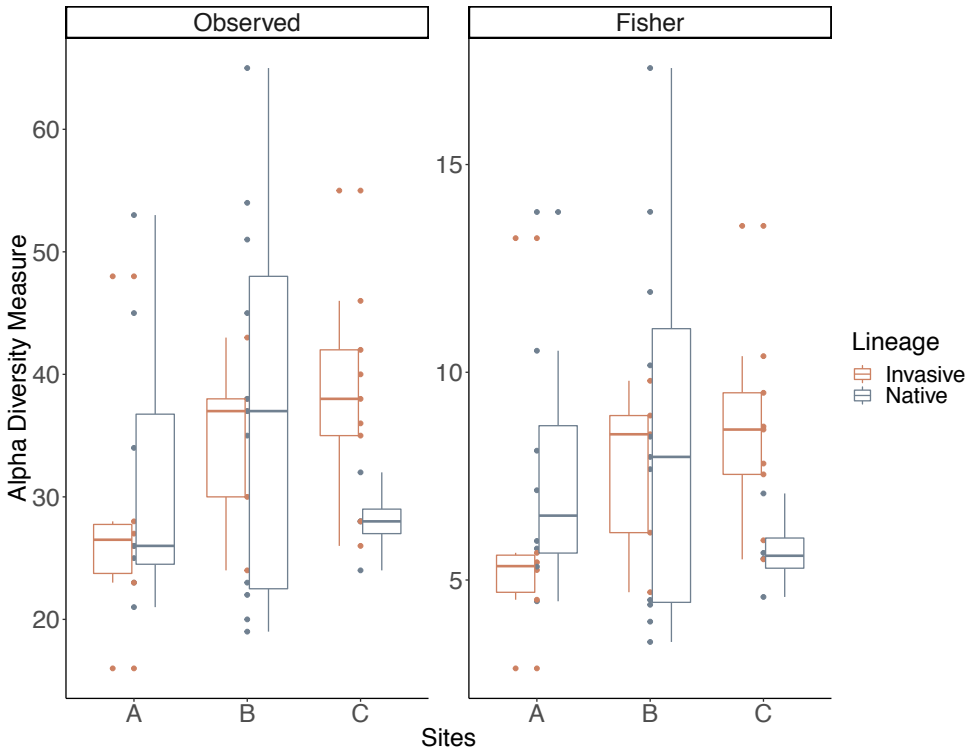
GG9	98.1%: Trematosphaeria hydrela genomic DNA sequence contains ITS1, 5.8S rRNA gene and ITS2, isolate F259	Isolated from *Arabis alpina* surface sterilized roots Taxonomy: Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Pleosporomycetidae; Pleosporales; Massarineae; Trematosphaeriaceae; Trematosphaeria	Almario, Juliana & Jeena, Ganga & Wunder, Jörg & Langen, Gregor & Zuccaro, Alga & Coupland, George & Bucher, Marcel. (2017). Root-associated fungal microbiota of nonmycorrhizal Arabis alpina and its contribution to plant phosphorus nutrition. Proceedings of the National Academy of Sciences. 114. Accession number: LT821517.1
GG3	97.2%: Cf. Phialocephala sp. AU_BD15 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	Taxonomy: Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Leotiomycetes; Helotiales; Helotiales incertae sedis; Phialocephala.	Phialocephala sp. strain AU_BD15 Culture collection: Gareth Griffith, Wales, UK Accession number: JN995646.1
GN, GG2	NA	Excluded from analysis due to poor sequencing quality	



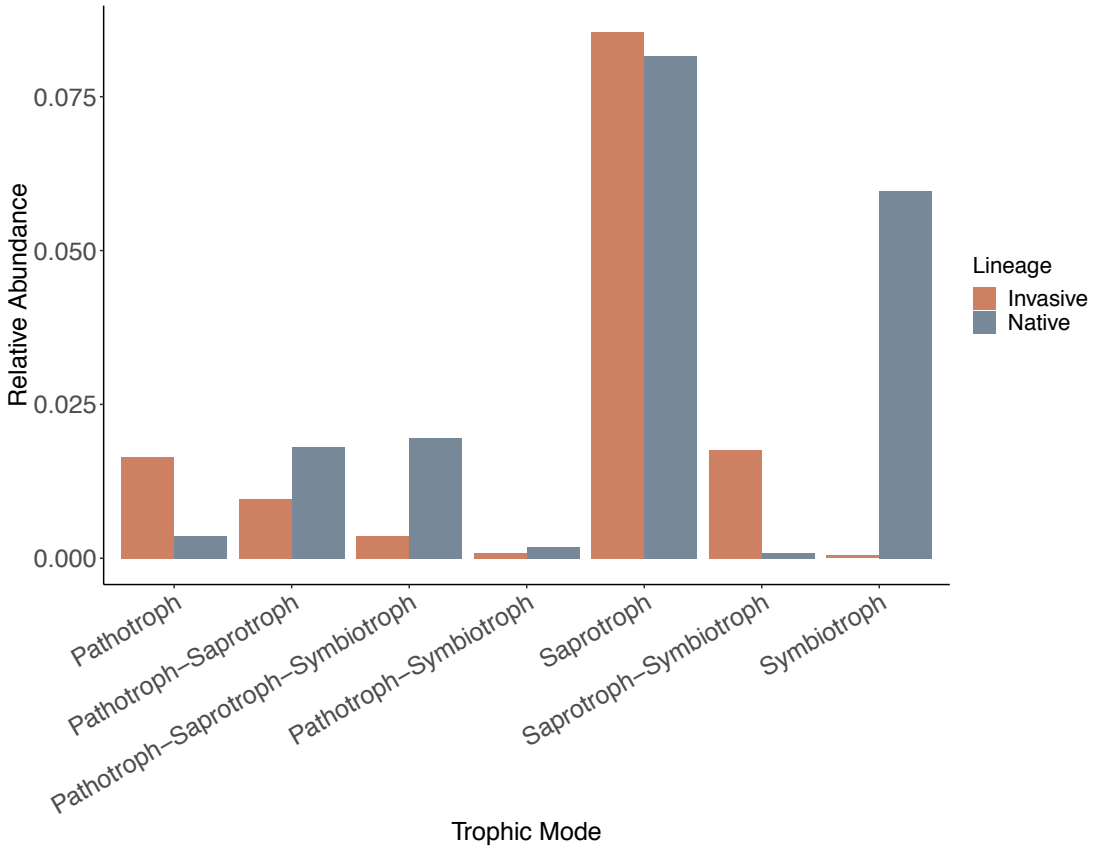
Supplementary Figure 1 Rarefaction curves for ITS Illumina sequences showing cutoff at 14,705 sequences.



Supplementary Figure 2: Relative abundance of the ten most abundant Orders of fungal root endophytes found in native and invasive lineages of *Phragmites australis*



Supplementary Figure 3: Alpha diversity of fungal root endophytes of native and invasive *Phragmites australis* across a salinity gradient (A=~0.7 ppt, B=~1.2 ppt and C=~3 ppt). Diversity was measured at each stand and is based on Illumina sequencing results.



Supplementary Figure 4: Trophic mode assignment for fungal root endophytes of native and invasive *Phragmites australis* based on the FunGUILD database. The figure is based on 85 of the 165 sequences that could be matched to the FunGUILD database.

Chapter 5: Conclusions

Tidal freshwater wetlands provide key ecosystem services, but their location near urban centers makes them susceptible to degradation from human activities and development. Inputs of contaminants and nutrients into these systems results in changes in microbial communities, biogeochemical cycles and native plant communities which have become increasingly threatened by non-native plant invasions. In this dissertation we first looked at overall differences between microbial communities in urban and rural wetlands, emphasizing how nutrient and pollutant inputs affect community structure and function. We then focused on different aspects of the invasion of *Phragmites australis* which is considered to be linked to increased anthropogenic disturbance, and specifically N enrichment in the Chesapeake Bay. We explored the potential use of a novel management approach using a carbon-rich amendment to immobilize N, and assessed its effect on the competitive interactions between native and invasive *Phragmites*. Lastly, we studied the role of plant-fungal interactions in the invasion process of *Phragmites*, and evaluated their role in salt tolerance of this plant.

Chapter 2 summary

In chapter 2 we looked at the microbial community structure across an urban gradient in tidal freshwater wetlands located in Washington D.C., USA and Buenos Aires, Argentina; two major cities located at similar latitudes north and south. We hypothesized that microbial communities would differ across the proposed gradient,

and between two plant species at each site, and that there were specific microbial taxa that could serve as indicators of urban wetlands. To test these hypothesis we identified an urban, suburban and rural wetland near each city and collected rhizosphere soil at each of those sites from either *Phragmites australis* and *Peltranda virginica* in the U.S., or from *Hymenachne grumosa* and *Sagittaria montevidensis* in Argentina.

16S rDNA gene sequencing confirmed that the microbial communities differed across the urban gradient at both locations, but we did not detect significant differences between plant species at each site. Nitrifying bacteria were identified as indicators of urban wetlands, and *Methanobacteria* that carry out methanogenesis were indicators of rural wetlands. We also identified a decrease in methanogens and an increase in ammonia-oxidizers across the rural-urban gradient, which was likely a result of higher nitrogen inputs into urban waters that increased the competition between denitrifiers and methanogens. Analysis of the KEGG functional profiles further revealed that nitrification and xenobiotic degradation pathways were more abundant in urban than rural sites. This was an interesting but expected pattern considering that these are known contaminants in both of the urban wetlands surveyed.

This chapter explored how N inputs and other anthropogenic disturbances can affect microbial structure and function in freshwater tidal wetlands, and identified similar patterns even in urban wetlands that were geographically distant. Based on these results we propose that microbial communities in urban wetlands might be

carrying out different functions that those in rural areas; and microbial communities could be used as indicators of wetland function.

Chapter 3 summary

This chapter evaluated the potential of sawdust application as a soil amendment to limit the growth of invasive *Phragmites australis* while improving the competitive ability of the native lineage. We set up a greenhouse competition experiment to assess the interaction between lineages under varying C:N ratios, and in monoculture or mixed planting arrangements.

We were surprised to find that plants growing in mixed arrangements showed facilitative interactions and had greater above and belowground biomass when compared to monocultures regardless of C:N treatments. This was unexpected considering that many reports in the literature suggest that invasive *Phragmites* competitively excludes the native lineage, especially under high N conditions. We propose that this was due to non-kin recognition that might promote effective niche partitioning of belowground resources resulting in greater biomass in the mixed treatments. Another interesting find was that both lineages allocated more resources to rhizomes than lateral roots in the mixed planting treatments across C:N, suggesting that persistence in the environment might be prioritized through investment in these belowground storage structures.

Our field observations also corroborate the lack of competitive exclusion of the native lineage in invasive dominated sites. We have identified neighboring stands of native and invasive *Phragmites* that have remained relatively unchanged for at

least 15 years, and have seen native *Phragmites* grow and expand when planted in the middle of a stand of the invasive lineage in one of our field sites. This supports the results from other studies that suggest that local extirpations of native *Phragmites* populations are likely mediated by disturbance and subsequent rapid growth of the invasive lineage, and is not just a result of competitive exclusion.

Concerning our C:N treatments, we found that urea addition overall increased aboveground and rhizome biomass in both lineages as expected, but sawdust addition did not limit the growth of the invasive lineage or favor the native as we initially hypothesized. Therefore at the levels studied, C additions would not be recommended as a management tool for *Phragmites*.

Chapter 4 summary

In chapter 4 we characterized root fungal endophytes in native and invasive *Phragmites australis*. We collected root samples throughout a growing season from contiguous stands of each lineage at three sites that varied in salinity, and used ITS sequencing to characterize the fungal communities, and microscopy to determine fungal colonization. Interestingly, we found that each lineage selected for specific endophytes in its roots and therefore harbored different fungal communities, even though the overall pool of available fungal species was almost the same. Our microscopy results revealed that invasive *Phragmites* had consistently higher colonization by dark septate endophytes (DSE) throughout the growing season, and colonization was not dependent on water level as has been seen in other plant-fungal systems. We also noted that colonization of the invasive lineage increased with

salinity, and hypothesized that DSE might have a role in salt tolerance of invasive *Phragmites.*

To explore the role of DSE in salt tolerance, we isolated fungal endophytes from the invasive lineage and set up a laboratory study where we inoculated the isolates into seedlings of either native or invasive *Phragmites*. Our results showed that seedling survival increased for both lineages, so we followed up with a greenhouse assay to assess the effect of one of the endophytes in salt-tolerance of the invasive lineage. Our greenhouse results showed that under mesohaline conditions invasive *Phragmites* had greater aboveground biomass when inoculated with a DSE supporting the role of these endophytes in plant stress tolerance.

These results suggest that fungal mutualists could be an additional, and yet unexplored mechanism that could allow invasive *Phragmites* to establish in brackish and salt marshes and improve its ability to compete against native vegetation by increasing its aboveground biomass under stress.

Final thoughts

Our results suggest that tidal freshwater wetlands in urban settings harbor distinct microbial communities that reflect the common environmental stressors in those areas. Nitrogen enrichment was one of the main stressors and both indicator taxa and functional profile analysis supported this. Future studies in other urban freshwater wetlands could further explore the relationship between microbial community structure and wetland function, specifically concerning processes like nitrification and pollutant removal.

Nitrogen inputs into the Chesapeake Bay are considered to be one of the causes of invasive *Phragmites* expansion. We explored whether immobilizing N through C additions could alter plant competitive interactions of native and invasive *Phragmites australis*. However, our results suggest that N immobilization through sawdust addition may not be an effective method for controlling the spread of this lineage. Native and invasive *Phragmites* responded similarly to sawdust addition, and at the levels tested it did not favor the native lineage as hypothesized. Regardless of treatment additions, our study found that native and invasive *Phragmites* established facilitative interactions, and we consider it unlikely that the invasive could competitivity exclude the native in the absence of disturbance. The mechanisms behind the observed facilitation could be further investigated to evaluate if niche partitioning or kin recognition play a role in that interaction. This could be achieved by growing native *Phragmites* with other wetland native species as well as with the invasive and evaluating differences in biomass production and resource allocation.

Our study also assessed the role of fungal endophytes in the expansion of the invasive lineage into brackish areas and found that DSE could increase aboveground biomass under moderate salt stress, possibly giving the invasive lineage a competitive advantage. Native *Phragmites* seedlings also benefited from fungal inoculation, so we suggest that DSE inoculations could be explored as a tool for restoration of this species or to improve its fitness in a context of sea-level rise. Furthermore, it would be interesting for future studies to look at the role of DSE in salinity tolerance of other grasses, and particularly crops in areas susceptible to sea level rise. For example, in eastern Maryland farmers are already experiencing problems growing

crops as their sources of freshwater are becoming increasingly more saline. If DSE inoculations prove to be effective in grass crops, they might provide at least a temporary solution to decreasing yields at moderate salt levels.

In conclusion human disturbances like increased nitrogen inputs into tidal freshwater wetlands have altered microbial and plant communities, changing the structure and function of these systems. Efforts to restore these wetlands should focus on preserving valued ecosystem functions such as N or contaminant removal in urban sites, while also maintaining a diverse plant community that can harbor the great biodiversity that characterizes freshwater wetlands. A better understanding of the role of plant-microbe interactions in plant fitness could be valuable for both control and restoration of wetland plant species.

Appendices

Chapter 2 Supplemental figures:

Figure 1: Set-up of experimental plots in Jug Bay Wetlands Sanctuary (MD,USA) to replicate C:N and planting treatments presented in Chapter 2. Vegetation in the established $1m^2$ plots was mowed and plots were covered with plastic for ~3 weeks before planting.

Figure 2: Photographs of plots showing four plantlets of either monocultures of each lineage of mixed plantings (2 of each lineage) in plots urea (left) or sawdust (right) as C:N treatments.

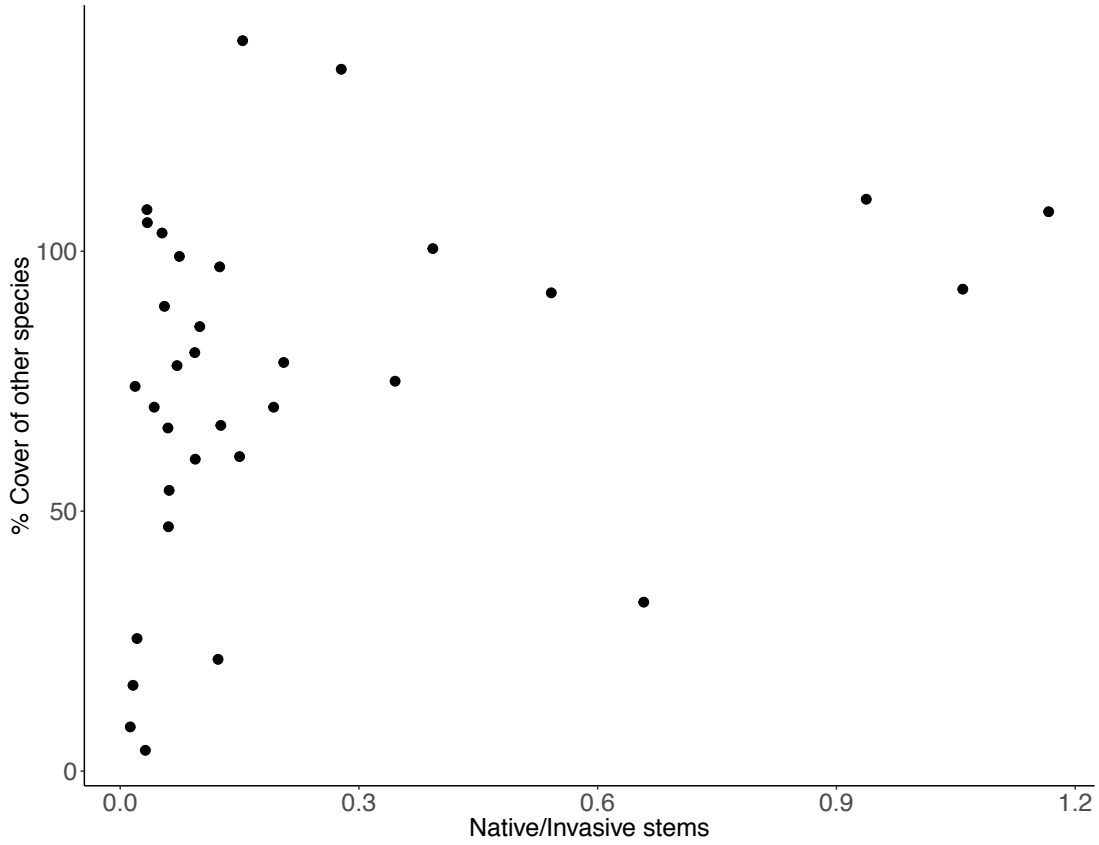


Figure 3: Percent cover of non-*Phragmites* species across different stem ratios of native and invasive *Phragmites* in mixed planting treatment plots established in Jug Bay Wetlands Sanctuary (MD, USA) after one year.

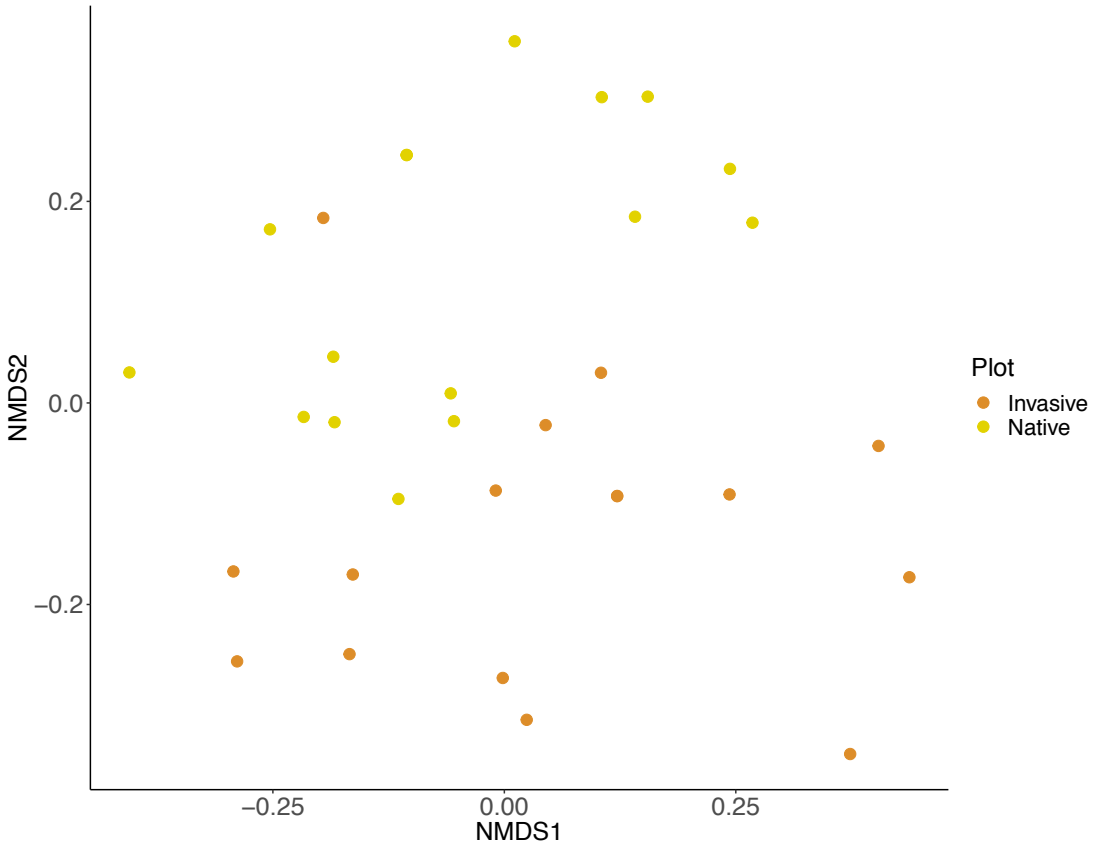


Figure 4: Results of non-parametric multidimensional scaling (NMDS) using Jaccard's index (presence-absence matrix) showing plant community composition of plots established in Jug Bay Wetlands Sanctuary(MD, USA) that had either only invasive *Phragmites* or both native and invasive stems. Stress=0.194

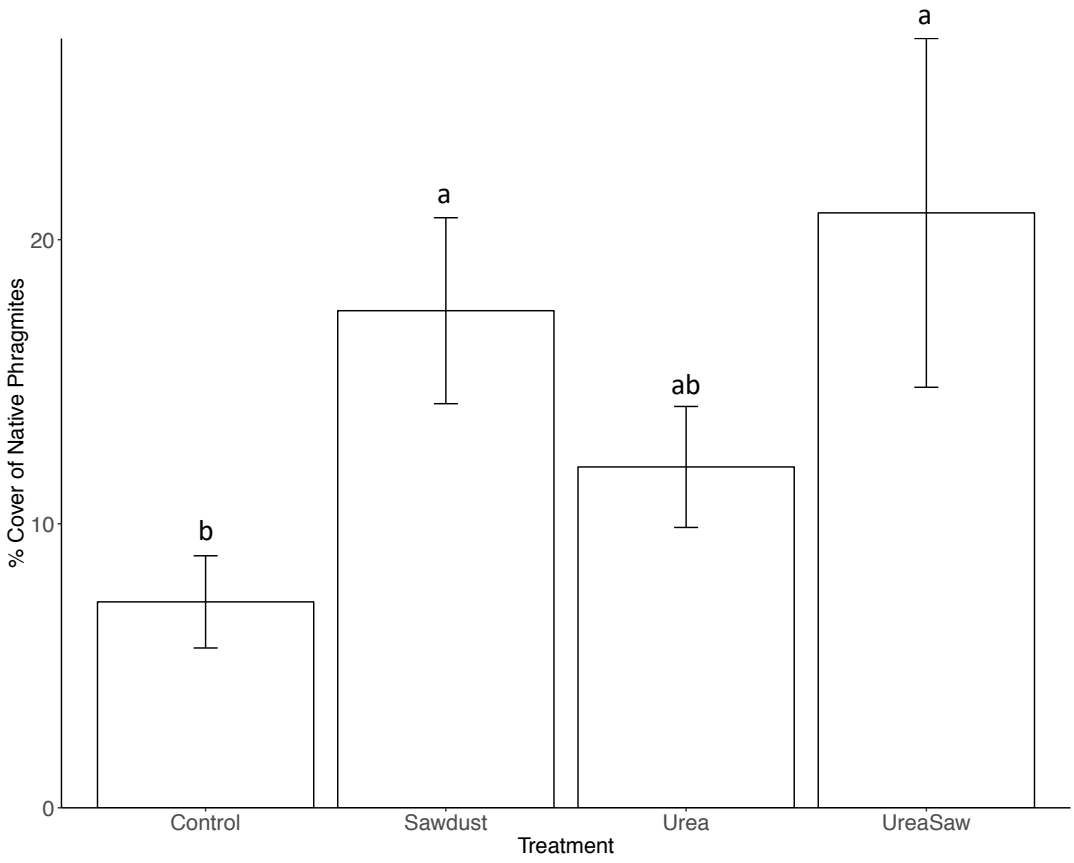


Figure 5: Mean percent cover of native *Phragmites* after one year of C:N treatment application in Jug Bay Wetlands Sanctuary, MD,USA. Different letters indicate significant differences (alpha=0.05).

Figure 6: Native *Phragmites* growing in between invasive *Phragmites* in Jug Bay Wetlands Sanctuary (MD,USA) after 4 years of planting.

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