PHRAGMITES AUSTRALIS SEEDLING RECRUITMENT IN A CHANGING WORLD

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ABSTRACT

Phragmites australis is a cosmopolitan C3 perennial grass that has become invasive in North American wetlands and now span across the continental United States. Its spread can be exacerbated by anthropogenic factors and the microbial communities it cultivates. The spread of *Phragmites* is mainly driven by seedling recruitment, which is influenced by reproductive output, seedling survival, and seedling performance. Here I study how *Phragmites* seedling recruitment varies across region by performing a survey of reproductive traits and seedling growth experiment of *Phragmites* spanning three regions in North America. We also assess how anthropogenic factors (CO_2 and nitrogen) affect seedling recruitment by collecting reproductive traits and performing a seedling growth experiment from *Phragmites* populations exposed to long-term global change factor. Lastly, we assess how *Phragmites* foliar microbial communities are recruited and how they vary between regions using a regional survey. We find evidence that *Phragmites* populations from the Southeast have a high potential for seedling recruitment, as they exhibit high reproductive output and seedling growth responds positively to nitrogen fertilization. We discover that long-term nitrogen fertilization increases reproductive output in *Phragmites*, and that elevated CO_2 has the potential to increase belowground growth in *Phragmites*. We uncover that lower leaf communities from older and more polluted sites may contribute to higher proportions in pathogenic foliar fungi in *Phragmites.* This work contributes to our current understanding of regional variation in both *Phragmites* seedling recruitment and foliar microbial communities as well as contributing to our understanding of *Phragmites* under future global change.

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INTRODUCTION

Human activity has unequivocally changed the abiotic and biotic landscape of earth. Anthropogenic activity is increasing nutrient pollution and greenhouse gas emissions which are the driving force behind the global change and are deemed Global Change Factors (GCF). These GCFs are altering the chemistry of the atmosphere, hydrosphere, and edaphic conditions across the globe. Globalization has also increased the rate of biological invasions bringing about negative economic and ecological ramifications. Recently, studies on the effect of GCFs on invasion have demonstrated that GCFs can facilitate the invasion of introduced species through direct effects such as growth promotion or indirect effects such as through changing microbial communities.

The Eurasian lineage of the cosmopolitan C3 grass, *Phragmites australis,* has become one of the most invasive wetland species in North America since its introduction in the 19th century, especially in the Northeast and Midwest region. *P. australis* has a high vegetative growth rate which is only increased in response to GCFs, making it highly competitive against native wetland plants. In addition, it has a high reproductive potential and is readily establishing new populations through seedling recruitment. Because of its widespread distribution and its positive response to GCFs, it offers a unique model to study regional phenotypic variation in fecundity in response to GCFs. As microbial interactions with plants can influence invasion outcomes it is important to assess the composition of microbiomes in invasive plants. Here I use widespread geographic fecundity surveys, seedling growth experiments, long-term ecological

research experiments, and microbiome analysis to explore the spatiotemporal patterns in in *Phragmites* fecundity and seedling performance.

In my first chapter I study the regional variation in fecundity and seedling performance in introduced European *Phragmites* (hereby *Phragmites*) across three regions (Northeast, Midwest, and Southeast) of the continental United States. These regions vary in intensity and history of *Phragmites* invasion, thus assessing variations in fecundity and seedling performance may inform these observed patterns. I also test for variations in seedling response to nitrogen to assess how different regions will respond under increased anthropogenic nitrogen pollution. I collected inflorescences from 34 sites across the three regions and measured seed and inflorescence traits to assess fecundity. I also performed a seedling growth experiment under two nitrogen levels to assess regional phenotypic variation in seedling performance under different nutrient regimes. I uncover region*nitrogen interactions which point to potential regional differences in seed viability and seedling response to nitrogen which may have implications on future invasion patterns. This study highlights regional variation to GCF response in invasive plants and their complex interactions in different life stages.

In my second chapter, I test the temporal effects of long-term exposure to two GCFs, nitrogen and CO_2, on *Phragmites* fecundity and seedling performance. I collected inflorescences at the Invasive *Phragmites* Experiment at the Global Change Research Wetland (GCReW) Long-term Ecological Research (LTER) site where *Phragmites* has been exposed to over 10 years of elevated/ambient CO_2+nitrogen. These inflorescences were used to assess fecundity and seedling performance under subsequent GCF exposure. I found that elevated nitrogen increased inflorescence size, leading to a higher potential to recruit seedlings. Seedlings displayed some level of acclimatization to long-term exposure to GCFs, however CO_2 consistently increased

belowground biomass. This study is the first to utilize the LTER network to study chronic GCF exposure on fecundity and seedling performance and highlights the dynamic changes in seedling recruitment under projected global change.

In my third chapter I explore foliar and litter fungi and bacteria associated with *Phragmites* by assessing composition, diversity, and trophic composition across *Phragmites* leaf/litter age across two focal invasion regions (New Jersey and Michigan). We find that *Phragmites* recruits most of its foliar fungi and bacteria as it ages and we find a positive association between leaf age and alpha-diversity. Pathogen accumulation patterns varied across our two regions pointing to possible influence of invasion history and anthropogenic factors. We uncover a decoupling of bacterial and fungal beta-diversity patterns across our two regions, where populations in New Jersey experience lower fungal beta-diversity. While most studies in *Phragmites* focus on soil microbes, our study explores the foliar component of the *Phragmites* microbiome, and we uncover spatial patterns in fungal and bacterial composition and recruitment.

CHAPTER 1. THE REGIONAL VARIATION IN *PHRAGMITES AUSTRALIS* REPRODUCTIVE TRAITS AND SEEDLING PERFORMANCE IN NORTH AMERICA

Introduction

Plants with cosmopolitan distributions often exhibit regional phenotypic variation across a variety of growth and reproductive traits (Weber & Schmid 1998; Garcia-Nogales et al. 2015; McAssey et al. 2016; Xiao et al. 2019; Leal-Saenz et al, 2020). Due to founder effects and inbreeding, invasive plants can often have lower genetic diversity than native range populations (reviewed in Hernández-Espinosa et al. 2022), however as the range of invasive plants expands, rapid evolution of growth and defense related traits also increases (reviewed in Felker-Quinn et al. 2013). Regional variation in traits aiding in expansion, invasiveness, and competitive ability can lead to invasion hotspots. Invasive plants such as *Spartina alterniflora* and *Phytolacca americana* display latitudinal variation in growth traits such as plant height and the culm/stem density (Liu et al. 2016; Xiao et al. 2018). Understanding regional variation in the traits of invasive species can allow us to better understand variation in invasive success and ultimately inform region-specific management plans.

Plant fecundity, germination, and early seedling performance are key traits that can determine invasiveness and that can exhibit regional variation. Fecundity traits can affect propagule pressure, which is one of the primary drivers in biological invasions (Colautti 2004; Simberloff 2010). For example, *S. alterniflora* (Liu et al. 2020)*, Ambrosia artimisiifolia* (Zhou et al. 2021)*, Ambrosia trifida* (Hovick et al. 2018), and *P. americana* (Xiao et al. 2018) exhibit regional variation in reproductive traits, which can lead to regional variation in seedling recruitment and differential rates of invasive expansion within the introduced range. Germination

ability and seedling establishment and survival have also been found to be an important characteristic of invasive plants (Pyšek and Richardson 2007). The seedling stage represents a particularly vulnerable stage in a plant's life, and a quick transition from relying on seed provisions to environmental sources of nutrients increases the chances of seedling survival (Leck et al. 2008; Silvertown 2008; Kettenring & Whigham 2018). Regional variation in germination and seedling performance has been observed across a variety of trees, forbs, and grasses and in both invasive and native plants (Jayasankar et al. 2003; Lamarque et al. 2015; Magni et al. 2019; Samis et al. 2019; Liu et al. 2020 Roman et al. 2022). One study also demonstrated that a species had higher seed provisioning in its invasive vs. native range, that translated into higher seedling performance in the invasive range (Hierro et al. 2013). Overall, understanding fecundity, germination, and early seedling performance across invasive ranges can help identify invasion hotspots and areas of potentially rapid expansion.

Global industrialization over the last 200 years has increased the rate of anthropogenic pollution resulting in excess deposition of nutrients into the environment (reviewed in Burkholder & Gilbert 2013). Anthropogenic nitrogen deposition is altering nitrogen dynamics in habitats worldwide, adding an additional layer of complexity to biological invasions (Canfield et al. 2010; Stevens et al. 2015). Increases in nitrogen can release plants from nutrient limitation and favor faster growing invasive plants that outcompete natives as seen with *Phalaris arundinacea* (Martina & Von Ende 2013) and *Molinia caerulea* (Tomassen et al. 2004). Wetlands often accumulate high concentrations of nitrogen from industrial and agricultural run-off and as a result can become areas of nitrogen saturation (Galloway et al. 2004; Withers et al. 2014; Fowler et al. 2015). This may explain why wetlands are disproportionately affected by biological invasions as they make up 6% of earth's landmass but are home to 24% of the world's

most invasive plants (Zedler & Kercher 2004). Because anthropogenic pollution is often linked to biological invasions, understanding the nature of their relationship can inform current invasion dynamics and help us predict how they may change in the future.

Common reed, *Phragmites australis* (Cav.) Trin. ex Steud. is one of the most widespread plants on earth. It is found on every continent except for Antarctica. In North America, the Eurasian lineage of *P. australis* (hereafter referred to as *Phragmites*) is rapidly becoming the most impactful invasive wetland plant (Chambers et al. 1999; Saltonstall 2002; Burdick & Konisky 2003; Buchsbaum et al. 2006; Lambertini et al. 2012). Although some studies suggest evidence of multiple introductions of *Phragmites* into North America (Saltonstall 2010; Meyerson & Cronin 2013), the earliest herbarium samples of *Phragmites* suggest that it was first introduced to the Mid-Atlantic region in the 19th century, and was first described in 1876, being found near ballast ground in Camden, New Jersey (Saltonstall 2002). *Phragmites* is now present across much of North America, but regional variation in distribution and invasion intensity exists (Saltonstall 2002; Kettenring et al. 2012). In the Northeast/Mid-Atlantic where the residence time of *Phragmites* is the longest, the invasion is also very intense, and evidence suggests most new populations are established through seedling recruitment (Saltonstall 2002; Kirk et al. 2011; Albert et al. 2015). In the Midwest, the invasion is relatively recent compared to the Northeast, but the intensity and impact of *Phragmites* on native wetlands has been mounting in recent years (Lynch & Saltonstall 2002; Wilcox et al. 2003; Price et al. 2014). While *Phragmites* has colonized as far as the west coast of North America and is becoming a problem in places like the Great Salt Lake in Utah (Kulmatiski et al. 2011; Long et al. 2017; EDDMapS 2024), the distribution of *Phragmites* in the Southeast and South-Central regions of the United States is rather sparse and patchy, and the invasion is comparatively benign (White 2004; Meyerson &

Saltonstall 2010; EDDMapS 2024). One common garden study found that *Phragmites* growth tended to decrease with latitude, but flowering frequency tended to increase, suggesting that *Phragmites* fecundity may be even higher in southern populations (Mozdzer et al. 2016). Furthermore, environmental suitability modelling suggests that *Phragmites* is highly climatically suited to living in the Southeast and South-Central regions of the United States (Guo et al. 2013). There is growing evidence that seedling recruitment is the primary mode of *Phragmites* expansion throughout much of North America. While *Phragmites* can disperse and establish new populations via vegetative propagules (rhizomes & culm fragments), these modes of dispersal are more stochastic and dependent on local hydrological conditions (Hudon et al. 2005; Meyerson et al. 2014). Studies suggest that the establishment of most new populations in the Northeast, Mid-Atlantic, and Midwest is through seedling recruitment (Brisson et al. 2010; Kirk et al. 2011; Albert et al. 2015; Kettenring et al. 2015; Fant et al. 2016). Once local genetic diversity is sufficient to overcome pollen limitation, *Phragmites* has a propensity for high sexual reproductive output (McCormick et al. 2010; Kettenring et al. 2011). *Phragmites* displays regional phenotypic variation in traits in both its invasive range in North American and its native range in Eurasia as common garden studies demonstrate variation in growth traits across regional scales (Clevering et al. 2001; Achenbach et al. 2012; Eller & Brix 2012; Ren et al. 2020). Despite the importance of seedling recruitment in the establishment of new *Phragmites* populations in the Midwest, Northeast, and Mid-Atlantic, comparisons of Phragmites fecundity and seedling performance across regions have not been explored (Brisson et al. 2010; Kirk et al. 2011; Albert et al. 2015; Fant et al. 2016; Kettenring & Whigham 2018).

Phragmites thrives in high nitrogen environments, which often shifts competitive interactions in its favor when competing against native wetland plants (Minchinton & Bertness

2003; Rickey & Anderson 2004; Mozdzer & Zieman 2010; Mozdzer & Megonigal 2012). Elevated nitrogen can boost *Phragmites* fecundity by increasing the mass of individual inflorescences (Rickey & Anderson 2004), the number of inflorescences per plant, and the number of florets per inflorescence (Kettenring & Whigham 2009; Kettenring et al. 2011). Chronic nitrogen pollution may increase maternal seed provisioning and increase seed quality, as seen across commercial and wild grass species (Torres et al. 2009; Ronnenberg et al. 2011; Wang et al. 2022; but see Kettenring & Whigham 2009; Kettenring et al. 2011). Increased nitrogen availability promotes *Phragmites* seedling growth across a wide geographic sampling in Europe (Clevering 1999). Unlike European studies, only localized studies on the effects of nitrogen on *Phragmites* fecundity and seedling performance exist in North America.

Several studies have suggested that regional variation in genetic diversity exists in *Phragmites.* In the Northeast and Mid-Atlantic (Chesapeake Bay), high levels of genetic diversity have been described in local *Phragmites* populations, most likely due to multiple introductions of *Phragmites* into the area (Belzile et al. 2010; Kettenring et al. 2012). Studies in the Midwest find higher genetic diversity in the introduced lineage compared to the native lineage (Tippery et al. 2020). This increase in genetic diversity is also correlated with the intensity of *Phragmites* invasion in the Midwest and the Atlantic, as *Phragmites* expansion in the Mid-Atlantic has increased by 25x over the last 40 years, and studies in the Saint Lawrence River and Rhode Island show that the introduced lineage has been expanding and dominating in native wetlands and estuaries (Lambert & Casagrande 2006; Belzile 2010; Kettenring et al. 2012). While there are multiple lineages of Eurasian *Phragmites* in the Mississippi river delta (White et al. 2004; Hauber et al. 2011), the distribution in the rest of the Southeast and South-Central region is rather sparse and the genetic diversity within the larger region is unknown. This

suggests that *Phragmites* in North America is likely not monomorphic and regional phenotypic variation may exist.

Here we determine the effects of *Phragmites* region of origin (Northeast, Midwest, and Southeast) and local watershed nitrogen load on *Phragmites* fecundity and germination by conducting a survey of reproductive traits across 34 populations in its invaded range in North America. Because of *Phragmites'* tendency to establish and flourish in nutrient enriched habitats, we hypothesize that H1.1) *Phragmites* populations experiencing **higher** nitrogen loads will have **greater** inflorescence length, inflorescence mass, spikelet number, average seed mass, % germination, and reproductive potential (defined as spikelet number * % germination) than populations experiencing lower nitrogen loads. The high intensity of *Phragmites* invasion in the Northeast is in some part due to the high rates of seedling recruitment; thus we also hypothesize that H1.2) populations from the Northeast will have **greater** inflorescence length, inflorescence mass, spikelet number, average seed mass, % germination, and reproductive potential than populations from the Southeast and Midwest.

Since seedling recruitment is determined by both fecundity and seedling establishment, we also examined variation in seedling growth by region and in response to elevated nitrogen by performing a seedling growth experiment using seeds collected from the Northeast, Midwest, and Southeast and grown under ambient and elevated nitrogen. We hypothesize that based on regional patterns of *Phragmites* invasion modes and seedling response to nitrogen H2.1) seedlings from the Northeast will have higher biomass, shoot height, and shoot number than seedlings from the Midwest and Southeast irrespective of nitrogen treatment, and H2.2) that elevated nitrogen will increase seedling growth, and this response will be more pronounced in populations with high local watershed nitrogen. In total, this research represents the first steps for

investigating the regional variation in seedling recruitment in *Phragmites* and will help predict the capacity for invasive spread of *Phragmites* in the future.

Methods

Sample collection

Inflorescences from 34 populations of *Phragmites* along a latitudinal gradient were collected between October - November 2021(Figure 1-1; Table 1-1). Populations sampled spanned 44° N (Quebec, Canada) to 31° N (Brunswick, Georgia, USA), and from 69° W (Cape Cod, Massachusetts, USA) to 87° W (Johnsonville, TN, USA) (Figure 1-1; Table 1-1). Sampling was conducted as follows: each collector would walk a 10m transect from the edge of the *Phragmites* stand and collect a single randomly selected inflorescence every meter for a total of 10 inflorescences per site (total N = 340) (Figure 1-1). Each inflorescence was subsequently laid out at room temperature for 48 hours before being placed in an individual paper bag and then shipped and stored at room temperature at Tulane University before the start of processing in spring of 2022.

Seed & inflorescence traits

The length of individual inflorescences from each population were measured from the base of the inflorescence where the first branches emerge (junction between first pedicel and rachis) to the tip of the last spikelet. The mass of the inflorescence was obtained by measuring the full inflorescence and subtracting the mass of the seeds extracted from the inflorescence. An estimate of spikelet number per inflorescence for each sampled location was calculated by counting total spikelet number on two random inflorescences per population and using the

relationship between average spikelet number and mass to estimate spikelet number for the other eight inflorescences from that same population.

Seeds were extracted from each inflorescence by rubbing inflorescences against a 1mm sieve followed by a 0.5mm sieve. The chaff was blown off and only mature naked seeds were retained. An aliquot of 50 seeds was taken from each inflorescence and weighed on a microbalance (Mettler-Toledo; Columbus, OH, USA) to calculate mean seed mass.

Each 50-seed aliquot was plated on 1% agarose (50 seeds/ 90mm*15mm petri plate). All seeds were first sterilized using a modified version of the surface sterilization protocol from Shearin et al. (2018), consisting of a 3-minute 70% ethanol followed by a 3-minute 3% sodium hypochlorite wash on day 1, an overnight incubation at room temperature, and another round of surface sterilization on day 2 using a 5-minute ethanol wash followed by a 20-minute sodium hypochlorite wash. After the second surface sterilization wash, the seeds were placed in a growth chamber with a 18/6 hour light cycle and 30/15°C day:night temperature cycle. Petri dishes were checked daily for mold and all germinated seeds were counted after two weeks. Percent germination was calculated by counting germinated seeds per plate divided by 50. To estimate the possible reproductive output of *Phragmites* we created the metric, reproductive potential, which is the % germination multiplied by the number of spikelets. Number of seeds was not used as a fecundity metric, as phenological differences between populations prevented us from collecting all the inflorescences before seed drop occurred.

Seed Germination and Seedling Growth experiment

Of the 34 populations sampled, we chose a subset of 24 populations (indicated in Table 1-1) to use in the seedling growth experiment, due to seed stock limitations. These 24

populations spanned at latitudinal range from Québec, Canada (N 46.832574º) to Brunswick, Georgia, USA (N 31.130608º) (Table 1-1). One seedling was grown from each of four randomly selected inflorescences collected from each of the 24 populations and was grown in two different nitrogen levels (elevated N; 25g.m^2.yr^{-1}, ambient N; 2g.m^2.yr^{-1}), totaling 192 pots (1 seedling * 4 inflorescences * 24 populations * 2 N treatments = 192 pots) (Figure 1-2).

Seedlings for this experiment were germinated from 50-seed aliquots from four randomly selected inflorescences per population and germinated following the protocol described above. After one week, germinated seedlings were planted into square form pots (13.34cm L x 13.34cm W x 15.24cm H) filled with Leafgro high humus soil conditioner (Leafgro, Maryland Environmental Service, Millersville, Maryland, USA). Pots were placed in flat trays (54.43 cm L x 27.79 cm W x 20 cm H), each holding up to 8 pots, at which time the trays were fertilized with either the elevated N treatment or the ambient N treatment. The programming for the growth chamber light and temperature regime were taken as the average day length in May (14/10 hour light cycle) and a 25/15°C day:night temperature cycle, representative of the mid-latitude (N 38°) climate. The amount of photosynthetically active radiation (PAR) at soil level was 250 umol/m2/s (for reference full sunlight ~2000 umol/m2/s). Trays were bottom watered to the lip of the tray twice per week and the corresponding N treatments were added to the trays every 2 weeks, at which time pots were rotated between trays receiving the same nitrogen treatment.

Seedling height and shoot number were collected throughout the growth experiment on days 7, 14, 28, and 42. Shoot number and shoot height within each pot was collected by counting the number of fully emergent shoots over 5mm in height. Seedling harvest commenced on day 56. Seedlings were clipped at the soil line and the third fully formed leaf of the tallest

shoot within each pot was collected for specific leaf area (SLA) measurements. Freshly collected leaves were then photographed next to a ruler for scale, converted to 8-bit black and white images and leaf area was measured using ImageJ (Schneider et al. 2012) area calculations based on scaled photographs of the SLA leaf sample. Both the SLA leaf sample and the rest of the aboveground biomass were dried separately to constant mass at 50°C. SLA was then calculated by dividing dry mass of the SLA leaf sample by the ImageJ measured area of the corresponding leaf. Belowground biomass was washed clean of soil using a 1mm sieve to retain detached fine roots, before belowground biomass was dried to constant mass at 50°C. A subset of the roots was stored for up to 7 days at 4°C while root imaging was performed prior to drying. Root images were processed using the Rhizovision software (Seethepalli & York 2020) to derive root length by root class (fine roots (<1mm), secondary roots (1-3mm), and rhizome (>3mm)), specific root length, rooting depth, and root volume by root class.

Nitrogen and climate data

Total nitrogen loads (kg/km^2) from the watershed corresponding to each site were obtained using datasets from the USGS SPARROW mapper (Ator 2020) which shows the annual nitrogen load in 2012. Mean growing season (March-October) temperature and precipitation were extracted from the NOAA National Centers for Environmental Information climate monitoring data which displays temperature and precipitation on the county level. Climatic metrics were collected to estimate the relative influence of climate on the observed fecundity traits.

Statistical analysis

To test the effects of region (Northeast, Midwest, and Southeast) and watershed level nitrogen on each of our response variables in the field survey (inflorescence length, inflorescence mass, spikelet number, average seed mass, and germination rate) we compared multiple linear mixed effects models and generalized least square models using the "lme" and "gls" functions in the R package "nlme" version 3.1-149 (Pinheiro et al. 2023) in R studio version 1.3.1093 (R Core Team 2023). We first created a base model for backwards selection by including all fixed effects (region*watershed-level nitrogen + temperature + precipitation) in a model explaining variance in a particular response variable. Population was used as a random effect in all models. Backwards selection was performed, and best-fit models were selected using AIC. Because populations displayed a wide range of variance, we tested for heterogeneous variances across different populations using AIC. We then ran a type III ANOVA on the best model to determine the significance of each of the explanatory variables. We performed model validation by plotting fitted values against residuals, each of the fixed effects against residuals, and a histogram of the residuals alone. Because germination rates are proportions, we used binomial regression models using the "glm" function in R package "stat" version 4.0.3 (R Core Team). Binomial regression models did not include the random effect and heterogeneous variances of population due to constraints of the "glm" function.

For the seedling growth experiment, the effect of watershed level nitrogen* region* nitrogen treatment were used as fixed effects in models of seedling growth response variables (total biomass, aboveground biomass, belowground biomass, aboveground:belowground biomass, SLA, total shoot height, shoot number, total root volume, total root length, specific root length, root depth, and the volume and length of each root class (fine roots (<1mm ∅), secondary roots (1-3mm ∅), and rhizomes (>3mm ∅)) using site as a random effect in multiple linear

mixed effects models described above. Tukey's HSD test were performed on significant fixed effects to elucidate significant pairwise differences among factor-levels within each categorical factor using the "lsmeans" function in the R package "lsmeans" (Lenth 2018) to extract least-square means and then using the extracted means to perform a Tukey's HSD test in compact letter design using the "cld" function in the R package "multcomp" (Hothorn et al. 2023). In the case of significant interactions between fixed effects, a new model was run combining the two fixed effects into a single factor so that the Tukey's test could be performed.

Results

Inflorescence and Seed traits

Inflorescence mass was significantly influenced by region (Table 1-2). Inflorescences from plants from the Northeast (3.681 mg ± 0.239) had 22% and 7.6% more mass than inflorescences from plants from the Midwest (3.146 mg ± 0.196) and Southeast (3.582 mg ± 0.271) respectively, although the Tukey's HSD post-hoc test was unable to tease apart significant pairwise differences between regions (Figure 1-3A). Inflorescence length and the number of spikelets on each inflorescence were not significantly related to local watershed nitrogen levels or region (Table 1-2, Figure 1-3B-C).

None of our explanatory variables significantly explained mean seed mass (Table 1-2, Figure 1-3D). However, the effect of local watershed nitrogen on percent germination depended on region (Table 1-2) (significant region x watershed nitrogen interaction), such that there was an inverse relationship between watershed nitrogen levels and percent germination only in populations sourced from the Southeast ($R^2 = 0.406$) (Figure 1-4). Regions differed significantly

in reproductive potential (Table 2), as plants from the Southeast (3623.988 ± 357.578) had 33% higher reproductive potential than plants from the Northeast (2721.936 ± 219.383) (Figure 3F).

Seedling growth traits

The effect of nitrogen on total shoot height and shoot number after 42 days of growth was dependent on region (significant N x region interaction) (Table 1-3). Tukey's HSD post-hoc test was unable to parse pairwise differences between treatment groups for total shoot height; however, Southeastern *Phragmites* responded positively to elevated nitrogen, with seedlings accumulating 22% more shoot height (2516.062 mm ± 252.963) than seedlings grown under ambient conditions (2070.875 mm ± 198.006) (Figure 1-5A). Conversely, seedlings from the Midwest had a negative response to elevated nitrogen, with seedlings grown under ambient nitrogen (2177.714 mm ± 136.036) accumulating 12% more shoot height than seedlings grown under elevated nitrogen (1946.683 mm ± 97.575) (Figure 1-5A). For shoot number, seedlings from the Southeast grown in elevated N (5.937 ± 0.504) had significantly more shoots (38%) than the same seedlings grown under ambient N (4.313 ± 0.350), whereas elevated nitrogen did not affect shoot number in Northeast and Midwest populations (Figure 1-5B).

The effect on nitrogen on total and belowground biomass also depended on region (significant N x region interaction) (Table 1-3). Although Tukey's HSD post-hoc test was unable to resolve significant pairwise differences, there were notable patterns in total biomass between the treatment groups. Namely, Southeastern *Phragmites* seedlings grown under elevated nitrogen (2.489 g ± 0.326) accumulated 31% more total biomass than seedlings grown under ambient conditions (1.901 g ± 0.227) (Figure 1-5C). The opposite pattern was observed in seedlings from the Midwest as seedlings grown under ambient nitrogen (1.904 g ± 0.177) accumulated 16%

more total biomass than seedlings grown under elevated nitrogen (1.648 g ± 0.128) (Figure 1-5C). The belowground biomass of seedlings from the Southeast grown under elevated nitrogen (0.692 g ± 0.095) was 63% higher than that of Midwest seedlings under the same conditions (0.423 g ± 0.041) (Figure 1-5C). There was a trend that the effect of nitrogen on aboveground biomass also depended on region (nearly significant N x region interaction) (Table 1-3); nitrogen only increased aboveground biomass of seedlings from the Southeast (Figure 1-5C). The effect of nitrogen on aboveground:belowground biomass ratio was also dependent on region (significant N x region interaction) (Table 1-3). Seedlings from the Southeast grown under ambient nitrogen (2.589 ± 0.242) had 20% lower aboveground:belowground biomass ratio than seedlings from the Midwest grown under elevated nitrogen (3.239 ± 0.194) (Figure 1-5D). Watershed-level nitrogen did not explain total ($F_{1,173} = 0.021$, n.s.), aboveground ($F_{1,173} = 0.077$, n.s.), or belowground ($F_{1,173} = 0.008$, n.s.) biomass under elevated and ambient nitrogen.

The only root traits significantly influenced by any of our explanatory variables were specific root length and secondary root volume, which varied significantly depending on region (Table 4). Seedlings from the Southeast (10,936.181 mm^3 ± 1602.859) had 68% higher secondary root volume than seedlings from the Midwest (6,527.244 mm^3 ± 500.898) (Figure 1-6) and seedlings from the Northeast (28.557 mm/mg ± 1.543) had 17% higher specific root length than seedlings from the Midwest (24.444 mm/mg ± 1.170) (Figure 1-6).

Discussion

The effects of local watershed nitrogen on seed germination

We hypothesized that *Phragmites* populations experiencing higher watershed N loads would have higher fecundity and germination. However, we found that in general watershed-level nitrogen did not affect any of the inflorescence traits, average seed mass, or reproductive potential. The only result we found was that watershed N only affected germination rates in the Southeast, where higher N decreased germination.

Phragmites populations from the Southeast were more sensitive to local variation in nitrogen loads than in other regions, at least with respect to percent germination. While regional variation in germination success in other plant species has been observed (García-Nogales et al. 2016; Mohl et al. 2023), few studies have related variation in germination success to maternal nitrogen exposure. One study found that Powell amaranth (*Amaranthus powellii*), a noxious weed in croplands, experiences lower germination success in populations exposed to conventional (inorganic N) vs. organic (organic N) fertilization practices (Brainard et al. 2006). Another study found that in the yew species *Taxus baccata,* long-term maternal nitrogen fertilization reduced percent germination in offspring (Pers-Kamczyc & Suszka 2022). These studies are in opposition to the observed effect in *Phragmites*, where more nutrient polluted regions (Northeast & Midwest) are less sensitive to local nitrogen levels than the historically less-polluted Southeast. Several experimental studies show that nitrogen fertilization decreases seed viability in *Succisa pratensis* (Vergeer et al. 2003), *Juniperus communis* (Gruwez et al. 2014), and a variety of subarctic plants (Milbau et al. 2017); however nitrate can increase seed viability (Ronnenberg et al. 2011; Baskin & Baskin 2014), and studies in other grass species generally find no effect on seed viability (Wagner et al. 2001; Torres et al. 2009 Tullos & Cadenasso 2016). Notably, our *Phragmites* populations from the Southeast experienced on average lower watershed-level nitrogen (1.257g/m^2/yr ± 0.073) than the Northeast (3.145g/m^2/yr

± 0.335) or the Midwest (1.761g/m^2/yr ± 0.0908). This suggests that either the Southeast experiences lower levels of nitrogen pollution and that perhaps populations there are not be adapted to high nitrogen levels. Like what has been observed in other grass species, localized studies in the Chesapeake have found that elevated nitrogen had no effect on *Phragmites* seed viability (Kettenring et al. 2011), however to our knowledge, studies on the effects of nitrogen pollution in other regions have not yet been explored. It is possible that low seed germination rates of Southeastern *Phragmites* populations established in nitrogen polluted areas may dampen its invasiveness in those areas, and this warrants more research into whether nitrogen pollution is influencing *Phragmites* invasion dynamics across different regions.

Regional variation in reproductive traits

In line with our predictions, inflorescences from the Northeast had higher mass than those from the Southeast and Midwest, even after accounting for weight of seeds. Increased inflorescence mass suggests that the plants from the Northeast are investing more resources into reproduction than other regions (Mullins and Marks, 1987). Considerable intraspecific variation in reproductive investment exists globally across various lineages of *Phragmites*, but the North American invasive *Phragmites* allocates the most to reproduction compared to populations from Europe and Asia (Pyšek et al. 2019). The increased inflorescence mass of Northeast populations mirrors the wide distribution and intensity of *Phragmites* invasion in the Northeast (Saltonstall 2002; Burdick & Konisky 2003; Kiviat 2010; EDDMaps 2024). This points to increased reproductive investment as a potential mechanism for its dominance in the Northeast, and warrants further research linking seedling recruitment and fecundity in the region,

While inflorescence mass varied regionally according to our hypothesis, seed traits (seed mass, germination, reproductive potential) did not demonstrate regional variation. The average seed mass from populations from the Northeast was the same as populations from the Midwest and Southeast. Furthermore, Northeast seeds had the lowest percent germination of any region, even considering the significant decrease in percent germination by Southeast populations with high levels of maternal N exposure. While regional variation in percent germination has not previously been studied in *Phragmites,* such variation has been observed in a variety of other invasive plant species. For example, invasive *Ambrosia artemisiifolia* in China exhibited lower germination in seeds from Northeastern latitude populations versus Southeast populations (Zhou et al. 2021). Invasive *Spartina alterniflora* populations in China displayed the opposite pattern, with populations in Northeastern China exhibiting a higher percent germination than populations from the Southeast (Cheng et al 2022). Thus, our results show that *Phragmites* populations from the Northeast invested more into inflorescences, but this investment did not translate into increased seed mass or germination. The reproductive potential of *Phragmites* from the Southeast was significantly higher than populations from the Northeast, which is opposite to our hypothesized patterns. These results suggested that seedling recruitment in the Southeast would be higher than in the Northeast. *Phragmites* habitat suitability modelling indicates that *Phragmites* can readily establish in the Southeast and South-Central regions of North America, yet the distribution of *Phragmites* in this region is sparse (Guo et al. 2013; EDDMapS 2024). Interestingly, a common garden study found that *Phragmites* grown in low-latitude climates tended to produce more inflorescence per plant, suggesting higher reproductive output in the South which is in-line with our findings (Mozdzer et al. 2016). Human activity is the strongest driver in the genetic structuring of *Phragmites* populations in North America (Guo et al. 2018).

Human activity can increase disturbance and nutrient pollution, both of which have been linked to increased invasion in *Phragmites* (Chambers et al. 1999; Bertness et al. 2002; Minchinton & Bertness 2003; Silliman & Bertness 2004). Therefore, the spread of *Phragmites* may potentially be due to the history of industrialization in North America, which has historically lagged in the South (Bateman & Weiss 1975; Guo et al. 2018). Several studies show that weak Allee effects can limit the local spread of *Phragmites*, as local genetic diversity must accumulate for outcrossing to occur and produce viable seed (McCormick et al. 2010; Kettenring et al. 2011). Although we cannot accurately determine the number of seeds produced on each inflorescence, it is notable that populations from the Southeast had a lower number of seeds than the other regions we sampled. sampled. Because our reproductive potential metric only includes spikelet number x germination rate, and spikelets can have variable numbers of seeds, our metric misses important information on seed set which is where Allee effects would manifest. More research is warranted into studying the dynamics of reproduction and identifying potential limits in seedling recruitment in the Southeast, as we found the potential for high seedling recruitment.

Regional variation in seedling response to nitrogen

We uncover inverse belowground seedling responses to elevated nitrogen between *Phragmites* populations in the Southeast and Midwest. Elevated nitrogen boosted belowground biomass in seedlings from the Southeast while elevated nitrogen decreased belowground biomass in seedlings from the Midwest. This pattern may be driven by differences observed in secondary root volume, as seedlings from the Midwest had significantly lower root volume than plants from the Southeast. As a result of the inhibitory effects of elevated nitrogen on belowground growth in

Midwestern seedlings, these seedlings had the highest aboveground to belowground biomass ratio observed in our study.

Ammonium (NH_4) and nitrate (NO_3) can act as signals for the proliferation or inhibition of lateral root growth, with both low N and high N thresholds for lateral root growth inhibition reported (Zhang et al. 1999; 2007). Other studies have also demonstrated a negative, neutral, and even positive relationships between sediment nitrogen levels and *Phragmites* belowground biomass, suggesting that there is considerable intraspecific variation in responses to nitrogen fertilization (Holdredge et al. 2010; Mozdzer & Megonigal 2012; Caplan et al. 2014; Chen et al. 2017; Gonzalez Mateu et al. 2021). These results suggest that the consequences of eutrophication on *Phragmites* belowground biomass may be very different from region to region. Elevated nitrogen decreased the belowground biomass of the native wetland grass *Spartina alterniflora* and is hypothesized to cause tidal marsh collapse through increased tidal erosion in *Spartina*-dominated marsh (Deegan et al. 2012; Hanley et al. 2021). This phenomenon may very well be reflected in *Phragmites*, and if so, increased eutrophication in Midwestern (but not Northeastern or Southeastern) *Phragmites* dominated wetlands may lead to marsh destabilization. Increases in belowground biomass may also increase the priming of deep carbon pools as *Phragmites* can increase soil aeration through its aerenchyma tissue leading to higher rates of decomposition and subsidence (Bernal et al. 2017, Bernal et al 2023).

Seedlings originating from the Southeast experienced a significant increase in shoot number when grown under elevated versus ambient nitrogen. Even though region*nitrogen treatment differences could not be resolved in the post-hoc test, total shoot height tended to increase with nitrogen fertilization in the Northeast and Southeast but decrease in the Midwest.

Although the expansion of *Phragmites* stands into adjacent wetland can be achieved through vegetative lateral expansion (Güsewell & Edwards 1999; Minchinton & Bertness 2003; Brisson et al. 2010), recent work has revealed that the advancing front of *Phragmites* stands can often be composed of multiple genets, highlighting the role of seedling recruitment in *Phragmites* patch expansion (Kettenring et al. 2016). Increased density in mature *Phragmites* stands correlates to lower native plant biomass and diversity (Holdredge & Bertness 2011; Elsey-Quirk & Leck 2021, but see Theuerkauf et al. 2017). Thus, increased shoot density of seedlings recruited near the edge of *Phragmites* stands may help in outcompeting native plants at the interface between native wetlands and invasive *Phragmites.* While seedling shoot density and biomass may not affect native plant communities as much as mature stands do, increased seedling vigor can help overcome seedling-stage mortality associated with flooding and competition and aid in successful establishment (Mauchamp et al. 2001; Saltonstall & Stevenson 2007).

Taken together, our results suggested that regional variation in seedling responses to nitrogen exists, as populations from the Southeast responded positively to increased nitrogen fertilization while Midwest populations showed the opposite pattern. Increased anthropogenic nutrient pollution may influence different regions in different ways and potentially promote the invasion of populations in the Southeast more than other regions. Regional variation in belowground patterns in response to nitrogen may also influence important wetland characteristics such as marsh integrity and rates of soil decomposition.

Conclusions

Here we demonstrated a high degree of intraspecific variation in fecundity and seedling performance in *Phragmites* across its invaded range in North America. There is a disconnect between the relatively low abundance of *Phragmites* in the Southeast and the high reproductive potential and high growth rates of Southeastern seedlings observed in this experiment. More research is needed to elucidate the mechanisms inhibiting Southeast *Phragmites* spread within the region. Anthropogenic nitrogen pollution has differential effects on belowground investment and shoot number of *Phragmites*. The differential belowground effects of elevated nitrogen in the Southeast versus the Midwest may have cascading effects in marsh stability and soil decomposition and may influence the ability of *Phragmites* dominated marshes in the Southeast to keep up with sea-level rise. Additionally, an increase in soil nitrogen promoted high seedling growth rate in Southeastern *Phragmites* which may increase the rate of seedling recruitment. Thus, Southeastern *Phragmites* populations represent a potential invasion hotspot in the future, which warrants further exploration into the factors limiting invasion in this region. This current study along with future research will help educate management in ways of combatting further spread of *Phragmites* into the Southeast through understanding regional invasion dynamics in *Phragmites*.

Table 1-1 Region, site name/ city/ state/province/ country of origin, coordinates, and watershed-level nitrogen of each *Phragmites* population used to source inflorescences and seed for inflorescence/seed trait analysis and seedling growth experiment (indicated by the *).

Region	Site (City, State/Province, Country)	Latitude	Longitude	Watershed-level nitrogen (kg/km^2/year)
Northeast	Québec City, QC, Canada *	46.832574	-71.278028	3.700
Northeast	Blainville 1, QC, Canada *	45.661025	-73.846928	8.176
Northeast	Blainville 2, QC, Canada *	45.661025	-73.846928	8.176
Northeast	Saco, ME, USA *	43.488616	-70.388192	0.366
Northeast	Biddeford, ME, USA	43.457593	-70.378077	0.366
Northeast	Rowley, MA, USA *	42.724972	-70.855639	3.421
Northeast	Boston, MA, USA *	42.343408	-71.092965	1.584
Northeast	Wellfleet, MA, USA *	41.902449	-69.979032	0.226
Northeast	Westport, CT, USA *	41.117198	-73.346232	1.550
Northeast	North Brunswick, NJ, USA	40.473528	-74.426250	2.123
Northeast	Hamilton, NJ, USA	40.230546	-74.678429	2.123
Northeast	Villanova, PA, USA	40.041639	-75.347556	1.979
Northeast	Philadelphia, PA, USA *	39.890278	-75.261278	9.932
Midwest	Novi, MI, USA *	42.487883	-83.495745	4.481
Midwest	La Porte, IN, USA *	41.691709	-86.718274	1.245
Midwest	Schererville, IN, USA	41.506281	-87.443019	4.531
Midwest	Lafayette, IN, USA *	40.446970	-86.868260	2.407
Midwest	Indianapolis, IN, USA *	39.791320	-86.169590	2.901
Midwest	Patricksburg, IN, USA	39.276672	-86.972608	1.843
Midwest	Lincoln City, IN, USA *	38.133506	-86.983347	2.073
Midwest	Henderson, KY, USA *	37.866148	-87.776730	1.644
Midwest	Central City, KY, USA *	37.289160	-87.104740	2.002
Midwest	Muhlenberg, KY, USA *	37.275330	-87.133110	2.002
Midwest	Graham, KY, USA	37.250730	-87.246450	2.002

Midwest	McNary 1, KY, USA *	37.236570	-87.327350	2.002
Midwest	McNary 2, KY, USA *	37.234950	-87.350610	2.002
Midwest	New Johnsonville, TN, USA *	36.035178	-87.987878	0.598
Southeast	Gloucester Point, VA, USA *	37.246361	-76.504528	0.565
Southeast	Kill Devil Hills, NC, USA	36.004500	-75.673667	0.845
Southeast	Wanchese, NC, USA *	35.870833	-75.642694	1.190
Southeast	Georgetown, SC, USA *	33.258353	-79.255025	0.843
Southeast	Savannah, GA, USA	32.071778	-81.076806	2.910
Southeast	Broadfield, GA, USA	31.326252	-81.445617	1.345
Southeast	Brunswick, GA, USA *	31.130608	-81.479963	1.451

Table 1-2 ANOVA degrees of freedom, F-value, and associated p-value from linear mixed effect models testing the effect of local watershed level nitrogen * region and environmental factors (precipitation and temperature) on inflorescence and seed traits collected from North American *Phragmites* in 2021.

	Inflorescence Mass			**Inflorescence Length**			**Spikelet Number**		
	numDF	**F-value**	**p-value**	**numDF**	**F-value**	**p-value**	**numDF**	**F-value**	**p-value**
Nitrogen	1	0.376	0.513	1	0.067	0.798	1	0.309	0.583
Region	2	3.704	0.038	2	0.288	0.752	2	2.215	0.130
Region:Nitrogen	2	2.211	0.129	2	0.265	0.769	2	0.419	0.662
	Mean Seed Mass			**Percent germination**			**Reproductive potential**		
	numDF	**F-value**	**p-value**	**numDF**	**Chi-squared**	**p-value**	**numDF**	**F-value**	**p-value**
Nitrogen	2	1.889	0.309	2	12.424	0.001	2	3.995	0.205
Region	1	1.080	0.172	1	10.211	0.002	1	1.700	0.032
Region:Nitrogen	2	0.407	0.670	2	11.767	0.003	2	1.526	0.239

Table 1-3 ANOVA numerator degrees of freedom, F-value, and associated p-value from linear mixed effect models testing the effect of nitrogen * region on seedling growth traits and biomass collected from *Phragmites* seedling growth experiment. P-value significance is reported, p <0.05(*), p <0.01(**), p<0.001(***).

	Total biomass			**Aboveground biomass**			**Belowground biomass**			**Aboveground: Belowground Biomass**		
	DF	**F-val**	**p-val**	**DF**	**F-val**	**p-val**	**DF**	**F-val**	**p-val**	**DF**	**F-val**	**p-val**
Region	2	1.053	0.367	2	0.900	0.422	2	1.247	0.308	2	2.700	0.090
Nitrogen	1	5.645	0.019 (*)	1	7.573	0.007 (**)	1	0.023	0.879	1	1.433	0.233
Region:nitrogen	2	3.456	0.034 (*)	2	2.500	0.086	2	3.625	0.029 (*)	2	4.072	0.019 (*)

	Specific Leaf Area			**Total Shoot Height (day 42)**			**Shoot Number (day 42)**		
	DF	**F-val**	**p-val**	**DF**	**F-val**	**p-val**	**DF**	**F-val**	**p-val**
Region	2	0.509	0.608	2	0.401	0.674	2	0.026	0.975
Nitrogen	1	1.092	0.298	1	2.619	0.108	1	5.878	0.017 (*)
Region:nitrogen	2	0.023	0.977	2	3.444	0.034 (*)	2	3.976	0.021 (*)

Table 1-4 ANOVA degrees of freedom, F-value, and associated p-value from linear mixed effect models testing the effect of nitrogen * region on root traits collected from *Phragmites* seedling growth experiment.

	Total Root Length			Fine Root Length		
	numDF	**F-value**	**p-value**	**numDF**	**F-value**	**p-value**
Region	2	3.162	0.063	2	3.131	0.065
Nitrogen	1	0.090	0.765	1	0.573	0.452
Region:nitrogen	2	0.731	0.485	2	0.898	0.413
	Secondary Root Length			**Rhizome Length**		
	numDF	**F-value**	**p-value**	**numDF**	**F-value**	**p-value**
Region	2	3.030	0.070	2	1.919	0.172
Nitrogen	1	0.634	0.429	1	0.001	0.970
Region:nitrogen	2	0.217	0.806	2	0.679	0.511
	Total Root Volume			**Fine Root Volume**		
	numDF	**F-value**	**p-value**	**numDF**	**F-value**	**p-value**
Region	2	2.696	0.091	2	2.669	0.093
Nitrogen	1	0.004	0.951	1	0.030	0.864
Region:nitrogen	2	0.451	0.639	2	0.977	0.382
	Secondary Root Volume			**Rhizome Volume**		
	numDF	**F-value**	**p-value**	**numDF**	**F-value**	**p-value**
Region	2	3.624	0.045	2	1.587	0.228
Nitrogen	1	0.297	0.588	1	0.392	0.533
Region:nitrogen	2	0.444	0.644	2	2.229	0.116
	Specific Root Length			**Root Depth**		
	numDF	**F-value**	**p-value**	**numDF**	**F-value**	**p-value**
Region	2	4.823	0.019	2	0.899	0.422
Nitrogen	1	1.586	0.212	1	1.061	0.307
Region:nitrogen	2	3.089	0.052	2	0.862	0.428

Figure 1-1 Location of *Phragmites* populations used for inflorescences collections in 2021. The points correspond to location of sites, the green points indicate populations designated as Northeast populations, blue points indicate populations designated as Southeast populations, and red points indicate populations designated as Midwest populations.

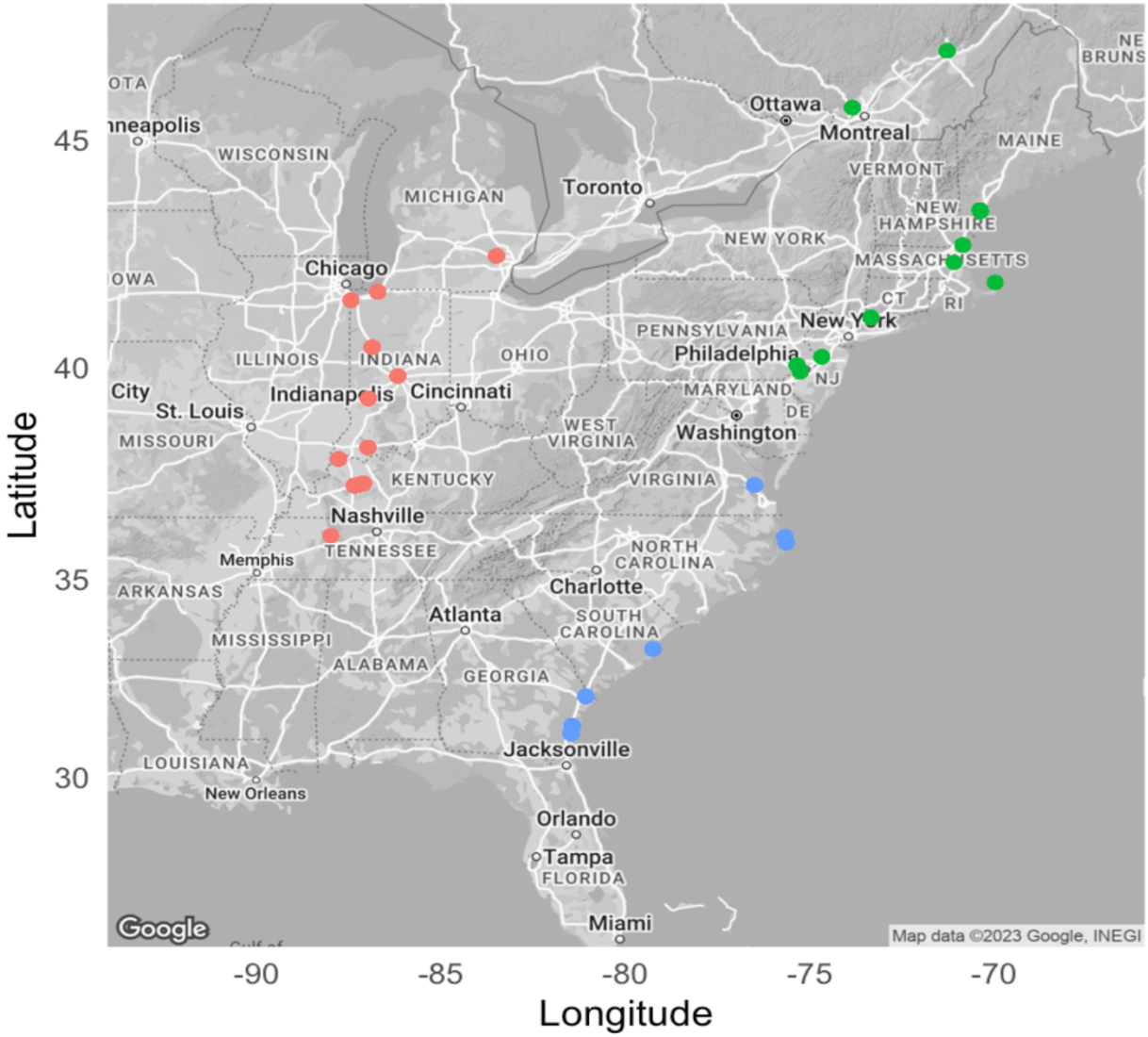


Figure 1-2 Growth set-up for the *Phragmites* latitudinal seedling growth experiment. The columns represent nitrogen treatment, with elevated nitrogen treatments on the left represented by the blue pots, numbers, and box and the ambient nitrogen treatments represented by the black box and grey pots. Each population is represented by the number (1-24), each pot represents an inflorescence within the population with each inflorescence contributing a single seedling.

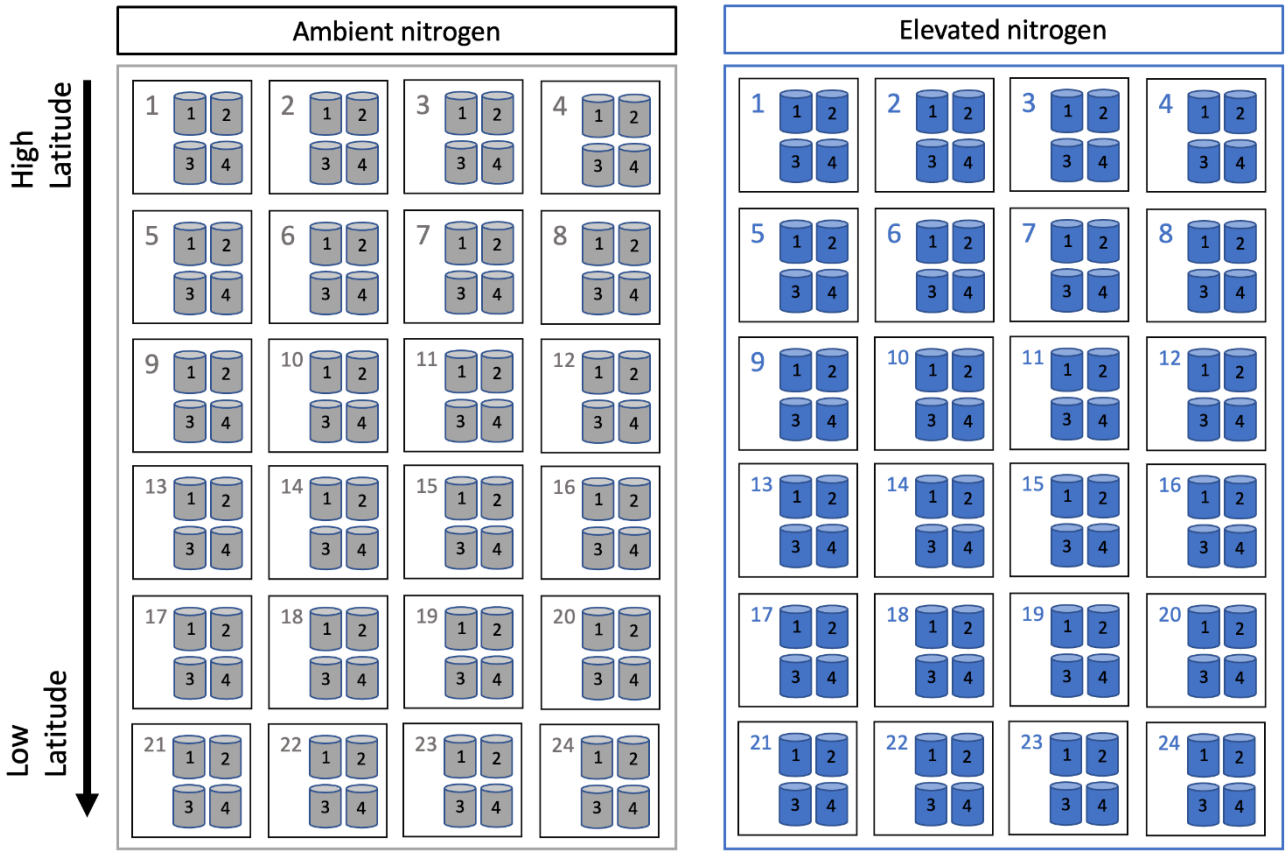


Figure 1-3 Mean of A) inflorescence mass, B) inflorescence mass, C) number of spikelets, D) average seed mass, E) percent germination, and F) reproductive potential by region of origin (Northeast, Midwest, and Southeast). Letters represent Tukey's HSD test displayed in compact letter design (CLD) showing significant pairwise comparisons between each region. The dark red diamond within each boxplot (A-D, F) represents the mean value, the upper and lower hinges of each boxplot represent the 25th and 75th quantile, the middle line represents the median value, the whiskers represent the 5th and 95th percentile, and outliers are represented by black points. The box and whiskers in the violin plot represent the 10th quantile (lower whisker), 25th quartile (lower edge of box), midline (median), 75th quartile (top edge of box), and 90th quantile (top whisker).

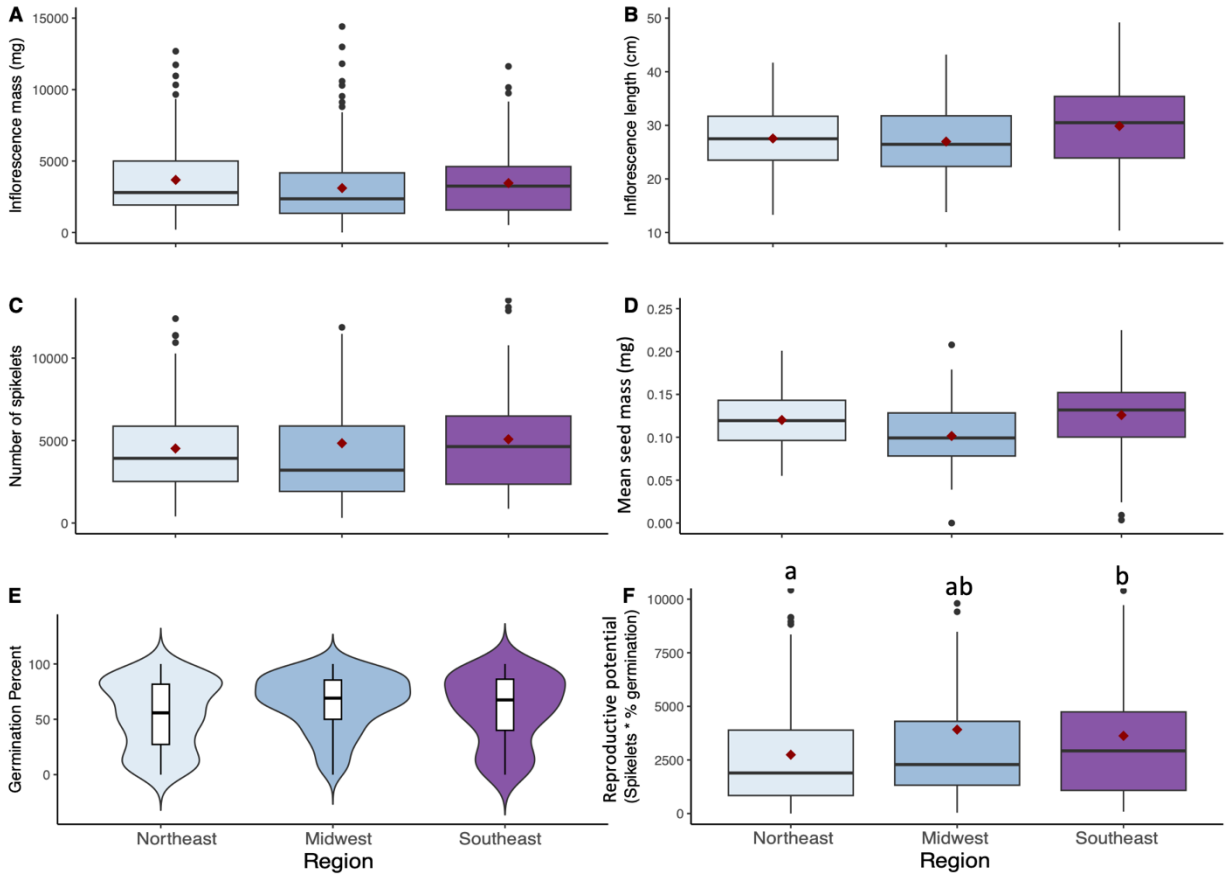


Figure 1-4 Effect of logarithm of watershed level nitrogen on percent germination across region of origin (Northeast, Midwest, and Southeast). Green points represent seeds from inflorescences sourced from the Northeast, blue points represent seeds from inflorescences sourced from the Southeast, and red points represent seeds from inflorescences sourced from the Midwest. Lines represent local regression and grey area represents the confidence interval.

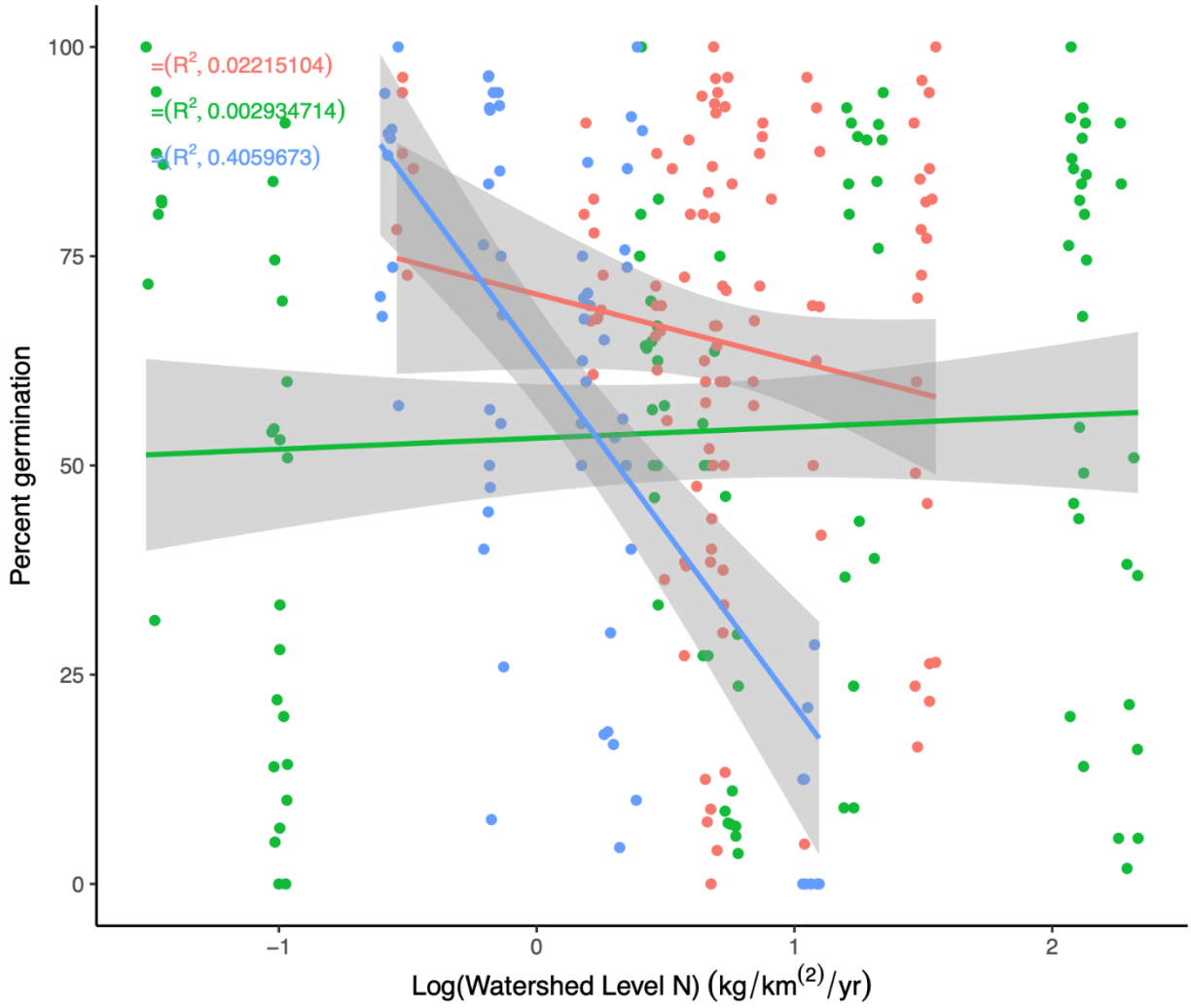


Figure 1-5 Mean of A) total shoot height at day 42, B) number of shoots at day 42, C) aboveground and belowground biomass, and D) aboveground:belowground biomass by region of origin (Northeast, Midwest, and Southeast) and nitrogen treatment (ambient and elevated nitrogen). Letters represent Tukey's HSD test displayed in compact letter design (CLD) showing significant pairwise comparisons between each region*nitrogen treatment (significantly different letters indicated in red). In plot "C" CLD letter below the x-axis represent comparisons in belowground biomass, and error bars represent standard error. The dark red diamond within each boxplot represents the mean value,the upper and lower hinges of each boxplot represent the 25th and 75th quantile, the middle line represents the median value, the whiskers represent the 5th and 95th percentile, and outliers are represented by black points.

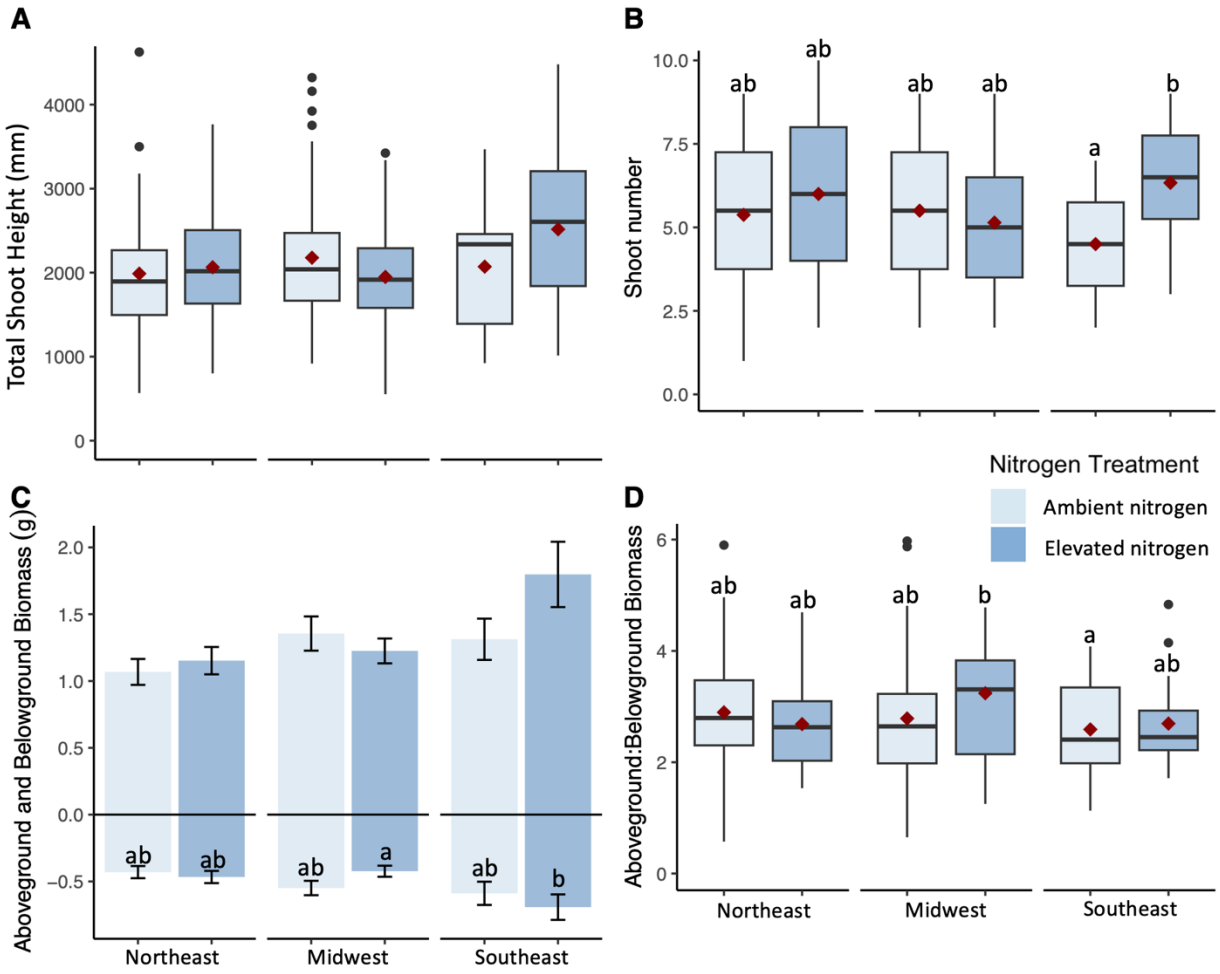
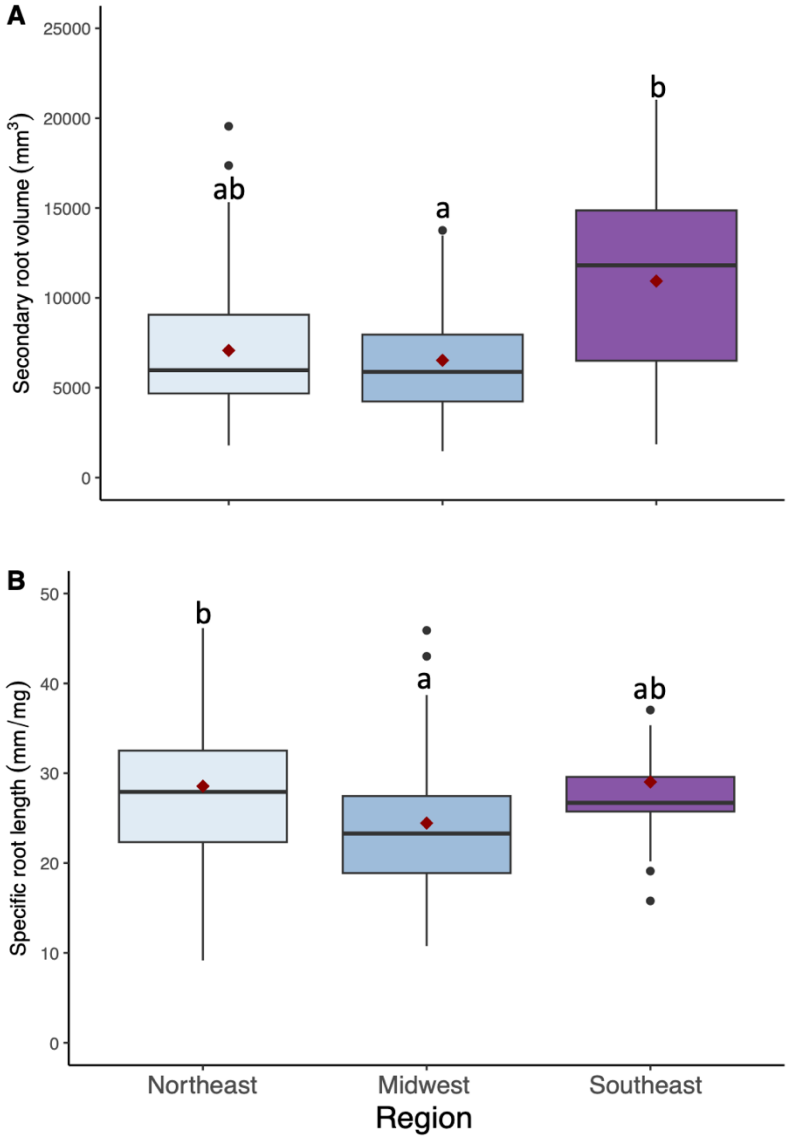


Figure 1-6 Mean of A) secondary root volume and B) specific root length by region of origin (Northeast, Midwest, and Southeast). Letters represent Tukey's HSD test displayed in compact letter design (CLD) showing significant pairwise comparisons between each region. The dark red diamond within each boxplot represents the mean value, the upper and lower hinges of each boxplot represent the 25th and 75th quantile, the middle line represents the median value, the whiskers represent the 5th and 95th percentile, and outliers are represented by black points. The box and whiskers in the violin plot represent the 10th quantile (lower whisker), 25th quartile (lower edge of box), midline (median), 75th quartile (top edge of box), and 90th quantile (top whisker).



CHAPTER 2. THE EFFECTS OF LONG-TERM CO_2 AND NITROGEN FERTILIZATION ON *PHRAGMITES AUSTRALIS* FECUNDITY AND SEEDLING PERFORMANCE

Introduction

Industrialization has increased the bioavailability of key plant nutrients such as CO_2 through industrial emissions and aquatic and edaphic nitrogen loads through aerial deposition and run-off (Galloway et al. 2004; Ekwurzel et al. 2017). Nitrogen fertilization through aerial deposition and runoff have been associated with promoting the growth of grasses across upland grasslands to tidal wetlands (Tyler et al. 2007; Čuda et al. 2021). An increase in atmospheric CO_2 can have a "fertilization effect" in C_3 plants because of RuBisCO-limited photosynthetic rates (Kirschbaum 2011). These global change factors (GCFs) have been shown to disproportionately benefit invasive plants in a variety of habitats ranging from arid upland systems to coastal wetlands (Tyler et al. 2007; Mozdzer & Megonigal 2012; Erskine-Ogden et al. 2016; Jabran & Dogan 2020). Most studies involving the effect of GCFs on invasive plants focus on the growth of adult plants while the effects of GCFs on invasive plant reproduction and fecundity are less well understood, despite the fact that propagule pressure (quantity, diversity, and frequency at which a species is introduced into a new area) can be a key determinant in the invasion success and spread of many invasive species (Colautti 2004; Ricciardi et al. 2010). GCFs like elevated CO_2 and nitrogen (N) can have a significant effect on the fecundity of invasive plants, which can in turn alter their rates of spread. However, the consensus on the direction and magnitude of elevated CO_2 effects on plant fecundity is mixed. While some studies have found a decrease in fecundity with increasing CO_2 (Case et al. 1998), others observe

increased fecundity through increased seeds and cones in coniferous trees (LaDeau & Clark 2001; Way et al. 2009), increased inflorescence mass in C3 grasses (HilleRisLambers et al. 2009). Other studies have found that elevated CO_2 had a negligible effect on plant fecundity (Chadha et al. 2020; Weller et al. 2020). Nitrogen fertilization has been implemented in agricultural systems for most of human history to increase crop yields, and modern N fertilization practices have increased crop yields 45% to 70% (Mueller et al. 2012). Many wild plants reflect the same pattern observed in agricultural crops, with increases in fecundity with increased N (Wander & Bouwmeester 1998; Drenovsky & Richards 2005; Jamieson et al. 2012; Li et al. 2016; Morrison & Questad 2019). In addition to increasing fecundity, elevated CO_2 and N have been demonstrated to increase seedling survival in some plant species, although this can be highly species specific (Bowler & Press 1993; Newbery & Wolfenden 1996; Sefcik et al. 2007; Clark & Tilman 2010). A quick transition out of the seedling stage and into an established plant could greatly increase the potential for the establishment of new populations, and in invasive species, could increase the rate of spread. Thus, increases in invasive plant fecundity in response to elevated CO_2 and nitrogen can translate into higher rates of seedling recruitment and exacerbate invasion under global change.

Most studies on the effects of CO_2 and N on invasive plants focus on short-term experiments (Jablonski 1997; Tyler et al. 2007; Kao-Kniffin & Balser 2007; Hager et al. 2016; Jabran & Dogan 2020) and manipulate a single GCF (Huxman et al. 1998; Edwards et al. 2001; Langley et al. 2009; Gao et al. 2020). While studying the effects of single-factor GCFs is useful in isolating singular effects, studies exploring the interactive effects of multiple GCFs are imperative in understanding their non-additive effects (Langley & Megonigal 2010; Mozdzer & Megonigal 2012; Farrer et al. 2014), which are critical for predicting global change impacts

under future climate scenarios. Furthermore, long-term studies on the effects of elevated CO_2 and N on plants provide the opportunity to study biological processes such as adaptive evolution and transgenerational effects that operate on generational timescales of years and decades. Survey studies have already found examples of adaptive evolution in invasive plants in response to GCFs in the field (Lau et al. 2008; Saban et al. 2019; Bernik et al. 2018; Clements & Jones 2021). Transgenerational studies on elevated CO_2 in plants using model and agricultural species have revealed increasing growth response to elevated CO_2 with subsequent generations (Bezemer 1998; Li et al. 2019; Panda et al. 2023). While some long-term studies of GCFs demonstrate adaptive evolution in plants, other studies show little evidence for adaptive evolution and some studies even show evidence that plants acclimatize to GCFs, growing less under GCF exposure than ambient conditions (Ward et al. 2000; Steinger et al. 2007; Leakey & Lau 2012; Drake 2014; Horgan-Kobelski et al. 2016). Large variations in plant response to long-term GCF exposure demonstrates the unpredictability of plant performance under global change and warrants species-specific GCF studies of ecologically significant plants.

Phragmites australis (Cav.) Trin. ex Steud. (hereafter referred to as *Phragmites*) is one of the most well studied and widely distributed C3 grasses on earth. In North America, both native and introduced subspecies are found (Saltonstall et al. 2004), and the introduced lineage has become one of the most prolific wetland invaders, having rapidly expanded across the continent for the last 150 years (Saltonstall 2002). Anthropogenic disturbance and increased eutrophication have been associated with the spread of *Phragmites* (Chambers et al. 1999; Minchinton & Bertness 2003; Saltonstall & Stevenson 2007; Guo et al. 2013), and numerous experiments have shown positive effects of CO_2 and nitrogen on the vegetative growth of adult *Phragmites* individuals (Mozdzer & Megonigal 2012; Eller et al. 2013; Mozdzer & Caplan

2018). Thus, the increased potential for *Phragmites* expansion under projected global change scenarios is probable through increased vegetative growth resulting in higher displacement of native plants.

Propagule pressure is an important driver in the expansion of invasive species. Increasing the number of propagules through boosting plant fecundity is one way to increase propagule pressure and the potential for successful seedling recruitment (Colautti 2004; Simberloff 2010). Although *Phragmites* can expand through vegetative growth, long-distance establishment of new populations and patch expansion is predominantly through seedling recruitment (Brisson et al. 2010; Kirk et al. 2011; Albert et al. 2015; Kettenring et al. 2016). Despite the importance of seedling recruitment in *Phragmites* invasions there has been little attention paid to the factors controlling fecundity and seedling performance. While there are no studies investigating the effect of CO_2 on *Phragmites* fecundity, elevated N tends to increase the number of inflorescences and florets per inflorescence in *Phragmites* (Kettenring et al. 2011; Lee et al. unpublished data). However, the increased vegetative growth of *Phragmites* under elevated CO_2 may translate into increased allocation of resources into inflorescences and seed provisioning, which has been shown to translate into increased seedling biomass (Lau et al. 2008; Mozdzer & Megonigal 2012; Eller et al. 2013; Mozdzer & Caplan 2018). Additionally, elevated CO_2 increases rates of seed germination in a variety of grass species (Edwards et al. 2001; Baruch & Jackson 2005; de Faria et al. 2015). It is also well established that nitrogen promotes *Phragmites* seedling growth, and produces more shoots of greater height and mass, as well as increasing belowground biomass (Saltonstall & Stevenson 2007; Deng et al. 2010; Kettenring & Whigham 2018). Overall, these results suggest that elevated CO_2 and nitrogen could have positive effects on fecundity and seedling recruitment.

In this study, we examined the cumulative effects of a 10-year long-term exposure to elevated CO_2 and nitrogen on seed and inflorescence traits of material sourced from the invasive *Phragmites* experiment at the Smithsonian Environmental Research Center's Global Change Research Wetland (GCREW) (Edgewater, Maryland). GCREW is part of the NSF Long-Term Ecological Research Network (LTER). GCREW is one of the longest running global change research facilities on Earth and is unique in focusing on global change effects on coastal wetland systems. Apart from measuring fecundity in the GCREW experiment, seedling performance was also assessed through a growth experiment to test whether seedlings of maternal plants grown under global change conditions perform differently from seedlings of maternal plants grown under ambient conditions. Assessing both adult plant fecundity and seedling growth addresses the two factors contributing to seedling recruitment and will allow us to detect if any adaptive changes have occurred in *Phragmites* under chronic exposure to global change factors.

Based on strong evidence of N boosting *Phragmites* fecundity and the mixed results of CO_2 fertilization effects on invasive plant fecundity, we hypothesize (H1) that elevated N, and to a lesser degree CO_2, will increase resource allocation to reproduction and increase inflorescence mass, length, number of spikelets, average seed mass, percent germination and fecundity potential. *Phragmites* affinity for establishing in nutrient polluted habitats and the positive growth effects of elevated CO_2 and N on *Phragmites* growth leads us to hypothesize (H2) that elevated CO_2 and N will promote *Phragmites* seedling growth which will be manifested in higher above/belowground biomass, shoot height, and number of shoots. Lastly, we predict that because of acclimatization and/or selection for increased growth under elevated CO_2 and N, (H3) seedlings from maternal plants grown under long-term exposure to elevated CO_2 and N will have

a greater growth response to elevated CO_2 and N which and have higher above/belowground biomass, shoot height, and number of shoots than seedlings from naïve maternal plants.

Methods

Invasive Phragmites experiment design

All plant material collected for this experiment was collected from the *Invasive Phragmites experiment* from the GCREW LTER site the Smithsonian Environmental Research Center (Edgewater, MD) which consists of twelve open-top chambers (OTCs) established in 2011 along the invasion front of *Phragmites* into native *Spartina patens*-dominated wetland. Each OTC receives a combination of elevated or ambient nitrogen (elevated; addition of 25g/m^2/year) and CO_2 (elevated; 700ppm). The OTCs (each 2.5 m in length x 1.24m in width x 4.5m in height) are part of a fully factorial experiment with ambient and elevated CO_2 treatments crossed with ambient and elevated N treatments (2 nitrogen treatments * 2 CO_2 treatments * 3 replicates = 12 chambers). Recent genotyping revealed the existence of multiple *Phragmites* genotypes within each OTC (McCormick et al. unpublished data).

Seed collection

Ten mature *Phragmites* inflorescences were collected at random in each OTC throughout October 2021. Inflorescences were monitored throughout the growth season and collected before seed drop whenever possible. Each inflorescence was removed from the shoot at the first emergent lateral pedicel. All inflorescences were stored at room temperature for 4-6 weeks while inflorescences traits were being measured before seed extraction. Mature seeds were stripped

from the inflorescence and all seed coverings were removed (palea and lemma) by rubbing inflorescences against a 1mm sieve followed by a 0.5mm sieve. The chaff was blown off and only mature naked seeds were retained. Naked seeds were stored at 4°C between seed measurements and germination trials.

Seed fecundity tests

Inflorescence/seed traits

Inflorescences were clipped at the point of the last pedicel and inflorescence length was measured from the tip of the apical spikelet to the point where the rachis meets the last pedicel, and the mass of each inflorescence mass was measured using a Standard ME Precision Balance (Mettler-Toledo, Columbus OH). The total number of spikelets were counted on three random inflorescences from each chamber and a mass:spikelet relationship was derived using the average of 3 inflorescences in each respective chamber to estimate the total number of spikelets for the remaining inflorescences in each respective chamber. The mass of 50 seeds was measured on a microbalance and averaged to calculate average seed mass per seed (Mettler-Toledo, Columbus OH).

The same 50-seed aliquots were plated on 1% agarose (50 seeds per 90mm*15mm petri plate). All seeds were sterilized using a modified version of the surface sterilization protocol from Shearin et al. (2018) consisting of two consecutive 70% ethanol/ 3% sodium hypochlorite washes over two days to maximize surface sterilization potential. After the second surface sterilization wash, the seeds were placed in a growth chamber with a 18/6 hour light cycle and 30-15°C day:night temperature cycle. Petri dishes were checked daily for mold and infected seeds were removed. Percent germination was calculated from the number of germinated seeds

per plate after 2 weeks. We constructed a fecundity metric we term fecundity potential, which is the number of spikelets on an inflorescence multiplied by the percent germination of seeds from that inflorescence which should give us a proxy of the potential reproductive output of that shoot.

Seedling growth experiment

Growth Set-up

Three *Phragmites* seedlings from each genotype (1-5 genotypes dependent on number of genotypes present in that chamber) from each of the 11 OTCs (no viable seeds were recovered from the elevated CO_2 "chamber 10") were grown under either ambient or elevated CO_2 and ambient or elevated nitrogen (3 seedlings*1-5 genotypes*11 chambers*2 CO_2*2 N treatments = 269 pots). Due to seed supply limitations within chambers 11 and 12, we were forced to use two or sometimes one seedling per genotype per experimental treatment. The number of genotypes between *Phragmites* populations can be highly variable, thus the variation between OTCs exhibited in our study reflects natural systems (Kettenring et al. 2016). Despite this we decided to replicate on the genotypic level rather than on the chamber level so not to overrepresent any one genotype within our sampling.

Growth regime

Seedlings were germinated using the methods described in the germination trials above. Seedlings were then transferred into pots (13.34cm L x 13.34cm W x 15.24cm H) filled with Leafgro high humus soil conditioner (Leafgro, Maryland Environmental Service, Millersville, Maryland, USA). Pots receiving the same nitrogen treatment were placed into corresponding flat

trays (54.43 cm L x 27.79 cm W x 20 cm H), each holding up to 8 pots, at which time the trays were fertilized with either elevated N treatment solution (555mg NH_4Cl; equivalent to 25g/m^2/year) or the ambient N treatment solution (66mg NH_4Cl; equivalent to 3g/m^2/year). These trays were then arranged on shelves located in one of two growth rooms where the elevated CO_2 room received an equivalent of ~900ppm of CO_2 and the ambient CO_2 room ~450ppm. The CO_2 levels in the elevated CO_2 growth chamber was controlled by an Arduino Mega 2560 microcontroller (Arduino, Somerville, MA, USA) connected to a Senseair K30 (Senseair, Delsbo, Sweden) CO_2 sensor which controlled an EV-2M-12-L valve (Clippard, Cincinnati, OH, USA). The rate of CO_2 flow through the valve was determined using an exponential growth and decay feedback loop on a 10-second sampling interval with a CO_2 setpoint of 900ppm. Temperature and humidity were logged using the Adafruit BME680 (Adafruit, New York, NY, USA).

Trays were bottom watered to the lip of the tray twice per week and the corresponding N treatment solutions were added to the trays every 2 weeks, at which time pots were rotated between corresponding N treatment trays. The CO_2 treatment was swapped between the two rooms midway through the growth period to account for differences in growth rooms. Briefly, the CO_2 was shut off in the growth chamber receiving elevated CO_2 and flushed to ambient CO_2 levels (~450ppm), during which the pots receiving elevated CO_2 were transferred to the growth chamber receiving ambient CO_2. Following this, the line feeding CO_2 was transferred to the growth chamber previously receiving ambient CO_2 and the CO_2 was turned on, establishing the new elevated CO_2 growth chamber and the plants receiving ambient CO_2 were placed in the previously elevated CO_2 growth chamber which was now under ambient CO_2.

Seedling shoot number and height were collected on days 7, 14, 28, 42, and 50 of the growth experiment. Shoot number of each plant was collected by counting the number of fully emergent shoots over 5mm in height. The rate at which shoots were growing (rate of shoot growth) was calculated by logarithm of the average shoot growth per day derived by dividing the difference in total shoot height at each timepoint by the days elapsed since the last timepoint (average log (total shoot height (mm) at TP_n – total shoot height at TP_{n-1})/(day at TP_n – day at TP_{n-1}). The rate at which seedlings were producing new shoots (shoot emergence rate) was calculated average (shoot number at TP_n – shoot number at TP_{n-1})/(day at TP_n – day at TP_{n-1}).

Seedlings were harvested over the next four days beginning on day 50. Seedlings were washed of soil and then clipped to separate aboveground and belowground biomass. The third fully formed leaf from the tallest shoot within each pot was collected for specific leaf area measurements. Biomass was dried to constant mass at 50°C, with the leaf sample collected for specific leaf area measurements was added back to aboveground biomass after drying. A subset of seedlings had their freshly harvested roots imaged before being dried. The *RhizoVision Explorer* software was used to acquire root length, specific root length, and root surface area (Seethepali et al. 2020). The root volume of each seedling was collected using volume displacement of the belowground biomass in a 50mL graduated cylinder.

Data analysis

We tested the effects of nitrogen, CO_2, and their interaction on each of our inflorescence and seed response variables (inflorescence length, inflorescence mass, spikelet number, average seed mass, percent germination, and fecundity potential) using multiple linear mixed effects models and generalized least square models using the “lme” and “gls” functions in the R package

nlme version 3.1-149 in R studio version 1.3.1093 (Pinheiro et al. 2023). An initial GLS base model was created for backwards selection, after which we created LME models by adding in chamber as a random effect using the REML approach. We used AIC to compare models, selecting the model with the lower AIC value. If the best-fit model included a random effect, we then tested whether including heterogeneous variances across chambers created a model with a lower AIC. Type III ANOVAs were then performed on the best model to determine the significance of each of the fixed effects. Model validation was performed by plotting fitted values against residuals, each of the fixed effects against residuals, and a histogram of the residuals alone.

For the seedling growth experiment, we tested the singular and interactive effects of the level of N and CO_2 experienced by the seedlings during the seedling growth experiment and the maternal treatment (N*CO_2*maternal treatment) for each response variable (total biomass, aboveground biomass, belowground biomass, aboveground:belowground biomass, SLA, total shoot height, shoot number, and a variety of root traits). We used the model selection process as described above, with the addition of adding in genotype (determined using microsatellite genotyping method as described in McCormick et al. 2016) as a random effect and including heterogeneous variances across genotype if it created the best-fit model.

Results

Inflorescence and Seed traits

Nitrogen significantly increased *Phragmites* inflorescence mass (Table 2-1) where plants grown under elevated N (2046.536 mg ± 108.648) had 124% higher inflorescence mass than plants grown under ambient N (917.607 mg ± 54.605) (Figure 2-1(B)). We also observed this

pattern for inflorescence length and spikelet number (Table 2-1), with plants grown under elevated N having 108% more spikelets (2046.536 ± 113.035) as well as 33% longer inflorescences (24.794 cm ± 0.047) than plants grown under ambient N (spikelet number: 984.710 ± 68.408, inflorescence length: 18.683 cm ± 0.039), respectively (Figure 2-1(A,C)). Nitrogen did not have a significant effect on *Phragmites* average seed mass, nor percent germination (Table 2-1). However, *Phragmites* grown under elevated N did have significantly higher fecundity potential (Table 2-1), with plants grown under elevated N having 91% higher fecundity potential (2126.825 ± 206.284) than plants grown under ambient N (1115.439 ± 140.945) (Figure 2-1(D)). Neither CO_2 alone nor the interaction between CO_2*N had any significant effect on any of the inflorescence and seed traits tested in this study (Table 2-1).

Seedling growth experiment: shoot height accumulation

Our results show that the interaction between N, CO_2, and maternal treatment significantly affected the variance in total shoot height of *Phragmites* seedlings (Table 2-2). Specifically, seedlings from maternal plants exposed to elevated CO_2+N (EN-maternal) exhibited a 58% increase in total shoot height under elevated N alone (2279.550 mm ± 242.038) compared to ambient conditions (1445.150 mm ± 103.850) (Table 2-3). Furthermore, while the interaction between N and maternal treatment significantly affected the rate of shoot height accumulation (Table 2-2), our Tukey's post-hoc test failed to resolve significant pairwise comparisons (Table 2-3).

Seedling growth experiment: shoot number

The interaction between N and maternal treatment significantly affected both the variance in the number of shoots and the rate of shoot emergence in *Phragmites* seedlings (Table 2-2). Notably, EN-maternal seedlings grown under elevated N produced 29% more shoots (5.537 ± 0.294) than when grown under ambient N (4.293 ± 0.255) (Figure 2-2(A)). Additionally, the rate of shoot emergence for EN-maternal seedlings under elevated N (0.175 shoots *day^{-1} ± 0.010) was 34% and 51% higher than the same seedlings grown under ambient conditions (0.131 shoots *day^{-1} ± 0.009) and seedlings from maternal plants exposed to elevated N (N-maternal) grown under elevated N (0.116 shoots *day^{-1} ± 0.012), respectively (Figure 2-2(B)).

Seedling growth experiment: specific leaf area

We found that the interaction between N, CO_2, and maternal treatment significantly influenced the variance in mean specific leaf area (SLA) of *Phragmites* seedlings (Table 2-2). Seedlings from mothers exposed to elevated CO_2 (E-maternal) grown under elevated N alone showed 53%, 48%, and 40% higher SLA (514.26mm^2 * mg^{-1} ± 65.22) compared to those grown under ambient (336.60 mm^2 * mg^{-1} ± 13.44), elevated CO_2 (347.17 mm^2 * mg^{-1} ± 16.48), and elevated CO_2+N (366.75 mm^2 * mg^{-1} ± 19.15) conditions, respectively (Table 2-3). Furthermore, these E-maternal seedlings had 57% higher SLA than N-maternal seedlings grown under elevated CO_2 (328.58 mm^2 * mg^{-1} ± 12.57) (Table 2-3).

Seedling growth experiment: biomass

The interaction between N, CO_2, and maternal treatment significantly influenced the variance in mean total and aboveground biomass of *Phragmites* seedlings (Table 2-2). Specifically, seedlings from maternal plants grown under ambient conditions (A-maternal) and

grown under elevated CO_2+N conditions (2.49 g ± 0.33) achieved 64% and 67% higher total biomass than when grown under elevated N alone (1.58 g ± 0.12) or ambient conditions (1.49 g ± 0.15), respectively (Figure 2-3). Moreover, the aboveground biomass for A-maternal seedlings under elevated CO_2+N (1.65 g ± 0.19) was 51% and 61% higher than under elevated N alone (1.10 g ± 0.09) or ambient conditions (1.03 g ± 0.11), respectively (Figure 2-3). We also found that in EN-maternal seedlings, seedlings grown under elevated N alone (1.32 g ± 0.18) had 79% greater aboveground biomass than the same seedlings grown under ambient conditions (0.74 g ± 0.10) (Fig. 2-3). We also found that elevated CO_2 significantly increased the belowground biomass (0.68 g ± 0.04) of *Phragmites* seedlings by 48% compared to ambient CO_2 conditions (0.46 g ± 0.03) (Figure 2-4; Table 2-2).

Seedling growth experiment: root traits

The variance in root volume of *Phragmites* seedlings was significantly affected by the interaction between N, CO_2, and maternal treatment (Table 2-2). Among A-maternal seedlings, we observed that seedlings grown under elevated CO_2+N (19.40 cm^3 ± 3.24) had 82% and 123% higher root volume than those grown under ambient conditions (10.68 cm^3 ± 1.23) and elevated N (9.86 cm^3 ± 1.46), respectively (Table 2-3). Additionally, elevated N significantly increased the specific root length (SRL) of *Phragmites* seedlings, though we could not resolve pairwise significance using Tukey's HSD test (Table 2-2).

Discussion

Here we demonstrate the positive effects that elevated CO_2 and N have on *Phragmites* fecundity and seedling performance. We observed that *Phragmites* produced inflorescences with higher numbers of spikelets, longer inflorescences and higher inflorescence mass, resulting in a higher potential fecundity (more viable seeds) when grown under elevated nitrogen compared to plants grown under ambient nitrogen. Unexpectedly, the increase in inflorescence size under elevated nitrogen did not translate to higher average seed mass nor did it significantly affect percent germination in plants grown under elevated nitrogen. Elevated CO_2 had a consistent belowground growth promoting effect across all *Phragmites* seedlings, regardless of maternal treatment. Although we observed some interactive effects between maternal treatments and experimental/nitrogen treatments in various aboveground traits, these effects were not consistent, and we found no evidence that seedlings grow better under maternal conditions than seedlings naïve to treatment conditions. Integrating both fecundity and seedling performance into estimating rates of seedling recruitment under projected global change scenarios accounts for the interactive effects of elevated CO_2 and N on *P. australis* fecundity and seedling performance. These results provide insight into how seedling recruitment dynamics under projected GCF exposure may influence *Phragmites* invasion into the future.

Increasing N supply to plants can boost both vegetative growth and reproductive output. *Phragmites* tends to establish in areas of high disturbance which are often areas that also experience high levels of N pollution (Silliman & Bertness 2004; King et al. 2007; Guo et al. 2018). There is a long-held assumption that nitrogen additions can increase the reproductive output of plants, and more recently this has been extended to numerous invasive plants (Wander & Bouwmeester 1998; Jamieson et al. 2012; Li et al. 2016; Morrison & Questad 2019). Our study reflects patterns found in other plant species as *P. australis* grown under elevated N

produced inflorescences with greater length, mass, and spikelet number than *Phragmites* grown under ambient N. Additionally, our study demonstrates that nitrogen fertilization effects persist even after over 10 years of exposure, suggesting that plants within the OTCs exposed to long-term elevated nitrogen do not acclimatize to excess nitrogen. The same pattern of sustained and even increased yields is reflected in other grass species, as demonstrated in long-term agricultural nutrient fertilization studies in *Oryza* spp. (Lee et al. 2008; Wang et al. 2013). Because neither average seed mass nor percent germination differed significantly between any of the GCF treatments, we can assume that the seed quality was not reduced in response to higher allocation to inflorescences. As reflected in the fecundity potential metric, higher *Phragmites* reproductive outputs in-turn increase propagule pressure. If the propagule threshold is not already saturated, the increased fecundity of *P. australis* under prolonged elevated N exposure can facilitate *P. australis* expansion (Lockwood et al. 2005; Ricciardi et al. 2011). In areas of dynamic flooding and salinity, increases in propagule production may help overcome seedling establishment limitations (Bart & Hartman 2003; Deng et al. 2010; Baldwin et al. 2010). In this study we could not accurately capture the number of viable seeds produced by the plants due to constraints of seed capture and the inherent pollen limitations associated with the OTC design. However, the fecundity potential metric (number of spikelets * percent germination) we constructed gives us a proxy for the number of viable seeds that could be produced on each *Phragmites* inflorescence. These results support previous field studies which have found that elevated nutrient loads can increase *P. australis* reproductive output (number of viable seeds, number of florets per inflorescence), contributing to the body of evidence that *Phragmites* invasion will intensify as nitrogen increases under global change (Kettenring et al. 2011).

Our results support the existing literature that elevated CO_2+N boosts *Phragmites* biomass in naïve plants (Mozdzer & Megonigal 2012; Martin & Moseman-Valtierra 2017; Mozdzer & Caplan 2018). We found that A-maternal seedlings grown under a combination of elevated CO_2+N had significantly higher total and aboveground biomass than the same seedlings grown under ambient conditions and elevated N alone. However, this pattern was not observed in any of the other seedlings from other maternal treatments (N-maternal, E-maternal, nor EN-maternal) which suggests some level of acclimatization from long-term exposure to one or both GCFs. In other long-term studies, the growth boosting effects of elevated CO_2 fertilization has been shown to be transitory and eventually a variety of plants will acclimatize to elevated CO_2 by decreased photosynthetic capacity, stomatal conductance, and relative growth rate (Tjoelker et al. 1998; Calfapietra et al. 2003; Ferguson & Nowak 2011; Drake et al. 2014). Although the rate of atmospheric CO_2 is increasing at a rapid rate, these manipulation studies introduce instantaneous changes in available CO_2. This suggests that if plants are acclimating to these instantaneous changes in atmospheric CO_2, that they will also be able to acclimatize to the relatively gradual increase of atmospheric CO_2 from real-word global change.

Unlike the observed patterns in aboveground biomass, where response to GCFs was influenced by maternal treatment, elevated CO_2 consistently boosted the belowground biomass of *Phragmites* seedlings, irrespective of maternal treatment. Our results support previous findings that elevated CO_2 promotes plant growth and that these effects were sustained even across generations (Rasse et al. 2005; Lau et al. 2008; Stiling et al. 2013; Maier et al. 2022). CO_2 fertilization is observed to promote the accumulation of belowground biomass across a wide variety of C3 wetland grasses (Langley et al. 2009; Drake et al. 2014; Zhu et al. 2022). Previous work involving the effects of elevated CO_2 on *Phragmites* belowground traits suggest that

elevated CO_2 disproportionately increases the belowground biomass and rhizome mass of the invasive lineage compared to the native lineage (Mozdzer & Megonigal 2012; Caplan et al. 2014). *Phragmites* invasion increases in soil accretion and sediment trapping compared to other co-occurring wetland plants (Rooth et al. 2003). Higher *Phragmites* belowground biomass under elevated CO_2 can increase the capability of *Phragmites* dominated wetlands keep up with relative sea-level rise, although this effect may drop-off as observed in other wetland plants such as *Schoenoplectus americanus* (Zhu et al. 2022). However, *Phragmites* aerenchyma have been shown to increase soil decomposition through soil aeration causing increased soil compaction and elevation loss (Bernal et al. 2017). Thus, opposing mechanisms of vertical accretion and soil decomposition make predictions of soil elevation changes hard to predict. In addition, increased seedling root growth has been observed to increase the competitiveness of a variety of invasive shrubs (Ming et al. 2018) and invasive grasses (Dong et al. 2014). Thus, an increase in belowground biomass in *Phragmites* seedlings under elevated CO_2 may increase seedling survival through increase competitive ability. The persistent belowground growth- promoting effects of elevated CO_2 provide the possibility of keeping up with relative sea level rise, but the interactions with decomposition and nutrient addition make the outcomes unclear.

One of the primary mechanisms of *Phragmites* invasion into surrounding native wetlands is through lateral expansion and shading out of competitors through the creation of tall dense monocultures (Güsewell & Edwards 1999; Minchinton & Bertness 2003; Brisson et al. 2010). The EN-maternal seedlings in our seedling growth experiment produced more shoots and produced new shoots at a faster rate under elevated N compared to ambient conditions, suggesting that under chronic exposure to elevated CO_2+N, mechanisms responsible for *Phragmites* invasive success will be bolstered. It is also notable that N-maternal seedlings

grown under elevated N had a significantly lower shoot emergence rate when compared to EN-maternal seedling grown under the same conditions, highlighting the importance of maternal effects in growth response under N fertilization. These results suggest that *Phragmites* shoot density may increase dramatically in nutrient polluted areas under elevated CO_2 if these observed differences in growth response between EN-maternal and N-maternal under elevated N are generalizable to wild populations. This pattern becomes increasingly pertinent when considered the distribution of *Phragmites* populations and their propensity to establish in areas of high pollution, which may lead to an increase in stand-level shoot density in many existing populations (Chambers et al., 1999; Amsberry et al., 2000; Minchinton and Bertness, 2003; Silliman and Bertness, 2004; Guo et al. 2013). These findings highlight the interaction between maternal conditions and elevated N in *Phragmites* and gives insight into the possibility for increased efficacy of *Phragmites* lateral expansion under global change.

Conclusions

Predicting invasion under projected climate change scenarios is paramount for designing and implementing effective management practices now and into the future. Our study represents the first to explore both pre-germination and post-germination traits of *Phragmites* relevant to seedling recruitment under various global change scenarios. While we were unable to reliably determine viable seed yield, we found that elevated N increased fecundity potential by increasing the size of *Phragmites* inflorescences, which could increase *Phragmites* propagule pressure in nitrogen polluted habitats. *Phragmites* grown under chronically elevated CO_2 and N acclimatized to GCFs and total biomass under GCFs was highest in naïve plants. However, when we

examined belowground growth, we found that seedlings grown under elevated CO_2 have higher belowground biomass than plants grown under ambient conditions, highlighting the possibility that elevated CO_2 may ameliorate the effects of sea level rise through increased soil accretion in coastal populations and overall increase in seedling survival. Elevated nitrogen increased EN-maternal seedlings shoot number, which suggests that the efficacy of *Phragmites* lateral expansion may improve as more shoots may translate into the greater shading of native plants. Our results highlight potential shifts in *Phragmites* invasion dynamics through seedling recruitment under projected global change and the importance of studying multiple components that contribute to invasiveness.

Table 2-1 ANOVA p-values of linear mixed effects models on the effects of elevated N and CO_2 on explaining the variation in various inflorescence and seed traits in *Phragmites* from the *Phragmites* invasion experiment at GCREW. P-values *<0.05, ** <0.001, ***<0.0001 are indicated in red.

	Inflorescence Length	Spikelet Number	Inflorescence mass
		F-value	
N	5.893 (*)	12.991 (**)	14.845 (***)
CO2	0.834	3.289	3.020
N*CO2	0.578	3.121	3.460
	Average Seed Mass	**% Germination**	**Fecundity Potential**
		F-value	
N	1.926	1.299	6.646 (*)
CO2	1.658	1.442	0.001
N*CO2	0.298	0.370	1.611

Table 2-2 ANOVA Chi-square values of linear mixed effects models on the effects of maternal and experimental treatment and singular global change factor exposure (N and CO_2) on explaining the variation in various *Phragmites* seedling biomass, growth traits, and belowground traits from the *Phragmites* seedling growth experiment. P-values *<0.05, ** <0.001, ***<0.0001 are indicated in red.

	Nitrogen	CO2	Nitrogen * CO2	Parental	Parental *Nitrogen	Parental *CO2	Nitrogen * CO2 * Parental
					Chi-squared		
Accumulated shoot height	0.009	4.420 (*)	0.509	1.671	11.647 (**)	2.822	8.001 (*)
Shoot number	0.509	0.784	0.216	5.428	13.688 (**)	2.993	4.708
Rate of shoot growth	0.723	0.035	0.027	1.824	7.932 (*)	3.152	3.255
Rate of shoot emergence	1.630	0.086	0.347	3.968	17.161 (***)	2.741	5.237
Total biomass	0.030	1.888	3.848 (*)	1.232	7.017	2.735	9.181 (*)
Aboveground biomass	0.251	0.717	3.743	1.940	8.669 (*)	3.996	11.227 (*)
Belowground biomass	0.155	3.963 (*)	2.883	0.516	3.126	0.688	4.473
Root Volume	0.768	2.503	5.604 (*)	0.439	5.470	1.142	8.779 (*)
Total root length	0.183	1.179	0.009	1.305	2.624	1.194	2.269
Fine root length	0.257	0.949	0.020	1.469	3.025	1.442	2.520
Secondary root length	0.120	1.089	0.001	1.545	2.091	1.185	2.120
Rhizome length	0.128	2.008	0.008	0.130	0.605	0.201	1.091
Specific root length	4.640 (*)	3.148	0.801	5.055	2.897	0.992	2.171
Specific leaf area	7.935 (**)	0.188	4.195 (*)	1.903	16.604 (***)	2.387	10.905 (*)

Table 2-3 Means, SE, and Tukey's post-hoc (CLD) of various *Phragmites* seedling total and aboveground biomass, root volume, specific leaf area, and total shoot height across maternal and experimental treatment from the *Phragmites* seedling growth experiment. CLD letters represent Tukey's HSD test across maternal and experimental treatments.

		Root Volume											
		Maternal Treatment											
		Ambient			**Elevated CO_2**			**Elevated N**			**Elevated CO_2*N**		
		Mean (ml)	**SE**	**CLD**	**Mean (ml)**	**SE**	**CLD**	**Mean (ml)**	**SE**	**CLD**	**Mean (ml)**	**SE**	**CLD**
Experimental Treatment	**Ambient**	10.682	1.234	a	9.250	1.759	ab	11.906	1.541	ab	8.400	1.364	ab
	Elevated N	8.705	1.129	a	8.222	1.949	ab	9.857	1.460	ab	12.700	2.446	ab
	Elevated CO_2	14.043	1.504	ab	14.727	1.478	ab	17.250	1.321	ab	14.575	2.103	ab
	Elevated CO^2 * N	19.400	3.235	b	14.556	2.155	ab	14.643	2.751	ab	13.452	2.356	ab
		Specific leaf area											
		Mean (mm^2 . mg^{-1})	**SE**	**CLD**	**Mean (mm^2 . mg^{-1})**	**SE**	**CLD**	**Mean (mm^2 . mg^{-1})**	**SE**	**CLD**	**Mean (mm^2 . mg^{-1})**	**SE**	**CLD**
Experimental Treatment	**Ambient**	364.102	14.787	ab	336.599	13.439	a	367.584	23.460	ab	411.995	17.314	ab
	Elevated N	430.384	19.815	ab	514.255	65.216	b	373.586	14.569	ab	449.816	14.861	ab
	Elevated CO_2	373.896	11.271	ab	347.172	16.481	a	328.582	12.573	a	395.311	14.939	ab
	Elevated CO^2 * N	372.390	16.722	ab	366.746	19.149	a	389.677	26.383	ab	405.060	22.923	ab
		Total shoot height											
		Mean (mm)	**SE**	**CLD**	**Mean (mm)**	**SE**	**CLD**	**Mean (mm)**	**SE**	**CLD**	**Mean (mm)**	**SE**	**CLD**
Experimental Treatment	**Ambient**	1850.500	117.389	ab	1775.875	278.076	ab	2059.941	122.040	-	1445.150	103.850	a
	Elevated N	1846.762	131.416	ab	1744.889	211.105	ab	2104.938	158.765	-	2279.550	242.038	b
	Elevated CO_2	2282.043	110.540	ab	2333.800	168.826	ab	2122.176	88.022	-	2144.952	212.847	ab
	Elevated CO^2 * N	2469.091	205.111	ab	2690.400	398.939	ab	1984.000	180.219	-	2120.571	170.509	ab

Figure 2-1 Barplot of (A) spikelet count, (B) inflorescence mass, (C) inflorescence length, and (D) fecundity potential by nitrogen treatment from the *Phragmites* invasion experiment collected in 2021. Light blue boxes represent the ambient nitrogen treated plants while the dark blue boxes represent the elevated nitrogen treated plants. Letters represent Tukey's HSD test displayed in compact letter design (CLD) showing significant pairwise comparisons between each nitrogen treatment. The dark red diamond within each boxplot represents the mean value, the upper and lower hinges of each boxplot represent the 25th and 75th quantile, the middle line represents the median value, the whiskers represent the 5th and 95th percentile, and outliers are represented by black points.

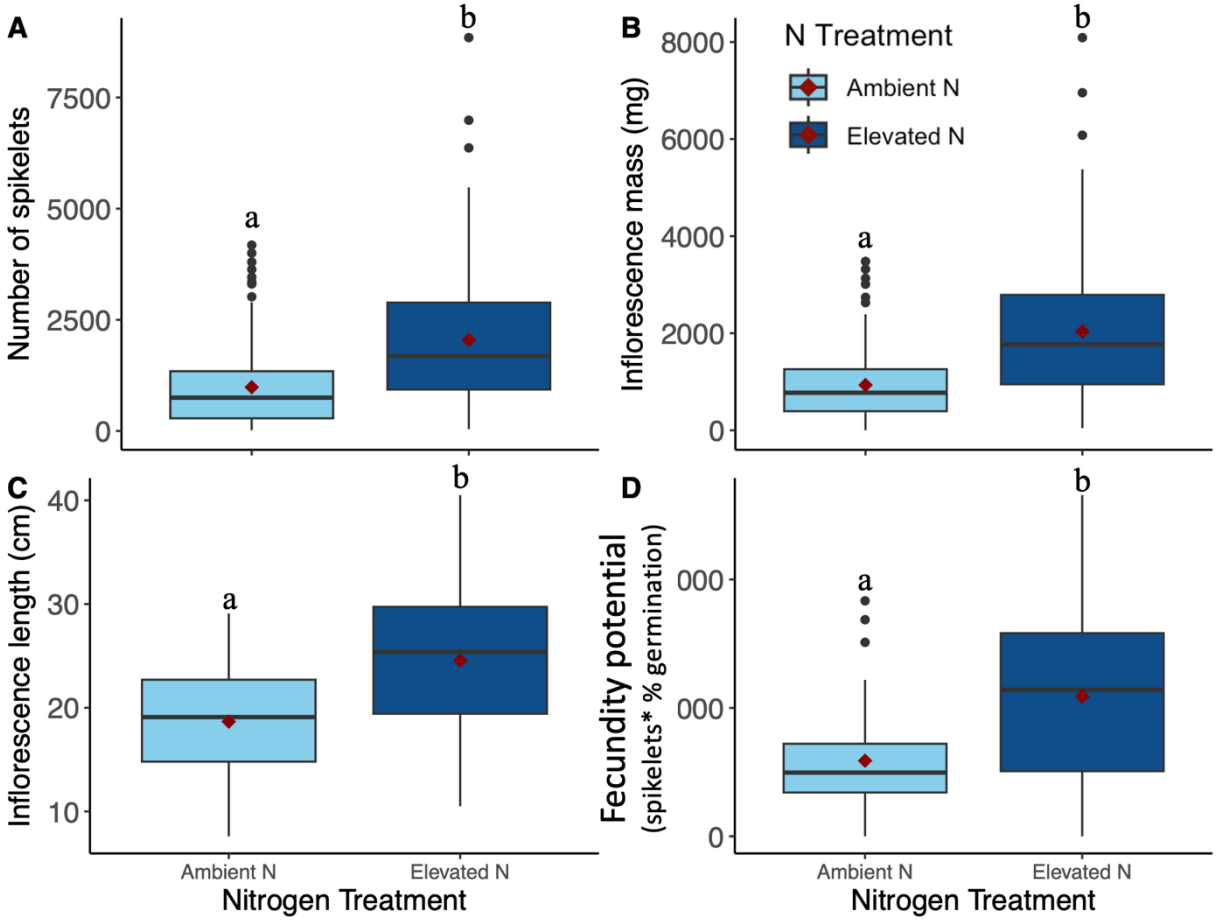


Figure 2-2 Barplot of (A) shoot number and (B) shoot emergence rate by nitrogen treatment and maternal treatment from the *Phragmites* invasion experiment collected in 2021. Light blue boxes represent the ambient nitrogen treated plants while the dark blue boxes represent the elevated nitrogen treated plants. Letters represent Tukey's HSD test displayed in compact letter design (CLD) showing significant pairwise comparisons between each nitrogen treatment*maternal treatment level. The dark red diamond within each boxplot represents the mean value, the upper and lower hinges of each boxplot represent the 25th and 75th quantile, the middle line represents the median value, the whiskers represent the 5th and 95th percentile, and outliers are represented by black points.

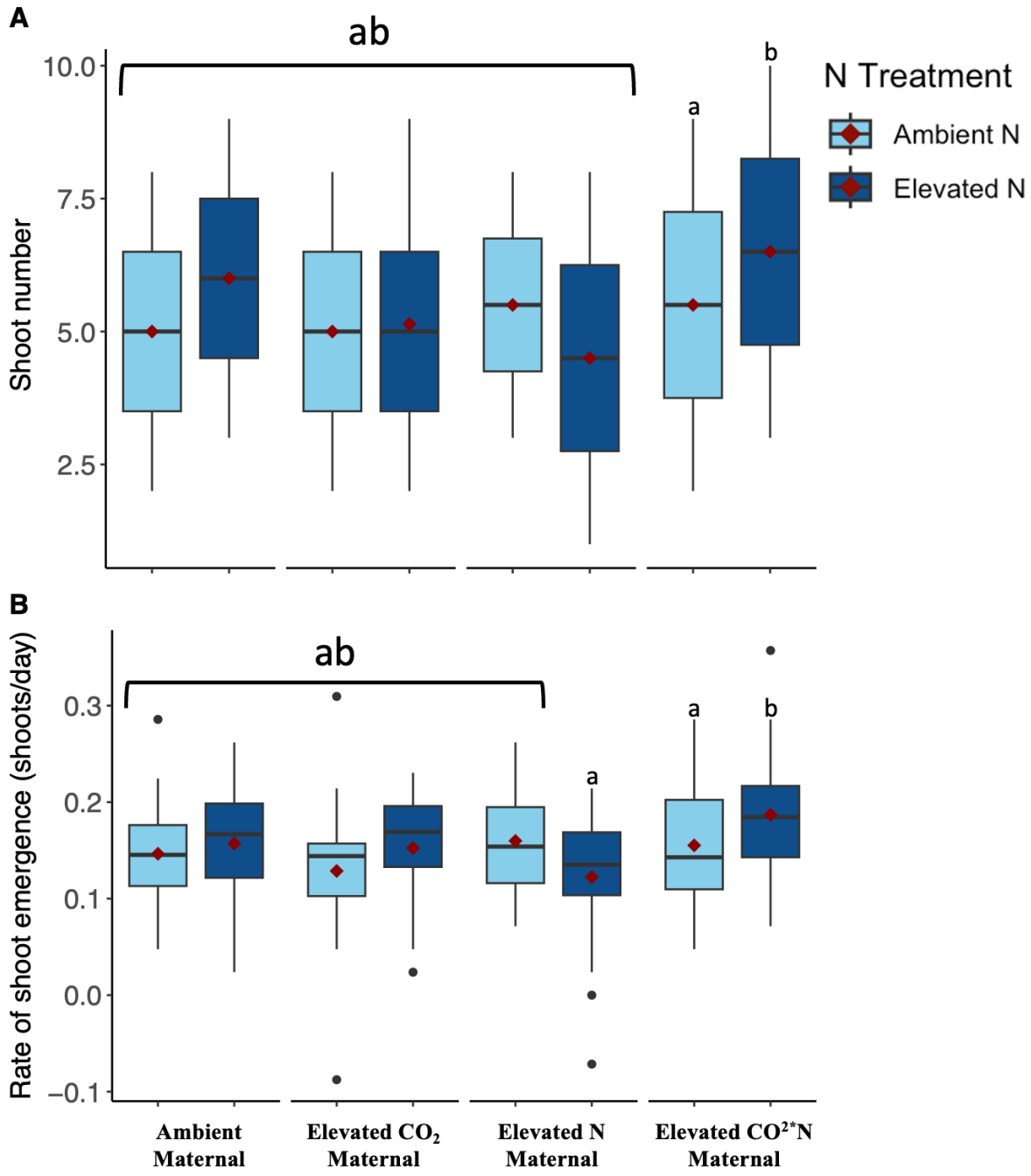


Figure 2-3 Barplot of *Phragmites* seedling above and belowground biomass within each maternal treatment by each experimental treatment from the *Phragmites* seedling growth experiment. Bars above the x-axis represent aboveground biomass while the bars below represent belowground biomass. CLD letters above each bar represent comparisons in total biomass biomass, and letters in each bars represent aboveground biomass and error bars represent standard error. *Phragmites* grown under ambient conditions are represented by grey bars, orange bars for elevated CO_2, blue bars for elevated N, and purple bars for elevated CO_2*N.

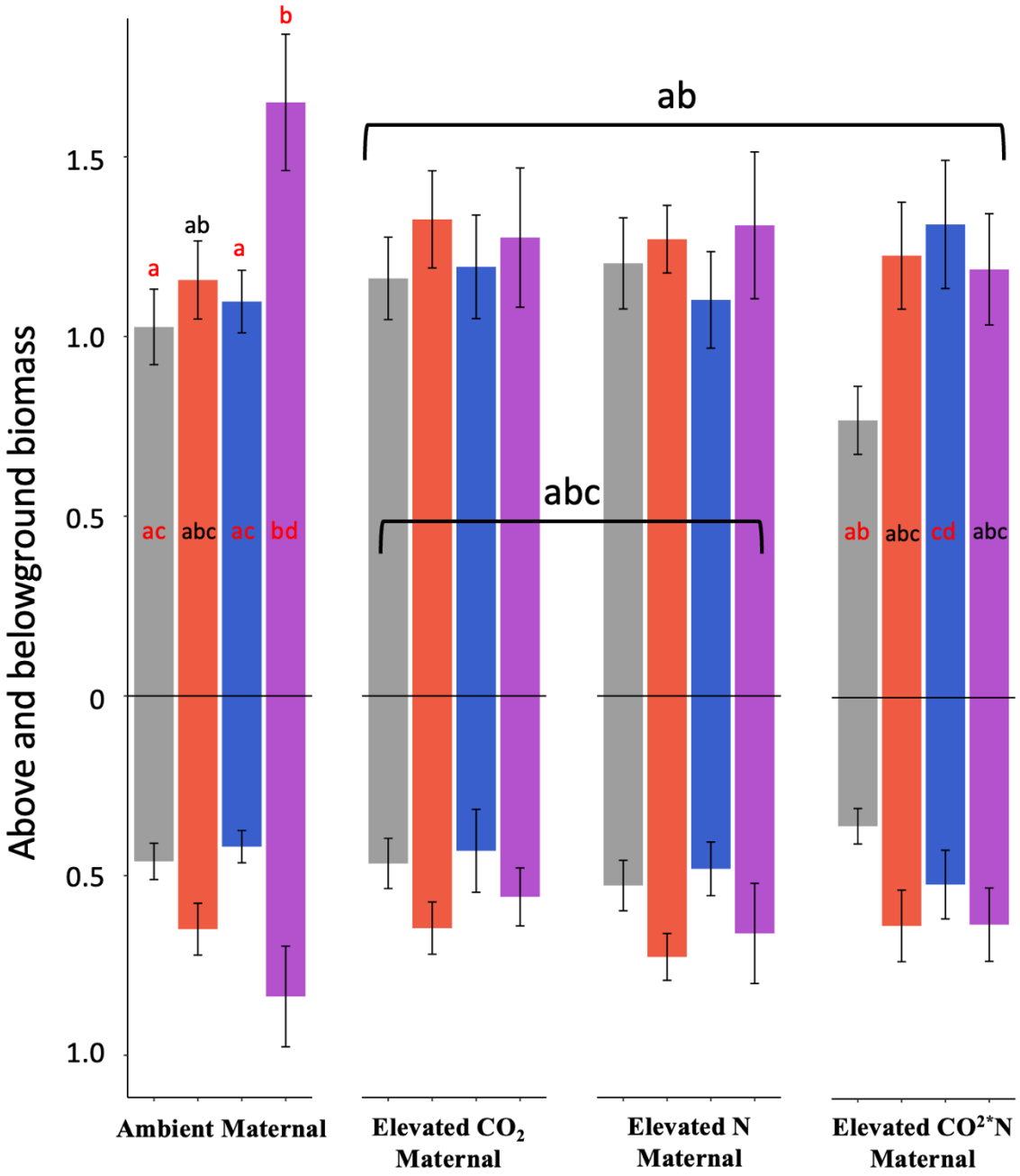
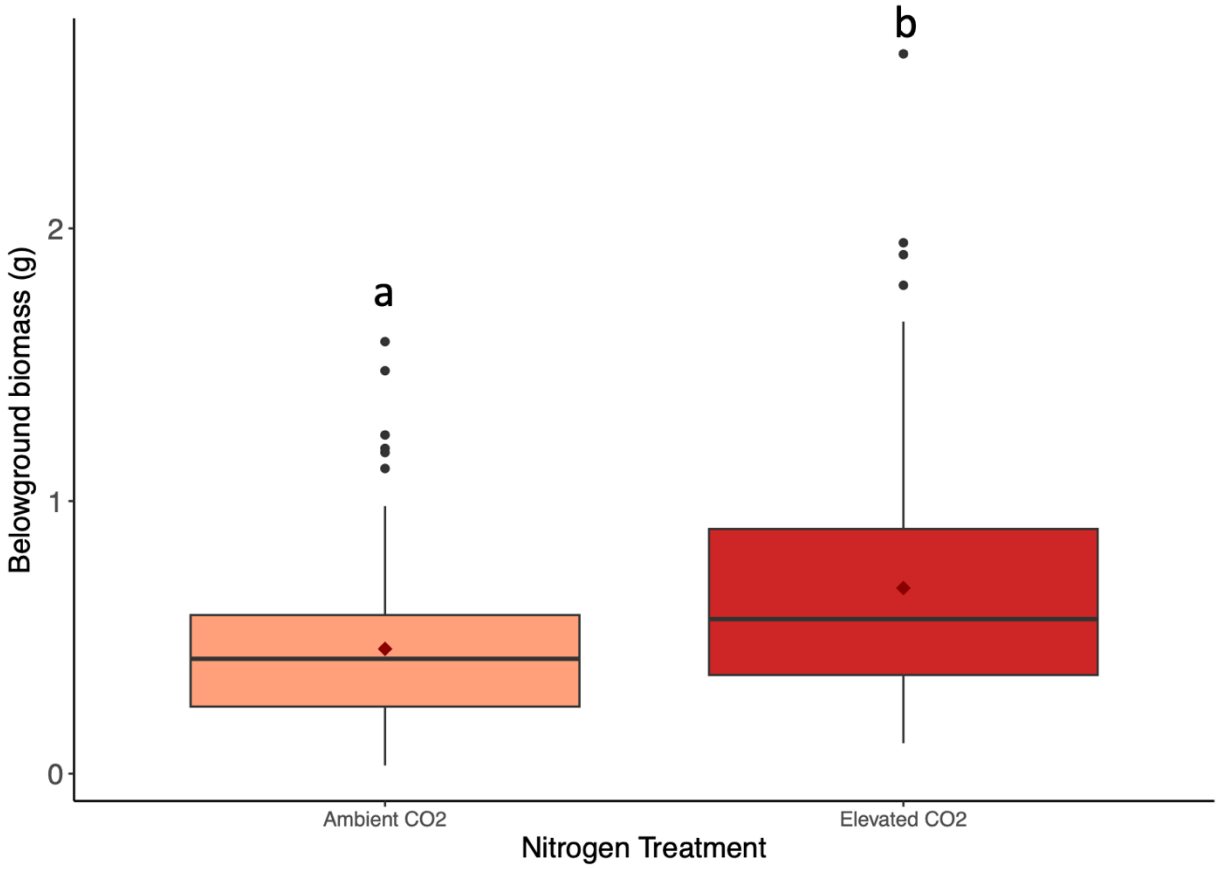


Figure 2-4 Barplot of the belowground biomass by CO_2 treatment from the *Phragmites* seedling growth experiment. Salmon boxes represent the ambient CO_2 treated plants while the dark red boxes represent the elevated CO_2 treated plants. Letters represent Tukey's HSD test displayed in compact letter design (CLD) showing significant pairwise comparisons between CO_2 treatment. The dark red diamond within each boxplot represents the mean value, the upper and lower hinges of each boxplot represent the 25^{th} and 75^{th} quantile, the middle line represents the median value, the whiskers represent the 5^{th} and 95^{th} percentile, and outliers are represented by black points.



CHAPTER 3. THE EFFECTS OF LEAF AGE AND PROVENANCE ON FOLIAR AND LITTER BACTERIAL AND FUNGAL COMMUNITY COMPOSITION IN *PHRAGMITES AUSTRALIS*

Introduction

The associations between plants and microbes span the spectrum of negative to positive interactions, from disease causing pathogens in wild plants such as in chestnut blight (*Cryphonectria parasitica*) and agricultural pathogens like soft rot (*Agrobacterium* spp.) to obligate N-fixing mutualisms in leguminous plants (Leonard et al. 2016; Rigling & Prospero 2018; Fahde et al. 2023). The pairwise interactions between plants and their associated microbial communities have in recent times been demonstrated to drive a variety of ecologically important phenomena, from forest succession, density-dependent coexistence, and success of biological invasions (Packer & Clay 2000; Reinhart & Callaway 2006; Gundale et al. 2014; Bauer et al. 2015). While much of the attention is paid to rhizopheric interactions between plant roots and microbes, plant-microbe interactions in the phyllosphere may be equally important for plant survival and fitness. A multitude of plant pathogens including bacteria, archaea, and fungi specialize on leaf tissues, leading to the destruction of photosynthetically active leaf tissue causing plants to allocate more resources into replacing/repairing damaged tissue, to become resource starved, and to becoming water stressed. Furthermore, symbiotic foliar bacteria and fungi have been shown to prevent infections by leaf pathogens, as well as regulate plant growth promoting hormones, and increase stress tolerance (reviewed in Dodds & Rathjen 2010 and Vorholt 2012).

Although some microbes can be vertically transmitted through seeds, most microbial colonization of leaf tissue is through horizontal transmission through airborne and soilborne

spores (Hodgson et al. 2014; Vorholt 2012; Smets et al. 2022). Thus, each new leaf recruits its own microbial community from environmental inputs, and phyllosphere microbial communities exhibit successional changes as leaves age and senesce (Copeland 2015; Barrett et al. 2015; Smets et al. 2022). Several studies in crop plants such as tobacco (*Nicotiana tabacum* L.) (Gao et al. 2023) and winter wheat (*Triticum aestivum*) (Comby et al. 2016; Grudzinska-Sterno et al. 2016; Sapkota et al. 2017) demonstrate succession of bacterial and fungal phyllosphere communities as leaves age and this is linked to functional and stoichiometric changes in leaf chemistry. As phyllosphere microbes can have wide ranging effects on plant performance, we need to assess how phyllosphere communities assemble and develop across many plant species beyond well-studied agricultural plants as a first step toward understanding the ecology of this diverse and important group of symbionts.

Much attention has been paid to exploring the role of microbes in driving ecological phenomena such as biological invasions over the last two decades. Positive plant-soil feedbacks and belowground enemy-release can facilitate the spread of invasive plants (Mangla & Calloway 2008; Reinhart et al. 2010; Gundale et al. 2014). However, as residence time increases and invasive plants become more abundant, there is also an increased likelihood of specialized mutualists/pathogens being introduced or native microbial spillover onto the invasive plant (Keane & Crawley 2002; Klironomos 2002; Hawkes 2007; Mitchell et al. 2010; Flory & Clay 2013). Several invasive plants display a positive relationship between negative intraspecific plant-soil feedbacks and residence time, suggesting increasing accumulation of *soil* pathogens over time (Nijjer et al. 2007; Diez et al. 2010; Lau & Suwa 2016; Stricker et al. 2016). Invasive plants like garlic mustard (*Allaria petiolata*) (Enright & Cipollini 2007), Japanese stiltgrass (*Microstegium vimineum*) (Flory et al. 2011; Flory & Clay 2013), kudzu (*Pueraria lobata*)

(Harmon et al. 2006), and *Ageratina adenophora* (Li et al. 2022) similarly harbor accumulated *foliar* pathogens which cause significant leaf damage.

Despite the growing knowledge of foliar pathogens in invasive plants, less is known about phyllospheric microbes that occupy a wider diversity of functional roles. Thus, a holistic approach of studying foliar bacterial and fungal communities is needed to assess their role in the success and expansion of introduced species (Vorholt 2012). Foliar microbial community composition comparisons both between native and invasive congeners (Aires et al. 2021) and native range and introduced range populations (Finkel et al. 2011; Lu-Irving et al. 2019) demonstrate distinct interspecific and intraspecific variation in microbial communities which may contribute to invasive success. The composition of foliar microbial communities needs to be explored in highly invasive plants as they represent an important unknown factor that could contribute to invasion.

Phragmites australis (Cav.) Trin. Ex Steud, is one of the most widely distributed plants on Earth. In North America, the cryptic invasion of the Eurasia lineage into the Mid-Atlantic around 150 years ago has caused the decline of many native wetland plants including the native subspecies, *Phragmites australis* subsp. *americanus* (Saltonstall 2002; Meadows & Saltonstall 2007; Kettenring et al. 2012; Hazelton et al. 2014). The spread of *Phragmites* is predicted to increase as it has a propensity to establish in anthropogenically disturbed areas, can tolerate high salinity, has multiple modes of dispersal, and can outcompete many native plants, making it one of the biggest threats to North American wetland diversity (Ailstock et al. 2001; Minchinton & Bertness 2003; Vasquez et al. 2005; Brisson et al. 2010; McCormick et al. 2010; Mozdzer et al. 2013; Kettenring et al. 2016). The distribution and abundance of *Phragmites* exhibits spatial heterogeneity reflecting this recent radiation as Atlantic populations, where invasion is most

intense, and are decades if not a century older than populations further west (Saltonstall 2002; Wilcox et al. 2003; Tulbure et al. 2007; Lambert et al. 2016).

Although *Phragmites* soil and foliar microbial communities have been studied across a variety of microbial functional groups there is still no clear consensus to whether they aid or inhibit *Phragmites* invasion (Nelson & Karp 2013; Crocker et al. 2015; Soares et al. 2016; Bowen et al. 2017; Allen et al. 2018; Shearin et al. 2018; Allen et al. 2020; Gonzalez-Mateu et al. 2020). Vertically-transmitted endophytic fungi and bacteria aid in *Phragmites* growth and germination (White et al. 2016; Shearin et al. 2018; Verma et al. 2018) and a majority of *Phragmites* foliar fungi are saprotrophic with very few isolates causing leaf damage (Allen et al. 2020; DeVries et al. 2020). Existing research on *Phragmites*-associated foliar microbes has focused on culturable fungi (Clay et al. 2016; Allen et al. 2020; Devries et al. 2020), which does not capture most existing fungal and bacterial diversity (reviewed in Peccia & Hernandez 2006; Alain & Querellou 2009; Yan et al. 2021). Thus, the entire phyllosphere community has yet to be fully explored in *Phragmites.*

Belowground studies provide a different perspective as plant-soil feedback studies in *Phragmites* find that the intraspecific feedbacks are generally negative, suggesting that pathogenic interactions dominate belowground (Allen et al. 2019; Bickford et al. 2022). Isolation of *Phragmites* rhizopheric fungi found an abundance of pathogenic oomycetes which reduced native plant growth (Nelson & Karp 2013; Crocker et al. 2015). Furthermore, fungicide applications increased native plant survival and growth, demonstrating the negative impact of *Phragmites*-associated soil fungi (Crocker et al. 2017). However, little is known about *Phragmites* litter-associated communities and their role in the belowground microbial dynamics.

Here we use 16S and ITS rRNA gene sequencing of *Phragmites* bacterial and fungal leaf/litter communities to study the effects of leaf and litter age across five sites in the Mid-Atlantic (New Jersey) and Midwest (Michigan) regions of North America. We hypothesize that (H1.1) as *Phragmites* leaves age, the overall diversity (α-diversity) will increase (higher Shannon and Chao1 indices), the community composition will change (leaves and litter of different ages will have significantly different bacterial and fungal communities). We also hypothesize that (H1.2) leaves will have a lower proportion of pathogens compared to litter (increased pathotrophs composition in FUNGuild and pathogenic bacterial and fungal indicators in litter layers). Additionally, we hypothesize that (H2) because of longer residence times of *Phragmites* in New Jersey, populations from New Jersey will harbor a higher proportion of pathogens due to an accumulation of pathogens than populations from Michigan and that bacterial and fungal communities will be significantly different between the two states. Exploration into the spatiotemporal patterns of foliar microbial community in *Phragmites* may give us insight into ways of combating *Phragmites* spread through identification of potential biocontrol agents.

Methods

Sample collection and storage

Samples were collected from two sites in New Jersey (Meadowlands and Milltown) by collaborators from Rutgers University (New Brunswick, New Jersey) and three sites in Michigan (Plymouth Parkway, Kuebler Trail, and Bandimere Park) by collaborators at the USGS Great Lakes Science Center (Ann Arbor, Michigan) on 9/29 and 9/30/2021 (Table 3-1). Five leaf samples per tiller were collected: youngest fully-expanded apical leaf, mid-level leaf, lower leaf starting to senesce, recently dehisced intact leaf at the base of each shoot (top litter), partially decayed litter under the top litter layer at the base of the shoot (lower litter). This sampling was

repeated for a total of five randomly selected and widely spaced tillers per site for a total of 125 samples across five sites. Samples were individually wrapped in a moist paper towel and stored in separate Ziploc bags and shipped overnight to Tulane University where they were immediately stored at -20˚C until extracted.

DNA extraction and sequencing

Two approximately 2.5 cm squares were cut from each leaf sample, one square was taken proximal to the leaf tip and one proximal to the base of the leaf, when possible sampling of the leaf edge was avoided and midrib was removed if present in the sample. Leaf squares were put in a tea strainer, and placed under running water for five minutes to wash away surface debris. Each sample was then surface sterilized by submerging the tea strainer for three minutes in 70% EtOH, one minute in 1:10 dilution bleach solution, one minute in 70% EtOH, and one minute in deionized water. All solutions were freshly prepared each day. The sample was then air dried and a middle square (~1 cm^2) of each sample was cut into small pieces for DNA extraction. DNA extractions were done using Qiagen DNeasy PowerPlant Pro Kit (13400) following manufacturer instructions (QIAGEN Inc.). Samples which yielded less than 2ng/uL were re-extracted using leaf material which was ground with liquid nitrogen before proceeding with the extraction kits. Samples were paired-end sequenced (300bp PE, Illumina Miseq) in two separate lanes at LSU Louisiana Cancer Research Center Translational Genomics Core using 16S rRNA (341F/805R) and ITS rRNA primer sets designed to target ITS1 region between the 18S and 5.8S rRNA genes as described in Bellemain et al. 2010 .

Bioinformatics

We used QIIME2 within a Miniconda3 environment for our bioinformatics pipeline, starting with demultiplexing our 16S bacterial and ITS fungal paired-end reads (Bolyen et al.

2019). Demultiplexed sequences were then trimmed of adapters and primers using cutadapt:QIIME2, and denoised using DADA2:QIIME2 to filter out low-quality reads, merge, and remove chimeric sequences. We filtered the feature table to remove ASVs with less than 5 reads across all samples and with a frequency of less 2 observations across all samples. Taxonomic classification for 16S rRNA sequences were classified using classifiers from the "Greengenes2" database trained on our 16S primer set (McDonald et al. 2022) and "UNITE ver. 9 release for Fungi2" ITS database (Abarenkov et al. 2023) trained on our ITS primer sets. Taxonomy tables were then extracted for downstream use. FUNGuild (ver. 1.1), run within a Miniconda3 environment, was used to assign trophic mode to fungal ASVs (Nguyen et al. 2016). Fungi were classified into three trophic modes, "pathotrophs" which are fungi that receive nutrients at the expense of the host cells and cause disease, "saprotrophs" which are fungi that receive nutrients by breaking down dead host cells, and "symbiotrophs" which are Ectomycorrhizal, Ericoid Mycorrhizal, Endophyte, and Epiphytes (Nguyen et al. 2016). Fungi that were classified as belonging to multiple trophic modes were counted as both (i.e., pathotroph-saprotroph would be both a pathotroph and a saprotroph). We retained fungi which had a confidence ranking of "highly probable" which were certain assignments, "probable" which were fairly certain assignments, and "possible" which were suspected but not proven assignments of trophic mode (Nguyen et al. 2016).

Data analyses

To examine microbial diversity, 16S and ITS taxonomy tables were converted to "phyloseq" objects using the "phyloseq" ver. 1.46.0 R package (McMurdie & Holmes 2013) and used to estimate both Shannon diversity and Chao1 using the "estimate_richness" function from

the same “phyloseq” package. We tested the effect of leaf age, state, and the interaction between the two in explaining the variation in Shannon and Chao1 indices using multiple linear mixed effects models and generalized least square models as described above.

To assess the effects of leaf age, site, and their interaction on community composition, Bray-Curtis dissimilarity was calculated from 16S bacterial and ITS fungal ASVs using the “vegdist” function from the “vegan” ver. 2.6-4 R package, and PERMANOVA was then performed using the “adonis2” function from the R package “vegan” (Oksanen et al. 2022). If site, leaf age, or the interaction of the two were significant, post-hoc pairwise multilevel comparisons were then performed on significant fixed effects or the interaction of each factor level using the “pairwise.adonis” function from the R package “pairwiseAdonis” ver. 0.4.1 (Arbizu 2020). To assess the variance in 16S and ITS community composition within each factor level, we used the function “betadisper” from the R package “vegan” to calculate the mean distance-to-centroid for each factor level in leaf age, site, and the interaction of the two, separately, and using the output to perform a permutation test (999 permutations) to test for significant differences in dispersion between factor levels within fixed effects and interactions using the “permutest” function from the R package “vegan”. We performed a Tukey’s HSD post-hoc test on mean distance-to-centroid of each sample to test if there were significant differences in dispersion between factor levels using the “TukeyHSD” from the “stats” R package ver. 4.3.2 in base R (R Core Team).

We tested the effect of leaf age, state, and the interaction between the two in explaining the variation in the mean percent composition of saprotroph, pathotroph, and symbiotroph in the fungal community using multiple linear mixed effects models using the “lme” function in the R package “nlme” ver. 3.1-164 (Pinheiro et al. 2023) in R studio ver. 2023.09.1+494 (R Core Team

2023). The random effect of site was applied to each model using the REML approach after which type III ANOVA were performed on the model to determine the significance of the focal fixed effect and interaction. Model validation was performed by plotting fitted values against residuals, each of the fixed effects against residuals, and a histogram of the residuals alone. Least-squares means were extracted from the best-fit model using the "lsmeans" function from the "emmeans" R package ver. 1.10.0 and used to perform a Tukey's HSD test in compact letter design (CLD) using the "cld" function from the R package "multcomp" ver. 1.4-25 (Hothorn et al. 2023; Lenth et al. 2024).

Indicator analysis on relative abundance data was used to identify taxa indicative of a certain leaf age and state. Each bacterial 16S and fungal ITS taxa were assigned an indicator value, 0 to 1 with 0 being no association to a given factor level and 1 being perfect concordance with a given factor level, and an associated p-value which indicates the confidence of the indicator value assignment using the "indval" function from the R package "labdsv" ver. 2.1-0 (Roberts 2023). ASVs were then filtered for ASVs with indicator values with associated p-values<0.05.

Results

Sequencing results

We obtained 8,813,017 16S sequence reads across 124 leaf/litter samples (P081 (Milltown top-litter sample rep 1) sample removed during feature table filtering step) with a sampling depth range from 30,496- 112,958 sequences per sample and 5,259,386 ITS sequence reads across 124 leaf/litter samples with a sampling depth range from 2,383- 134,193 sequences per sample. The mean read number per sample was 71,598 ± 1176 (16S) and 42,414 ± 2478

(ITS). There were 876 unique bacterial 16S ASVs and 377 unique fungal ITS ASVs (≥5 sequences across all samples, with frequency ≥2 observations).

Alpha diversity

The alpha-diversity (Shannon and Chao1) of bacterial 16S and fungal ITS communities were significantly influenced by leaf age but not state nor the interaction between leaf age and state (Table 3-2). As leaves aged, both the Shannon index and Chao1 increased in bacterial 16S communities. However, the Shannon-diversity of bacterial communities in the youngest and middle leaves was not significantly different, and the estimated number of species (Chao1) of bacterial communities within middle leaves was not significantly different than young leaves or lower leaves (Figure 3-1A). For fungal ITS communities, the estimated number of species (Chao1) generally increased with leaf age. However, the number of estimated fungal species in young leaves was not significantly different than middle leaves (Figure 3-1B). Shannon indices of fungal communities between young, middle, lower leaves were significantly different than lower litter, but not top litter (Figure 3-1B).

Beta diversity

The effect of leaf age on beta-diversity in both bacterial 16s and fungal ITS communities depended on the state (significant leaf age x state interaction) (Table 3-3). In New Jersey the leaf bacterial communities were significantly different from the litter communities, however in Michigan, the lower leaf communities were not significantly different than the top-litter community (Figure 3-2A). Bacterial communities within leaf age across the two states were not significantly different from one another aside from lower leaf communities (Figure 3-2A).

Aside from young leaves, fungal communities across leaf ages in New Jersey were not significantly different from one another (Figure 3-2B). Fungal communities within leaf age across the two states were not significantly different from one another aside from middle and lower leaf communities (Figure 3-2B).

The effect of leaf age on mean dispersion (distance-to-centroid) was dependent on state for both bacterial 16S and fungal ITS communities (Table 3-4). The bacterial communities found in the lower litter of Michigan *Phragmites* was significantly less dispersed than lower and middle leaf communities in Michigan and lower leaf communities from New Jersey (Table 3-4). Fungal communities from the lower leaves of New Jersey *Phragmites* had significantly lower mean distance-to-centroid than communities from New Jersey lower-litter, young leaves from Michigan, and lower-litter from Michigan (Table 3-4).

Trophic mode

Percent saprotroph and symbiotroph composition was significantly influenced by age of *Phragmites* leaves and litter (Table 3-5). Middle leaves had the highest proportion of saprotrophs followed by lower leaves, both of which had significantly higher proportions of saprotrophs than youngest leaves, top litter, and lower litter (Figure 3-4A). Symbiotroph composition increased with leaf age, with the youngest leaves having the lowest composition of symbiotrophs, which was significantly lower than top litter and lower litter (Figure 3-4A). Middle and lower leaves also had significantly lower composition of symbiotrophs compared to lower litter (Figure 3-4A).

The effect of leaf age on percent pathotroph composition depended on state (significant state x leaf age interaction) (Table 3-5). The only significant pairwise difference was observed

between youngest leaves and lower leaves within *Phragmites* populations sourced from New Jersey (Figure 3-4B).

Indicator analysis

Lower litter had the highest number of bacterial indicator species with a probability <0.05 at 376 species, followed by top litter with 90 species, middle leaves with 2, lower leaves with 3 species, and youngest leaves with 4 (Table 3-6). Populations from Michigan had 66 bacterial indicator species with a probability <0.05 compared to only 14 species among populations from New Jersey (Table 3-6).

Like bacterial communities, lower litter had the highest number of fungal indicator taxa with a probability <0.05 at 60 of which 40 were saprotrophs, 16 were pathotrophs, and 6 were symbiotrophs. Top litter had 19 indicators with 16 saprotrophs, 9 pathotrophs, and two symbiotrophs. Middle leaves had 4 indicators with one saprotroph and one pathotroph. Young leaves had 4 indicators, 3 of them being saprotrophs, 3 pathotrophs, and one symbiotrophs. Finally, lower leaves had 3 indicators with two pathotrophs, and one saprotroph (Table 3-7). Populations from Michigan had 30 fungal indicator taxa with a probability <0.05, 5 of them being symbiotroph, 17 saprotrophs, and 18 saprotrophs. This is compared to only 23 taxa among populations from New Jersey, 4 of them being symbiotroph, 16 saprotrophs, and 16 saprotrophs. (Table 3-7).

Notably, the top two fungal indicators for both lower leaves and New Jersey were *Stagonospora forlicesenensis* and *Mycosphaerellaceae*, the former a known foliar pathogen known to infect *Phragmites* leaf tissue (Allen et al. 2020; but see Ernst 2003, Gao & Mendgen 2006), and the latter a family of sac fungi known to cause leaf blot (Marín et al. 2003; Taylor et

al. 2003; Simon et al. 2009; Videira et al. 2017). Several taxa found in litter across both states are associated with heavily polluted habitats such as *Dothideomycetes* sp (NJ), and *Hypocreales* (MI), (Soares et al. 2016; Sim et al. 2018) and bacteria like *Roseateles depolymerans* (MI), *Micromonosporaceae* (MI), *Caulobacteraceae* (NJ), and *Kineosporia* sp (NJ) (Cortes-Lorenzo et al. 2013; Shah et al. 2013; Trujillo et al. 2014; Gschwendtner et al. 2016).

Discussion

Here we present the first comprehensive study of the diversity and distribution of *Phragmites* foliar and litter bacterial and fungal communities. We examined the changes in microbial community composition across leaf age from the apical young leaves to progressively older leaves from the same tiller to recently dehisced older leaves and then semi-decayed leaf litter on the ground. In addition, we compared microbial diversity between populations in the Mid-Atlantic (New Jersey) and Great Lakes (Michigan), which are two focal regions of *Phragmites* invasion in North America. In line with what is expected, there was a positive relationship between a-diversity and leaf age as leaves recruit a more diverse microbial community as they age. We found a surprising amount of overlap (low b-diversity) between bacterial and fungal communities within each leaf/litter age across New Jersey and Michigan, suggesting microbial community conservation over large geographic distances. In accordance with expected dynamics of pathogen accumulation with increased residence time and abundance, populations from New Jersey accumulated a higher proportion of pathogens as leaves aged, which was not observed in populations from Michigan (Klironomos 2002; Hawkes 2007; Mitchell et al. 2010; Flory & Clay 2013). Our indicator analysis identify pathogenic fungal taxa which inform patterns in New Jersey lower leaf and Michigan litter and reveal evidence of

environmental pollution structuring litter communities in both Michigan and New Jersey bacterial and fungal communities.

Alpha and beta diversity across leaf age

We find that in line with H1.1, both Shannon and Chao1 indices increased with leaf age, indicating that foliar microbial communities are rapidly recruited. Surprisingly, we found that leaf age was the strongest driver of microbial community composition as most leaves and litter were not significantly different than the corresponding leaf age in the other state (New Jersey and Michigan), suggesting the patterns in foliar microbial recruitment in *Phragmites* are similar across large geographic distances. While one study (Bowen et al. 2017) found that *Phragmites* soil bacterial communities exhibit high spatial turnover (high b-diversity) across large geographic distances compared to the native lineage, other studies such as Schroeder et al. (2020) and Bickford et al. (2022) found no significant difference in soil microbial spatial turnover compared to native Phragmites.

Fungal and bacterial communities displayed different trends in beta-dispersion across leaf age. Bacterial community dispersion generally increases as leaves age, before becoming less heterogenous as leaves become litter; while in fungal communities, older leaves display lower dispersion than younger leaves and litter, especially in New Jersey. Similarly, Stone & Jackson (2019) found that leaves from *Magnolia grandiflora* lower canopy exhibited higher bacterial community beta-dispersion compared to communities from the upper canopy, although litter microbial communities were not sampled. A study comparing young and mature leaf fungal communities in tea (*Camellia sinensis*) and *Amorphophallus albispathus* found higher dispersion in young leaf communities than in mature leaves (Unterseher et al 2018). Our results seem display a decoupling of bacterial and fungal community beta-dispersion patterns, where older

leaves harbor less conserved bacterial communities than litter communities, in concordance with existing literature, while in fungal communities, older leaves tend to display lower beta-dispersion.

The indicator analysis is in concordance with our a-diversity and beta-dispersion results, where we find fewer bacterial and fungal indicators in the living leaves than in the litter (545 litter indicators: 19 foliar indicators). This suggests that foliar microbial communities were generally less distinct and fewer taxa were strongly associated with the *Phragmites* phyllosphere than *Phragmites* litter. The top fungal indicators in New Jersey, *Stagonospora forlicesenensis* and *Mycosphaerellaceae* were also top fungal indicators in lower leaves overall. These two taxa are both strongly associated with lower leaf fungal community in New Jersey populations and may contribute to the high levels of fungal community conservation observed in New Jersey lower leaf fungal communities. Our results agree with previous studies on spatial patterns in *Phragmites* soil bacterial communities which suggest that there is high spatial conservation and maintenance of a core soil microbial community, although the invasive lineage tends to have higher community turnover compared to the native lineages (Bowen et al. 2017; He et al. 2022).

Patterns in fungal pathogen composition and conservation

We observe different trends in fungal pathotroph recruitment between New Jersey and Michigan *Phragmites* populations. In New Jersey, there was an increase in the proportion of pathotrophs as leaves aged, and a decrease as leaves become litter. However, in Michigan, we found a positive linear trend in increasing pathotroph composition as leaves age and turn into litter, although not statistically significant. Based on our results, we do not find support for H1.2: *that leaves will have a lower proportion of pathogens compared to litter (increased*

pathotrophs composition in FUNGuild and pathogenic bacterial and fungal indicators in litter layers). As we hypothesized, we observed lower levels of beta-diversity in New Jersey fungal communities compared to those found in Michigan but there was no discernable pattern in bacterial communities. Although we did not find that New Jersey *Phragmites* populations harbored a higher proportion of pathotrophs than populations in Michigan, we do note the interaction between leaf age and state on pathotroph composition as mentioned above. Thus, we partially confirm H2: *because of longer residence times of Phragmites in New Jersey, populations from New Jersey will harbor a higher proportion of pathogens due to an accumulation of pathogens than populations from Michigan and that bacterial and fungal communities will be significantly different between the two states.*

Indicator analysis gives us insight into which fungal taxa may be driving these patterns in pathotroph composition in New Jersey. Although there the relative proportion of saprotroph/pathotroph/symbiotrophs did not vary much between New Jersey and Michigan, a closer inspection revealed that the top fungal indicator in New Jersey, *Stagonospora forlicesenensis,* belongs to a genus of fungi which has been isolated from diseased *Phragmites* leaf tissue and experimental inoculation of which causes leaf necrosis (Allen et al. 2020). However, other studies have identified *Stagonospora sp.* as a beneficial seed endophyte in *Phragmites,* so variation in symbioses exist within the genus (Ernst et al. 2003; Gao & Mendgen 2006). The fungal taxa with the second highest indicator value in New Jersey, *Mycosphaerellaceae*, is a family of ascomycetes that cause a range of foliar diseases in *Trifolium, Proteaceae* and is responsible for Black Sigatoka disease in banana (Marín et al. 2003; Taylor et al. 2003; Simon et al. 2009; Videira et al. 2017). These two fungal taxa are also the top fungal indicators in lower leaf fungal communities, suggesting that these two taxa are strongly

associated with lower leaves in New Jersey populations. There are also several fungal pathogens highly associated with *Phragmites* populations from Michigan such as *Nigrospora sp.* (Bumby & Farrer 2022), a known foliar fungal pathogen in *Phragmites* and *Devriesia pseudoamericana,* which causes sooty blotch disease in apples (Frank et al. 2010). As these fungi are associated with the Michigan litter layers, these indicators suggest increasing pathotroph composition as leaves age and turn into litter. The close association of these taxa may be driving the higher fungal community conservation observed in New Jersey lower leaves and evidence of endemism of specific fungal pathogens in *Phragmites* foliar communities in New Jersey. The residence time of *Phragmites* in New Jersey is much longer than Michigan, as it is suspected that the cryptic invasion of *Phragmites* originated in and around New Jersey circa mid-1800's (Saltonstall 2002; Wilcox et al. 2003; Tulbure et al. 2007). This is in line with the idea that invasive plants have a higher chance of accumulating specialized pathogens as residence time increases (Klironomos 2002; Hawkes 2007; Mitchell et al. 2010; Flory & Clay 2013; Gioria et al. 2023). While disease-mediated *Phragmites* mortality has been observed in Europe (Reed die-back syndrome) (Brix 1999; Fogli et al. 2002) and in the Gulf Coast *Phragmites* (scale insect attack) (Knight et al. 2020; Farrer & Bumby 2022), mortality in Eurasian invasive *Phragmites* is not common. However, *Phragmites* has been observed to act as a reservoir for aboveground herbivores, leading to apparent competition with native *Phragmites* lineages (Bhattarai et al. 2017). *Phragmites* may also act as a potential reservoir of fungal pathogens which can spillover onto native wetland plants. This phenomenon has been observed in in a variety of invasive plants and is suspected to contribute to the decline of native plant populations (reviewed in Flory & Clay 2013; Najberek et al. 2022). Thus, pathogen accumulation in *Phragmites* foliar communities may spillback onto native plants and mediate *Phragmites* invasion.

Although FUNGuild assigns function to large fungal sequencing datasets, its classifications do not capture the strength of symbioses only a prediction of the direction of the symbioses, nor does it consider microbe-microbe interactions (Johnson et al. 1997; Liu & He 2021). Additionally, plant-microbe interactions can be highly species-specific and FUNGuild classifications do not account for the focal host species, and fungi within the same genus may be given the same general trophic classification.

Saprotroph and symbiotroph composition across leaf age

Notably, we found that *Phragmites* middle and lower leaves had significantly higher percent saprotroph composition than young leaves and both litter layers. Other studies of *Phragmites* foliar fungal communities have also found an overwhelming dominance of saprotrophs in foliar fungal communities (Allen et al. 2020; Devries et al. 2020; Likar et al. 2022). Previous studies have demonstrated this trend could be due to the proliferation of latent foliar endophytic saprotrophs in leaf tissue post-collection, inflating proportions of saprotrophic fungi, or could in fact reflect or mis-categorization of endophytes as saprotrophs based on trophic mode observed on other species by FUNGuild (Osono 2002; Lindahl et al. 2007; Devries et al. 2020). Additionally, surface sterilization of leaf tissue before sequencing may have influenced the relative proportion of fungi with different trophic modes as epiphytic fungi would have been largely depleted following this process.

There was a consistent increase in fungal symbiotrophs with increasing leaf and litter age. Although there is some evidence that there are beneficial vertically transmitted endophytes than can boost growth, it appears that the majority of *Phragmites* symbiotrophs are recruited during the development of leaves (White et al. 2016; Shearin et al. 2018; Verma et al. 2018). While the

existing literature on plant-soil feedbacks in *Phragmites* suggests that they cultivate neutral or net-negative intraspecific feedbacks (Allen et al. 2018; Gonzalez-Mateu 2020; Bickford et al. 2022), we do not know the relative contribution of mutualists and pathogens in this feedback. Plants interact with both mutualists and pathogens simultaneously and this interaction results in a net effect to the plant (Reviewed in Jiang et al. 2020). There is evidence that dark-septate endophytes can help *Phragmites* combat the effects of abiotic stressors such a high-salinity (Gonzalez-Mateu et al. 2020). We find that the abundance of symbiotrophs increases in litter communities, which warrants further research into role of litter symbiotrophs in mitigating negative belowground biotic and abiotic effects.

Foliar and litter community across nutrient gradient

The sites in New Jersey and Michigan differ in several aspects, from climatic differences in temperature (MAT; Washtenaw County, Michigan (2021): 9.94 °C; Bergen County, New Jersey (2021): 12.33 °C) and precipitation (MAP; Washtenaw County, Michigan (2021): 96.09 cm; Bergen County, New Jersey (2021): 138.66 cm) (NOAA 2024). In addition, the New Jersey sites are tidal with the Meadowlands reporting mesohaline conditions ranging from 6.1-12.0 PSU (Artigas et al. 2021) while the inland Midwest is largely freshwater. Aside from these other factors, variation in nutrient loads between New Jersey and Michigan could contribute to observed patterns in pathotroph composition and beta-diversity. New Jersey populations have higher nitrogen loads than the populations from Michigan, and phosphorus loads are also 4-5x higher in the Meadowlands (NJ) than the other populations. Nutrient pollution in New Jersey wetlands is also coupled with decades of anthropogenic disturbance through alterations in hydrology via impoundment, diking, and mosquito ditching (Sipple 1972; Quinn 1997; Raichel

et al. 2003). Both anthropogenic disturbance and increased nutrient fertilization has been associated with an increase in phyllosphere and rhizosphere fungal pathogen abundance as well as increasing the likelihood of novel pathogen introduction (Liu et al. 2017; Lekberg et al. 2021; Bi et al. 2022; Burgess et al. 2022; Hu et al. 2022; Maywald et al. 2022). While we cannot directly attribute the patterns in pathotroph composition observed in New Jersey versus Michigan to anthropogenic disturbance and pollution, their co-occurrence warrants further exploration into whether these anthropogenic factors drive patterns in pathotroph composition in *Phragmites* populations.

Our indicator analysis also revealed several bacterial (Cortes-Lorenzo et al. 2013; Shah et al. 2013; Trujillo et al. 2014; Gschwendtner et al. 2016) and fungal taxa (Soares et al. 2016; Sim et al. 2018) have been documented as being closely associated with environments containing pollutants like heavy-metals, nutrients, and other toxins. Thus, in both Michigan and New Jersey, these microbial communities are composed of members that thrive in anthropogenically impacted environments, suggesting that pollution may be structuring *Phragmites* microbial communities across various regions in North America.

There was high fungal community similarity between leaves and litter of different ages in New Jersey *Phragmites* populations. This pattern was not observed in fungal communities in Michigan *Phragmites* nor was it observed in bacterial communities. Recent efforts into observing the effects of intensifying land use have revealed an increase in microbial community homogenization in grasslands (Gossner et al. 2016; but see Zhou et al. 2020). However, studies regarding the effects of nitrogen fertilization and other nutrients seem to indicate that there is a positive relationship between nitrogen and b-diversity, suggesting that microbial community turnover would increase with pollution (Zhou et al. 2020 & 2022). However, when partitioning

bacteria and fungi, one study found that while nitrogen increased bacterial b-diversity, fungal β-diversity remained the same as nitrogen increased. Thus, *Phragmites* associated microbial communities may be responding in a similar way, where bacterial community b-diversity may be more sensitive to changes in nutrient additions than fungal communities, although there is still no existing support for anthropogenic factors reducing b-diversity in fungal communities.

Conclusion

Our study represents the first to explore the variation in *Phragmites bacterial and fungal* microbial communities as leaves age across two geographic regions. Although changes in α-diversity seem to be influenced solely by leaf age, other variables such as variation in pathogen composition, β-diversity, and β-dispersion vary as a function of leaf age and geographic origin. Geographic variation in fungal pathogen recruitment is especially notable as it can influence the invasion dynamics within each region. While geography may explain some of the patterns observed in our study, New Jersey and Michigan also varied in the amount of anthropogenic disturbance and pollution. Closer inspection of indicator taxa reveals members closely associated with pollution. Thus, environmental factors including pollution and also abiotic differences between regions (salinity, temperatures, precipitation, etc.) may also be important in explaining foliar and litter microbial community diversity and structuring. More research is needed to detangle and resolve the relative importance of spatiotemporal variables versus environmental and anthropogenic variables in explaining the patterns in the *Phragmites* microbiome.

Table 3-1 Sampling location of *Phragmites* leaf and litter in 2021 including site name, latitude, longitude, state the site is located, phosphorus load, and nitrogen load.

Site Name	Latitude	Longitude	State	Phosphorus load (kg/km2)	Nitrogen load (kg/km2)
Bandimere Nature Area (Bandimere)	42.299068	-83.742836	Michigan (MI)	67.217	513.253
Plymouth Parkway (Plymouth)	42.291981	-83.735937	Michigan (MI)	91.847	498.066
Kuebler Langford Nature Area (Kuebler)	42.298851	-83.751468	Michigan (MI)	67.217	513.253
New Jersey Meadowlands (Meadowlands)	40.816128	-74.037483	New Jersey (NJ)	365.322	2854.997
Mill Pond Park (Milltown)	40.453319	-74.437046	New Jersey (NJ)	61.981	964.347

Table 3-2 Chi-sq. and significance (p-value: >0.05 (n.s.), <0.05 (*), <0.001 (**), <0.0001 (***)) of linear mixed effect models of Shannon and Chao1 bacterial 16S and fungal ITS diversity across leaf age, state, and the interaction. Mean Shannon and Chao1 diversity, standard error (SE) and Tukey's HSD in compact letter design (CLD) of bacterial 16S and fungal ITS communities across leaf age from the *Phragmites* leaves and litter collected in 2021.

Bacterial 16S						
	Shannon Diversity			**Chao1**		
Factor	**numDF**	**F-value**	**p-value**	**numDF**	**F-value**	**p-value**
Leaf age	4	55.850	<.0001	4	95.677	<.0001
State	1	0.521	0.522	1	0.002	0.968
Leaf age * State	4	0.827	0.511	4	2.021	0.096
Fungal ITS						
	Shannon Diversity			**Chao1**		
Factor	**numDF**	**F-value**	**p-value**	**numDF**	**F-value**	**p-value**
Leaf age	4	89.797	<.0001	4	54.208	<.0001
State	1	0.002	0.963	1	0.522	0.522
Leaf age * State	4	1.829	0.128	4	1.196	0.317

Table 3-3 Numerator degrees of freedom (numDf), sum of squares, R^2, F-value, and adjusted p-value of bacterial 16s and ITS fungal community PERMANOVA across leaf age, state, and their interaction from the *Phragmites* leaves and litter collected in 2021.

Bacterial 16s Community					
Factor	**numDf**	**Sum of Sqs**	**R^2**	**F-value**	**p-value**
Leaf age	4	12.812	0.254	10.793	0.001
State	1	1.224	0.024	4.123	0.001
Leaf age * State	3	2.363	0.047	2.654	0.001
Fungal ITS Community					
Factor	**numDf**	**Sum of Sqs**	**R^2**	**F-value**	**p-value**
Leaf age	4	6.364	0.118	4.237	0.001
State	1	1.818	0.034	4.841	0.001
Leaf age * State	3	3.032	0.056	2.019	0.001

Table 3-4 Numerator degrees of freedom (numDf), F-value, and p-value of linear mixed effect models of beta-dispersion (distance to centroid) in 16S bacterial and ITS fungal communities across leaf age, state, and the interaction. Mean distance to centroid, standard error (SE) and Tukey's HSD in compact letter design (CLD) of 16S bacterial and ITS fungal communities across levels of state*leaf age from the *Phragmites* leaves and litter collected in 2021.

Bacterial 16s community				**Fungal ITS community**			
Factor	**numDf**	**F-value**	**p-value**	**Factor**	**numDf**	**F-value**	**p-value**
Leaf age	4	12.264	2.21E-08	Leaf age	4	2.537	0.044
State	1	1.006	0.318	State	1	0.001	0.970
Leaf age * State	9	3.145	0.002	Leaf age * State	9	3.227	0.002
State * Leaf age	**Mean (mean distance to centroid)**	**SE**	**CLD**	**State * Leaf age**	**Mean (mean distance to centroid)**	**SE**	**CLD**
New Jersey:Youngest	0.460	0.046	-	New Jersey:Youngest	0.602	0.015	-
New Jersey:Middle	0.523	0.035	-	New Jersey:Middle	0.609	0.009	-
New Jersey:Lower	0.591	0.018	b	New Jersey:Lower	0.514	0.032	b
New Jersey:Top litter	0.532	0.022	-	New Jersey:Top litter	0.569	0.020	-
New Jersey:Lower litter	0.486	0.033	-	New Jersey:Lower litter	0.622	0.016	a
Michigan:Youngest	0.517	0.024	-	Michigan:Youngest	0.606	0.019	a
Michigan:Middle	0.560	0.027	b	Michigan:Middle	0.536	0.021	-
Michigan:Lower	0.554	0.031	b	Michigan:Lower	0.589	0.016	-
Michigan:Top litter	0.463	0.025	-	Michigan:Top litter	0.563	0.024	-
Michigan:Lower litter	0.426	0.029	a	Michigan:Lower litter	0.617	0.014	a

Table 3-5 Chi-sq. and significance (p-value: >0.05 (n.s.), <0.05 (*), <0.001 (**), <0.0001 (***)) of linear mixed effect models of % saprotroph, pathotroph, and symbiotroph composition across leaf age, site, and state. Mean % composition, standard error (SE) and Tukey's HSD in compact letter design (CLD) of fungal community trophic mode assignments from FUNGuild classifications across leaf age, site, and state from the *Phragmites* leaves and litter collected in 2021.

	Saprotroph			Symbiotroph		
	numDF	**F-value**	**p-value**	**numDF**	**F-value**	**p-value**
Leaf age	4	8.311	<.0001	4	8.651	<.0001
State	1	3.469	0.065	1	1.987	0.254
Leaf age * State	4	1.617	0.175	4	0.802	0.526

	Pathotroph		
Factor	**numDF**	**F-value**	**p-value**
Leaf age	4	1.321	0.267
State	1	2.664	0.201
Leaf age * State	4	3.229	0.015

Table 3-6 Top 10 bacterial 16s indicators taxa by leaf age, site, and state. Indicator taxa were selected on based on indicator value probability p-value < 0.05.

Genus/species	Leaf Age	Indicator value	p-value	Genus/species	State	Indicator value	p-value
Sphingomonas azotifigens	Youngest	0.387	0.001	*Roseateles depolymerans*	Michigan	0.543	0.001
Agrobacterium spp	Youngest	0.362	0.001	*Sinobacteraceae*	Michigan	0.517	0.019
Sphingomonas asaccharolytica	Youngest	0.344	0.001	*Micromonosporaceae*	Michigan	0.492	0.001
Kineosporia spp	Youngest	0.331	0.005	*Methylobacterium adhaesivum*	Michigan	0.491	0.004
Sphingomonas spp	Middle	0.376	0.001	*Cystobacterineae*	Michigan	0.481	0.001
Clavibacter michiganensis	Lower	0.237	0.017	*Curtobacterium spp*	Michigan	0.447	0.029
Nocardioides spp	Lower	0.164	0.024	*Erwinia spp*	Michigan	0.444	0.030
Bosea genosp	Top litter	0.686	0.001	*Enterobacteriaceae*	Michigan	0.438	0.008
Salinibacterium spp	Top litter	0.621	0.001	*Stenotrophomonas retroflexus*	Michigan	0.433	0.009
Azospirillum spp	Top litter	0.611	0.001	*Sphingobacteriaceae*	Michigan	0.421	0.006
Janthinobacterium spp	Top litter	0.597	0.001	*Kineosporia spp*	New Jersey	0.618	0.014
Methylobacterium komagatae	Top litter	0.566	0.001	*Caulobacteraceae*	New Jersey	0.279	0.001
Frigoribacterium spp	Top litter	0.542	0.001	*Pseudomonas viridiflava*	New Jersey	0.260	0.001
Pedobacter cryoconitis	Top litter	0.542	0.001	*Erythrobacteraceae*	New Jersey	0.193	0.028
Erwinia spp	Top litter	0.523	0.001	*Micromonospora spp*	New Jersey	0.160	0.001
Methylobacterium spp	Top litter	0.520	0.001	*Holosporaceae*	New Jersey	0.145	0.021
Methylobacterium adhaesivum	Top litter	0.489	0.001	*Staphylococcus sciuri*	New Jersey	0.126	0.029
Flavobacterium spp	Lower litter	0.879	0.001	*Cupriavidus spp*	New Jersey	0.107	0.025
Cellulomonas spp	Lower litter	0.869	0.001	*Segetibacter spp*	New Jersey	0.100	0.010
Stenotrophomonas spp	Lower litter	0.857	0.001	*Nocardioidaceae*	New Jersey	0.097	0.022
Sphingobacterium spp	Lower litter	0.854	0.001				
Kineococcus spp	Lower litter	0.840	0.001				
Prosthecobacter debontii	Lower litter	0.839	0.001				
Janthinobacterium lividum	Lower litter	0.837	0.001				
Luteibacter spp	Lower litter	0.837	0.001				
Methylopila spp	Lower litter	0.836	0.001				
Cellulomonas xylanilytica	Lower litter	0.823	0.001				

Table 3-7 Top 10 fungal ITS indicators taxa by leaf age, site, and state. Indicator taxa were based on indicator value probability p-value < 0.05.

Genus/species	Leaf Age	Indicator value	p-value	Genus/species	State	Indicator value	p-value
Magnaporthaceae	Youngest	0.163	0.026	*Nigrospora sp*	Michigan	0.877	0.001
Naganishia globosa	Youngest	0.160	0.011	*Hypocreales*	Michigan	0.672	0.001
Dissoconium sp	Youngest	0.155	0.034	*Devriesia pseudoamericana*	Michigan	0.616	0.003
Cladosporium dominicanum	Youngest	0.120	0.031	*Dothideomycetes*	Michigan	0.520	0.001
Apenidiella foetida	Middle	0.472	0.003	*Phaeosphaeria sp*	Michigan	0.504	0.005
Paradissoconium narthecii	Middle	0.371	0.001	*Neodevriesia poagena*	Michigan	0.478	0.002
Pseudomassaria corni	Middle	0.340	0.010	*Neodevriesia sp*	Michigan	0.473	0.023
Dothideomycetes	Middle	0.336	0.027	*Phaeosphaeriaceae sp*	Michigan	0.455	0.001
Stagonospora forlicesenensis	Lower	0.530	0.009	*Funiliomyces sp*	Michigan	0.446	0.001
Mycosphaerellaceae	Lower	0.348	0.045	*Pseudomassaria corni*	Michigan	0.375	0.002
Keissleriella caraganae	Lower	0.120	0.038	*Stagonospora forlicesenensis*	New Jersey	0.791	0.004
Pleosporales sp	Top litter	0.564	0.030	*Mycosphaerellaceae*	New Jersey	0.718	0.001
Devriesia sp	Top litter	0.535	0.001	*Dothideomycetes sp*	New Jersey	0.461	0.028
Sordariomycetes	Top litter	0.482	0.008	*Myrmecridium schulzeri*	New Jersey	0.219	0.001
Periconia sp	Top litter	0.417	0.040	*Schizothyrium wisconsinense*	New Jersey	0.199	0.001
Hypocreales	Top litter	0.405	0.011	*Lophiostoma japonicum*	New Jersey	0.199	0.001
Funiliomyces sp	Top litter	0.400	0.004	*Lophodermium sp*	New Jersey	0.195	0.005
Neodevriesia poagena	Top litter	0.359	0.047	*Keissleriella poagena*	New Jersey	0.180	0.001
Sarocladium subulatum	Top litter	0.358	0.010	*Pyrenochaetopsis kuksensis*	New Jersey	0.179	0.001
Phaeosphaeriaceae sp	Top litter	0.334	0.006	*Hypocreaceae sp*	New Jersey	0.169	0.004
Mycosphaerellales	Top litter	0.235	0.024				
Ascomycota sp	Lower litter	0.851	0.001				
Sordariomycetes sp	Lower litter	0.676	0.001				
Devriesia pseudoamericana	Lower litter	0.520	0.001				
Stachybotryaceae	Lower litter	0.512	0.001				
Tetraploa sasicola	Lower litter	0.500	0.001				
Dothideomycetes sp	Lower litter	0.462	0.013				
Pleosporales	Lower litter	0.438	0.001				
Teratosphaeriaceae sp	Lower litter	0.402	0.007				
Orbiliaceae sp	Lower litter	0.361	0.001				
Conioscypha sp	Lower litter	0.360	0.001				

Figure 3-1 Boxplots of Shannon and Chao1 indices across leaf age of (A) bacterial 16s communities and (B) fungal ITS communities in *Phragmites* leaves and litter collected in 2021. Letters represent Tukey's HSD test displayed in compact letter design (CLD) showing significant pairwise comparisons between CO_2 treatment. The middle line represents the mean value, the upper and lower hinges of each boxplot represent the 25th and 75th quantile, the whiskers represent the 5th and 95th percentile, and outliers are represented by black points.

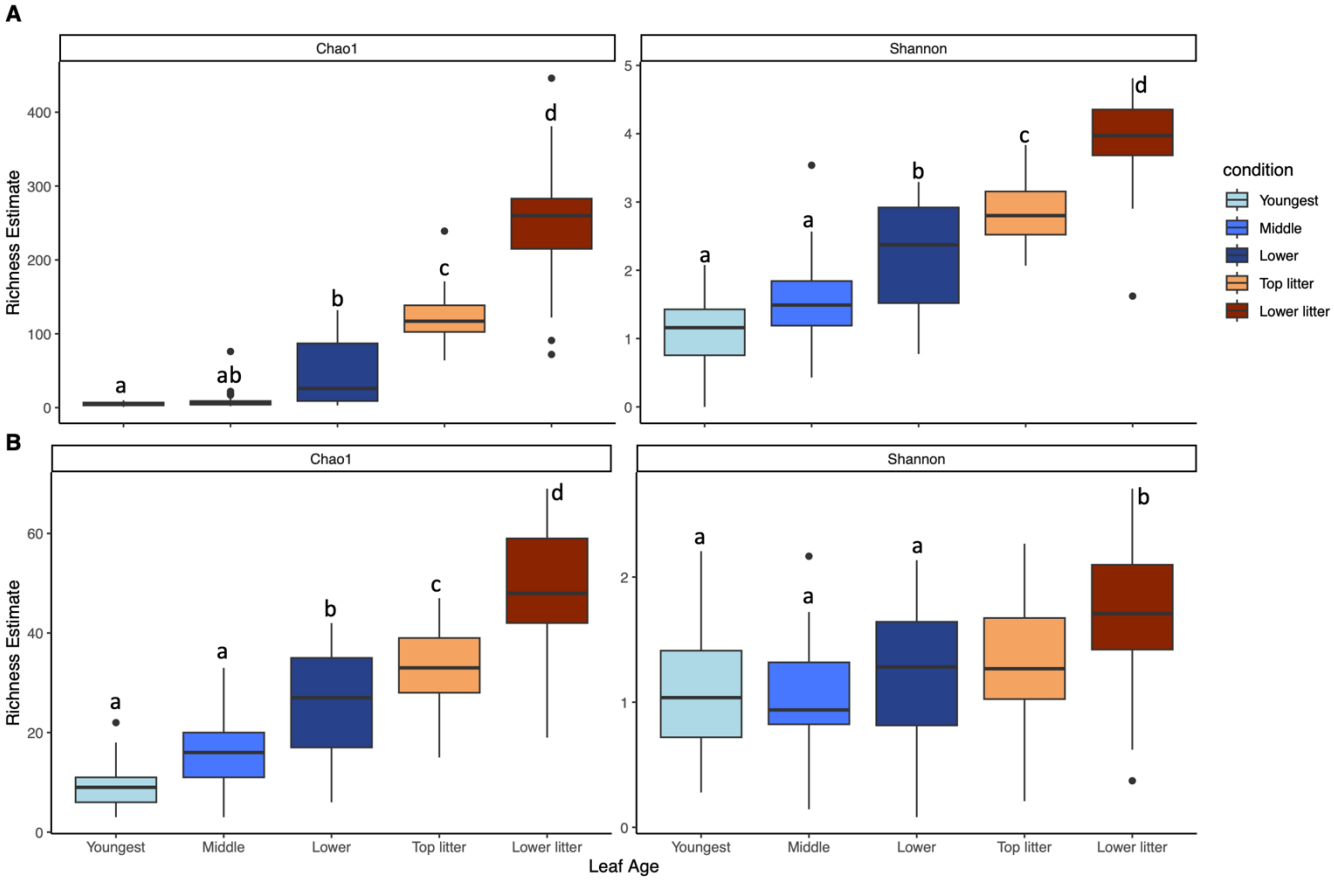


Figure 3-2 Non-metric Multidimensional Scaling (NMDS) plots of *Phragmites* (A) bacterial 16S communities and (B) fungal ITS communities collected in 2021 across state*leaf age. Letters represent Tukey's HSD test represented in compact letter design (CLD) within each microbial functional group across state*leaf age.

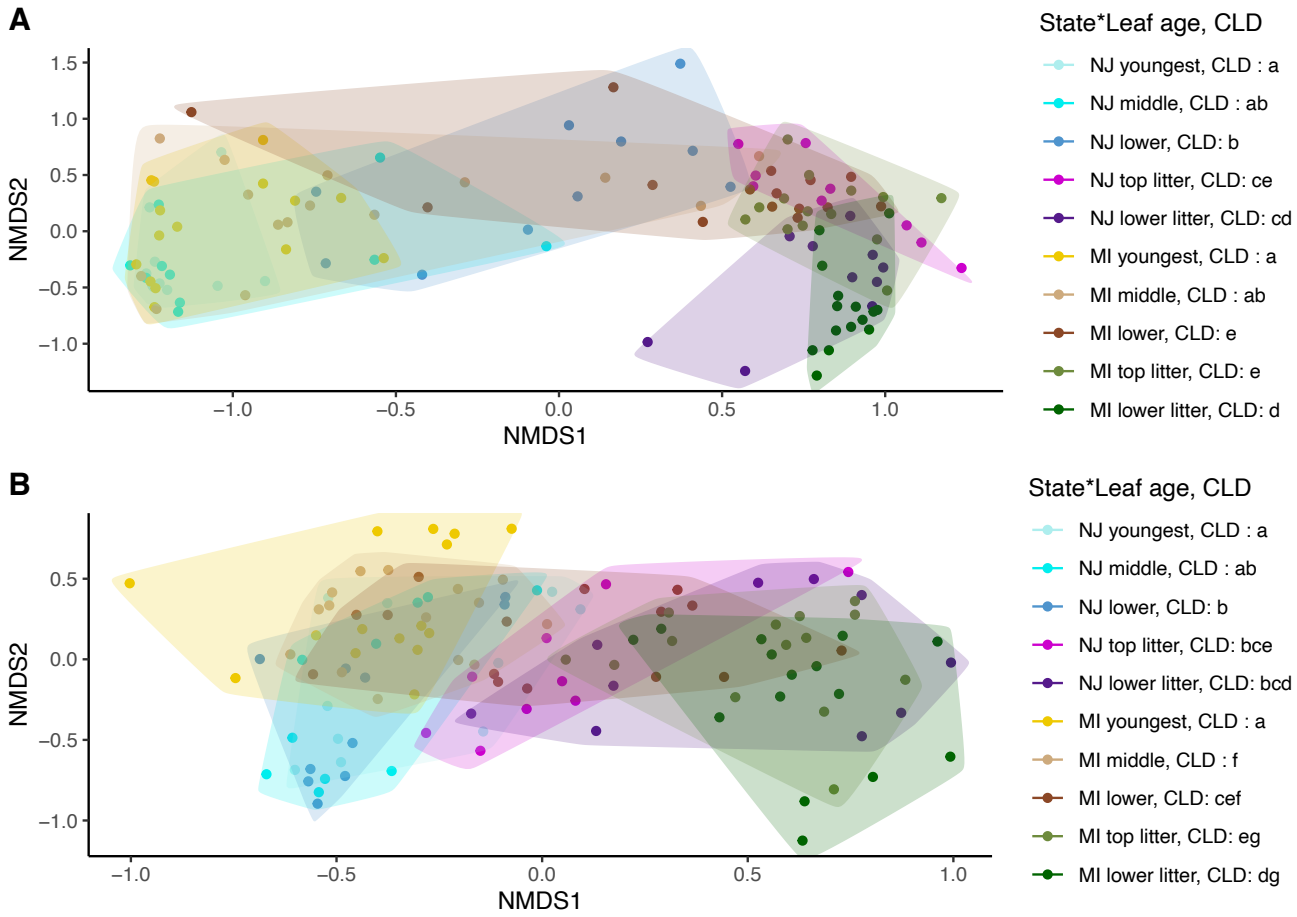
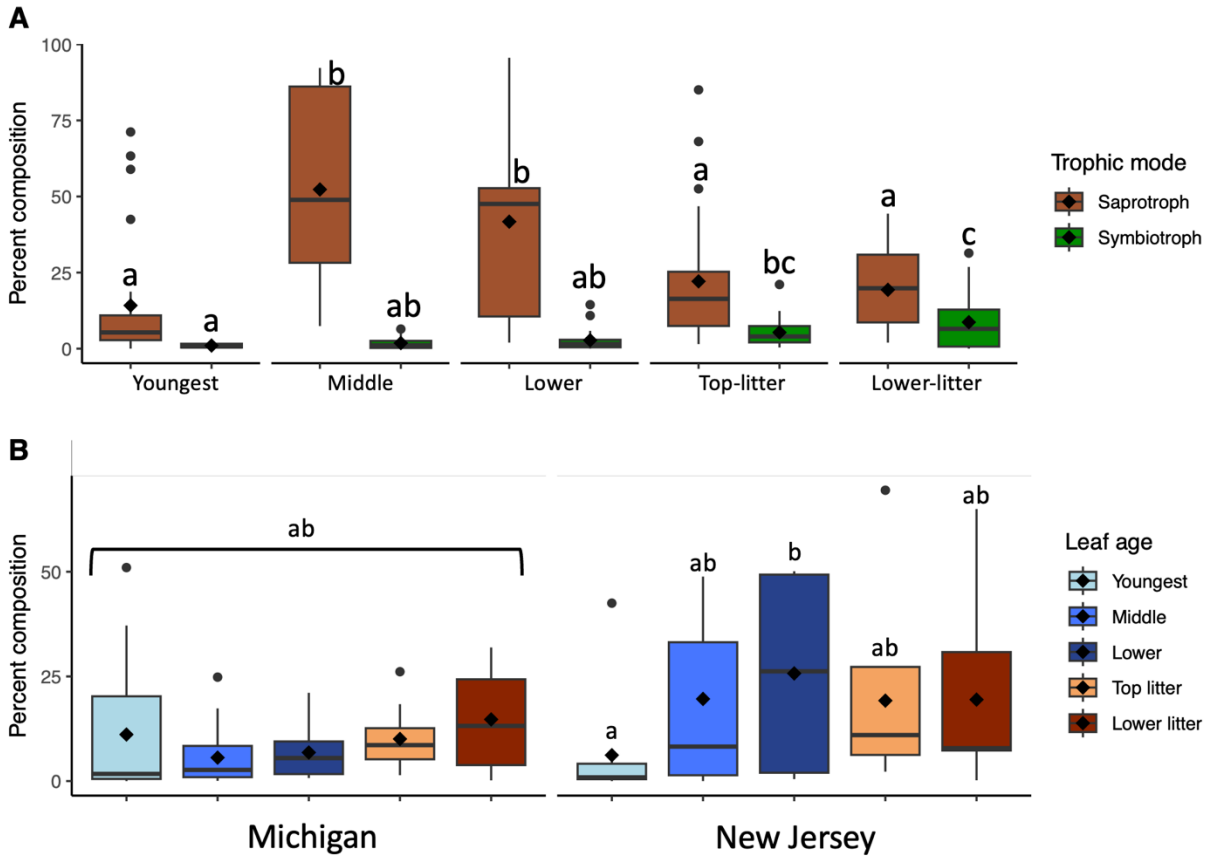


Figure 3-3 Boxplot of (A) percent saprotroph and symbiotroph composition across leaf age and (B) percent pathogen composition across leaf age and state from *Phragmites* leaves and litter collected in 2021. Letters represent Tukey's HSD test represented in compact letter design (CLD) within each trophic mode. The black diamond represents the mean value, the middle line represents the median value, the upper and lower hinges of each boxplot represent the 25th and 75th quantile, the whiskers represent the 5th and 95th percentile, and outliers are represented by black points.



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