1 Title: Influences of reduced iron and magnesium on growth and photosynthetic performance of
2 *Phragmites australis* subsp. *americanus* (North American common reed)

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14 **Abstract**

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16 Studies of the North American native wetland plant *Phragmites australis* (Cav.) Trin. ex Steud.
17 subsp. *americanus* Saltonst., P.M. Peterson & Soreng have been hindered by chlorosis – tissue
18 yellowing due to reduced chlorophyll production – while grown in controlled conditions,
19 resulting in reduced growth. This study tested the effects of reduced iron [Fe(II)] and magnesium
20 [Mg(II)], both important nutrients for chlorophyll production, on photosynthetic performance
21 and growth of North American *Phragmites*, a plant of interest for wetland restoration and
22 management. Plants were exposed to five treatments in a 13-week greenhouse experiment. Four
23 of the treatments consisted of a factorial arrangement of Fe(II) and Mg(II) treatments (0.0002 M
24 FeSO₄, 0.0002 M MgSO₄, both, or none, in supplied water), supplemented with a slow-release

fertilizer. A fifth treatment received no fertilizer, Fe(II), or Mg(II). Treatments that received added Fe(II) had significant increases in biomass, stem height, leaf and root production, chlorophyll B, and fluorescence parameters compared to non-Fe(II) treatments. Adding Mg(II) did not significantly improve plant health (growth, morphology, or resource capturing) compared to the fertilized control, and suppressed the stimulatory effect of Fe(II), possibly by interfering with root Fe(II) uptake. Under greenhouse conditions and normal fertilization practices, Fe(II) deficiency can sharply reduce growth and photosynthetic performance of *Phragmites australis* subsp. *americanus*. Supplementing plants with freshly prepared solutions of Fe(II) will alleviate limitation and facilitate experimental study and propagation under controlled conditions. The strong effect of iron limitation on native *Phragmites* suggests there could be a link between Fe(II) limitation and plant competitive interactions in some wetlands.

Keywords: wetland plants; propagation; nutrient limitation; chlorosis; Fe(II); Mg(II)

1. Introduction

Effective plant propagation for food, nursery production, or research has long been an important area of scientific exploration (Allen and Pearsall 1963, Marsh et al. 1963, Steucek and Koontz 1970, Wei et al. 1994, Thoiron et al. 1997, Rout 2015). Considerable research has focused on growth effects of secondary macronutrients and micronutrients such as magnesium (Mg) and iron (Fe) (Marsh et al. 1963, Steucek and Koontz 1970, Thoiron et al. 1997, Overman and Scholtz 2004, Rout 2015). When nutrient requirements are not met or are poorly understood, growth under controlled conditions may prove difficult or impossible.

Studies on nutrient limitation in wetland plants have primarily focused on macronutrients, especially nitrogen, under natural conditions in the field or under simulated conditions in the greenhouse (Baldwin 2013). Fewer studies have analyzed the effects that secondary nutrients (e.g., Mg) and micronutrients (e.g. Fe) have on growth of wetland species. Limited Mg may hinder wetland plant growth, as indicated by Ding et. al (2006), who found that Mg deficiencies were linked to reduced shoot growth and chlorophyll concentration. Rozbrojová and Hájek (2008) studied nutrient levels – including Fe – in a mountain fen, and found that poor vegetation growth was correlated with high levels of Fe (above 150 µg/g in vegetation tissue). Of the limited studies published on the relationship between wetland plant growth and Fe or Mg, only a few have focused on their effects on *Phragmites australis* (Snowden and Wheeler 1993; Weisner 1996, Vretare-Strand and Weisner 2002, Batty and Younger 2003, Gigante et al. 2014).

Phragmites australis, the common reed, is a perennial wetland grass and one of the most widespread plant species in the world (Haslam 1972, Clevering and Lissner 1999). It reproduces sexually through seed dispersal and asexually by rhizomal expansion, and can be found in many

fresh and brackish water wetlands, including fens, lake and river shorelines, and marshes (Saltonstall and Stevenson 2007, Ward 2010, Kettenring et al. 2011). Three taxonomic lineages of *Phragmites* have been described in North America: *Phragmites australis* (Cav.) Trin. ex Steud. subsp. *australis* (Eurasian, invasive); *Phragmites australis* subsp. *americanus* (native to USA and Canada); and *Phragmites australis* (Cav.) Trin. ex Steud. subsp. *berlandieri* (E. Fourn.) Saltonst. & Hauber (native to the USA subtropics and southward) (Saltonstall et al. 2002, Saltonstall 2004). This study specifically examined *Phragmites australis* subsp. *americanus*, hereafter referred to as native *Phragmites*.

Differences in morphological and physiological traits affect the competitive abilities of the native and invasive lineages. Native *Phragmites* is shorter and exhibits lower stem density than invasive Eurasian *Phragmites*, which has a longer growing season as well as greater primary productivity, nitrogen uptake, and leaf litter build-up (League et al. 2006, Saltonstall and Stevenson 2007, Mozdzer and Zieman 2010, Holdredge and Bertness 2011, Price et al. 2014). These traits, along with invasive *Phragmites*' tendency to proliferate and be stimulated by anthropogenic factors such as disturbance and nutrient pollution have allowed it to invade wetlands formerly dominated by native plants, including native *Phragmites* (Saltonstall et al. 2002, Mozdzer and Zieman 2010, Guo et al. 2013, Hazelton et al. 2014). The invasive lineage decreases biodiversity in wetlands through competitive displacement (Weisser and Parsons 1981, Marks et al. 1994, Ailstock et al. 2001), whereas the native lineage allows for more diversity due to its scattered shoot and root growth pattern (Meyerson et al. 2000, Hershner and Havens 2008, Price et al. 2014). Soil microbial communities also differ between the native and invasive lines (Yarwood et al. in press), suggesting differences in biogeochemical function. Aside from the differential impacts on biodiversity, both lineages have phytoremediatory and soil stabilization

properties, which are highly valued in constructed and restored wetlands (Hershner and Havens 2008, Faure et al. 2012).

Genetic confirmation of the existence of native *Phragmites* (Saltonstall 2002, Saltonstall et al. 2004) has led to greater interest in research on its biology and ecology. However, propagating it under controlled conditions for experiments or planting trials has proven more difficult than propagating Eurasian *Phragmites*. The invasive lineage produces prolific lateral shoots from stems placed horizontally in tanks of water and has high productivity if supplemented with commercial fertilizers in greenhouse pots and tanks ('Personal observation'; Achenbach et al. 2013). Native *Phragmites*, in contrast, does not produce abundant lateral shoots under flooded conditions ('Personal observation' and F. Eller, 'Personal communication'). When grown in the greenhouse, chlorosis, a lack of chlorophyll production in plants that leads to the yellowing of the leaves and stems, develops even when pots are supplemented with commercial fertilizers or Hoagland's solution (Marsh et al. 1963, Wei et al. 1994, 'Personal observation'). Hoagland's solution contains a suite of macro- and micronutrients, including Mg(II) and chelated Fe(III).

Other researchers studying native *Phragmites* have reported "apparent nutrient deficiency" (Mozdzer and Megonigal 2013) or alleviated chlorosis using reduced Fe(II) (Eller et al. 2013) or reduced Mg(II) (as Epsom salts, MgSO₄; K. Saltonstall, 'Personal communication'). Chlorosis due to Fe limitation has been studied in rice (*Oryza sativa*), another wetland plant in the same family (Poaceae). This limitation is thought to be due to an inability of rice to reduce oxidized Fe(III) to Fe(II) in the rhizosphere, because of radial oxygen leakage (Jones et al., 1982, Mori et al. 1991; Abadia et al. 2011). Magnesium deficiency has also been shown to reduce

growth and chlorophyll content in rice, which can be aggravated by increased potassium (K) supply (Jones et al. 1982, Ding et al. 2006).

The objective of this study was to understand how two nutrients necessary for chlorophyll production, iron and magnesium, affected the growth of native *Phragmites* from rhizomes to a healthy, non-chlorotic, adult stage. Magnesium sulfate (MgSO_4) and iron sulfate (FeSO_4) were used as supplements based upon anecdotal and published evidence suggesting some success in reducing chlorosis in native *Phragmites* and other wetland plants (Vretare-Strand and Weisner 2002, Saltonstall and Stevenson 2007, Eller et al. 2013). We hypothesized that plants given additional Fe(II) and/or Mg(II) would produce more aboveground and belowground biomass and have little or no evidence of chlorosis or other impairment of photosynthetic performance.

2. Methods

2.1. Experimental setup

A greenhouse experiment was established in a randomized block design of five treatment combinations of Fe(II), Mg(II), and fertilizer. Four of the treatments consisted of a factorial arrangement of Fe and Mg treatments (0.0002 M FeSO_4 (Eller 2013), 0.0002 M MgSO_4 (Saltonstall and Stevenson 2007), both, or none, in tap water). Pots receiving these treatments were supplemented once before the start of the experiment with a slow-release pelletized fertilizer at the recommended application rate (Osmocote[®], Scotts, Marysville, OH, N:P:K = 15:9:12, about 27.5 grams per pot). The fifth treatment was an unfertilized control consisting

only of unamended tap water. The five treatments are abbreviated as: Fe (only Fe(II) added); Mg (only Mg(II) added); MgFe (both Mg(II) and Fe(II) added); FC (fertilized control; no Fe(II) or Mg(II) added); and UC (unfertilized control; no Osmocote, Fe(II) or Mg(II) added). Five replicate blocks were established in the University of Maryland Research Greenhouse to test for any potentially confounding light or humidity gradients. Each block contained six pots (the experimental unit) planted with one native *Phragmites* rhizome in each pot. Each of the five blocks contained one Fe, Mg, MgFe, FC, and UC treatment, applied randomly to experimental units within each block, totaling five replicates.

The rhizomes were collected from tidal freshwater and oligohaline wetlands on the Choptank River, in the U.S. state of Maryland, in October 2014 (N38° 42' 8", W76° 41' 48", map datum: WGS84). After rinsing the rhizomes in the field, they were kept refrigerated (4 °C) and moist for 4 months. Prior to planting, 5.6-liter circular pots with a surface area of 410.43 cm² were prepared for facilitated water drainage by drilling three holes halfway up the side of each pot. On 6 February 2015, rhizomes with five or more nodes were then planted 3 cm below the surface of a 1:1 peat:sand mixture and the pots were placed inside of a 19-liter bucket. Water levels were maintained 5 cm below the soil surface. Each week, the plants were watered through the following procedure: the pot was removed from the bucket, the remaining bucket water was poured through the pot (to flush soil of precipitated solutes), and allowed to completely drain; the pot was placed back into the bucket and half of a 3-L solution of treatment water (mixed immediately prior to application) was poured onto the surface of the growth media; and the other half was poured into the exterior bucket. As the experiment progressed, additional water was needed due to the increasing evapotranspiration rates, but the total Fe and Mg loads remained the same. Evapotranspiration rates in individual experimental units were not tracked. On 12

February 2015, Osmocote was added to the fertilized treatments. The plants were allowed to sprout and acclimate for one month before treatments were added to the weekly-applied water, beginning on 12 March 2015.

Total nitrogen loading rates were 303 g/m²/year (following Saltonstall et al. 2007). The MgSO₄ was added at a rate of 0.1044 grams/pot/week in supply water (104 g/m²/year) and FeSO₄ was added weekly at 0.1354 grams/pot/week (156 g/m²/year). Greenhouse temperature was maintained above 26 °C and supplemental lighting simulated a 16-hour day. Water pH was checked periodically using pH papers to ensure the plants were not experiencing drastic changes in pH as this could impact the chemical speciation of the added Fe(II) and Mg(II). The pH remained circumneutral during the experiment so no adjustment was necessary.

2.2. Plant measurements

To measure plant growth nondestructively during the experiment, the height of each stem in each pot was measured twice weekly from the soil surface to the tallest collared (flat-bladed) leaf. The total number of stems per pot was also recorded.

As an indicator of stress to photosynthetic machinery due to environmental constraints, chlorophyll fluorescence was measured after 8 and 13 weeks following treatment initiation using a PAM-2100 Chlorophyll Fluorometer (Walz, Effeltrich Germany). Quantum yield (Y) of photochemical energy conversion of Photosystem II reaction centers was measured during the day. The Y parameter indicates the proportion of light absorbed by PSII chlorophyll used in photochemistry (i.e., not given off as light or heat), and is strongly related to carbon fixation (Maxwell and Johnson 2000). Maximum (or potential) quantum yield (F_v/F_m) is the maximum

fluorescence following a saturating pulse of light when all PSII reaction centers are open (i.e., after dark adaptation), which was measured at night. This parameter is a sensitive indicator of plant photosynthetic performance (Maxwell and Johnson 2000). Measurements were made using the PAM-2100 leaf chamber on the second highest collared leaf on two stems per pot, and the two values averaged.

2.3. Final measurements and harvest

The experiment was ended 13 weeks after initiating treatments, a duration chosen as enough time for treatment effects to be expressed but not so long as for root binding to occur, based on our previous experience with *Phragmites* in greenhouse studies. On 11 June 2015, final height measurements, stem count, and basal diameter were taken and plant leaves, stems, and roots were harvested. Leaves on every stem in each plot were stripped, starting from the lowest leaf on the stem to the top in order to keep the sheath attached to the blade. The leaves from each pot were weighed to the nearest 0.1 g, counted, and their total projected area measured using an LI-3100C Area Meter (LI-COR, Lincoln, Nebraska, USA). Four fresh leaves were stored at 4 °C for chlorophyll content analysis and remaining leaves dried to a constant mass at 35 °C. Stems were clipped at the soil surface, weighed wet, and dried to a constant mass. The rhizomes and lateral roots in each pot were removed from the growth media by rinsing with tap water over a 5-mm mesh sieve. Lateral roots were then stripped from the rhizomes, counted, and their wet and dry mass determined. The total length and average diameter of rhizomes (n=5) in each pot were measured before drying.

The sum of the projected areas of the leaves for each pot were divided by the dry mass of the leaves in that pot to determine specific leaf area (SLA), an index of the thickness of leaves, and thus their photosynthetic capability per leaf unit area (Evans and Poorter 2001). The sum of the projected areas of the leaves in each pot were divided by the total dry mass of all above and belowground plant material to determine the leaf area ratio (LAR), an index correlated with relative growth rate (Poorter and Remkes 1990). The total belowground dry mass (lateral roots + rhizomes) was divided by the total aboveground dry mass (stems + leaves) in the pot to determine the root:shoot ratio. The root:shoot ratio reflects the fraction of resources the plant allocates for nutrient uptake belowground as opposed to aboveground growth.

2.4. *Chlorophyll analysis*

The four leaves that were taken from each pot for total chlorophyll assay were cut into smaller pieces for a total wet weight of at least 300 mg (Zhongfu et al. 2009). The leaf pieces were crushed in a mortar and pestle with added liquid nitrogen until a powder formed. This powder was transferred into a 15-mL Falcon tube filled with 5 mL of 80% acetone solution and mixed for 30 minutes in the dark (to prevent chlorophyll degradation). The tubes were then centrifuged for 15 minutes at 3,000 rpm. After centrifugation, the supernatant was transferred to a cuvette and kept in the dark. This process was repeated three times for every sample, resulting in approximately 15 mL of supernatant. The supernatant's absorbance was measured using a Hach DR/4000U spectrophotometer (Hach, Loveland, Colorado) using 80% acetone as a blank control. Absorbances were recorded for wavelengths 645 nm and 663 nm. We then calculated the

milligram per gram of wet plant weight of chlorophyll a, chlorophyll b, and chlorophyll a + b (total weight).

2.5. Data analysis

Data were analyzed using SAS v. 9.2 for Windows (SAS Institute, Cary, North Carolina) using two analysis of variance (ANOVA) model structures, in accordance with the experimental design described previously. The data from the four fertilized treatments were analyzed using a two-way ANOVA to examine main effects of Fe and Mg and their interactions. A one-way ANOVA was used to test for differences across the one unfertilized and four fertilized treatments. For both models, the block was treated as a random variable. One- and two-way ANOVA models were used for dependent variables measured only once during the experiment (final biomass fractions, counts and measurements of leaves, roots, and stems) and repeated measures ANOVA was used for variables measured multiple times throughout the experiment (stem height, stem count, fluorescence). Data were checked for normality and variance homogeneity and were found to meet assumptions for ANOVA. Probability values from statistical tests were considered significant for $P < 0.05$ but tests indicating $P < 0.1$ were also noted. The Tukey procedure was used to separate least squares means at $P < 0.05$ significance, and arithmetic means and standard error were presented.

3. Results

3.1. Biomass fractions

In fertilized pots receiving Fe or Mg treatments, the addition of Fe increased the final above and belowground dry biomass ($P < 0.01$), but Mg did not ($P > 0.1$; Figs. 1A and 1D, Table 1). Total biomass likewise increased significantly due to Fe addition (Table 1; results were not plotted). However, the combined addition of Fe and Mg resulted in smaller means than Fe alone, resulting in an Mg x Fe interaction that was significant at the $\alpha = 0.1$ level for belowground biomass (Table 1, Fig. 1D), indicating a suppressive influence of Mg on Fe-stimulated growth. Shoot, leaf, rhizome, and lateral root fractions also showed a similar pattern of significant positive effects of Fe on biomass, along with Mg x Fe interactions that were significant for lateral roots ($P < 0.05$, Fig. 1F) and leaves (only at $P < 0.1$, Fig. 1C). Plants receiving additional Mg did not significantly differ from the FC for any biomass fraction, while plants receiving Fe had approximately 2-5 times more biomass as compared to the fertilizer-only treatment. Rhizomes died in two of the five pots receiving the Mg treatment during the experiment.

The UC plants were significantly lower in every biomass fraction (aboveground, belowground, lateral root, leaf, rhizome, shoot) from Fe-only treatments, but did not differ significantly from the Mg and FC treatments (Tukey test; Fig. 1). The block effect was significant at the $\alpha = 0.1$ level for most of the biomass fractions, indicating that blocking was helpful in removing variation in treatment effects associated with greenhouse gradients (Tables 1 and 2).

3.2. Morphological variables

The morphological data measured repeatedly over the course of the study (stem height and count) showed similar trends to the biomass fractions (Fig. 2, Tables 3 and 4). Significant height differences were observed between the treatments after eight weeks, with accelerated growth recorded as more shoots sprouted from the rhizomes (Fig. 2A). The Mg x Fe interaction was significant for both cumulative height and stem count, with the Fe-only treatment having the highest totals of both variables, followed by MgFe treatment, and then Mg-only treatment, which averaged a third fewer stems than Fe pots (Fig. 2B, Table 4).

Leaf area, lateral root count, and rhizome length also exhibited similar Mg x Fe interactions, with MgFe pots showing intermediate effects on growth as compared to Mg-only and Fe-only treatments, suggesting a suppressive effect of Mg on Fe response in root and leaf production (Fig. 3, Tables 1). Lateral root numbers per rhizome length and total leaf count were significantly different between the fertilized (mean $\pm$ SE: 0.90 ± 0.09 and 55.50 ± 7.67 , respectively) and unfertilized plants (0.50 ± 0.07 and 12.20 ± 1.50 , respectively, $\alpha = 0.05$ level), but no significant differences were seen within the fertilized treatments, signifying that neither Fe nor Mg induced greater growth in the sprouting of leaves or lateral roots per unit space on the shoots and rhizomes respectively (Table 1).

Between fertilized and unfertilized pots, significant changes were also seen in lateral root count (mean $\pm$ SE: 89.82 ± 14.79 vs. 13.60 ± 4.17 , respectively), leaf area ($2248.10 \text{ cm}^2 \pm 332.53$ vs. 127.02 ± 40.54), rhizome length ($98.15 \text{ cm} \pm 14.69$ vs. 25.80 ± 4.44), shoot basal diameter ($31.28 \text{ cm} \pm 4.04$ vs. 4.40 ± 0.81), and shoot count (5.88 ± 0.77 vs. 2.00 ± 0.32 , Table 2). Lateral root count and leaf area were also significantly greater in Fe-only treatments as compared to Mg-only and FC treatments (Figs. 3A, 3C, Tukey test, Table 1). No significant differences were seen in the shoot, leaf, or total percent water between any of the fertilized treatments, but shoot

percent water was significantly different between fertilized and unfertilized treatments (mean $\pm$ SE: 80.50% $\pm$ 2.34 vs. 66.30 $\pm$ 3.54, respectively, Tables 1 and 2). The lack of differences in percent water content between fertilized treatments indicates that nutrients do not affect water uptake (Tables 1 and 2).

3.3. Resource capture and allocation

Fluorescence tests produced different outcomes from biomass and morphological results. Though Fe treatments produced significantly higher F_v/F_m and yield than the other fertilized pots, unfertilized plants had similar fluorescence to Fe plants (Fig. 4, Table 3). Fe pots were the only treatments with significantly increased concentrations of chlorophyll B (mean $\pm$ SE: 1.48 mg/g $\pm$ 0.12) compared to the FC (0.59 $\pm$ 0.28), but produced no significant difference in chlorophyll A or total chlorophyll mass (Table 1). Fertilized treatments were significantly different to unfertilized treatments for chlorophyll B at the $\alpha = 0.1$ level (Table 2). Additional Mg did not result in any differences within fluorescence tests as compared to the FC treatments (Fig. 4 A and C, Table 4). No Mg x Fe interaction occurred for leaf chlorophyll concentration, indicating that additional Mg did not increase stress within photosynthetic processes in the chlorophyll, but did not alleviate chlorosis (Table 4).

Specific leaf area was significantly higher – indicating broader and thinner leaves – in the UC than the FC and the Fe treatments (Fig. 4B). The LAR, in contrast, was significantly higher in the fertilized treatments than the UC, signifying more resources were allocated to leaf biomass per unit total biomass as compared to the unfertilized treatments (Table 2, Fig. 4D). The root:shoot ratio best shows the change in resource allocation between fertilized and unfertilized

treatments, where treatments with fertilizer averaged below a 1:2 ratio between belowground and aboveground growth, while treatments without fertilizer added had an inverse ratio of 2:1, quadruple the amount of belowground biomass as compared to aboveground (Fig. 4E). No interactions were observed between Mg and Fe in any of the resource capture and allocation data collected in the experiment (Table 4).

4. Discussion

To our knowledge, this is the first study to experimentally examine Fe and Mg requirements for *Phragmites australis* subsp. *americanus* in a greenhouse setting. In studying the effects of two fertilizers on native and Eurasian *Phragmites* seedling growth, Saltonstall et al. (2007) supplemented their seedlings with micronutrients – including Mg(II) – to prevent micronutrient deficiencies. They observed trends in plant growth between unfertilized and fertilized native *Phragmites* similar to our study, with significant increases in aboveground and belowground biomass and cumulative stem height. Other studies alluded to nutrient deficiencies affecting their experiments, which were resolved by adding more nutrients to the soil (Eller et al. 2013, Mozdzer and Megonigal 2013). Eller et al. (2013) specifically mentioned supplementing the plants with Fe(II) along with other nutrients and fertilizers for approximately 52 days before harvesting, but did not mention chlorotic issues.

The most successful growth conditions were observed with Fe(II) additions. In general, added Fe(II) had a strong positive effect on root and shoot growth – in biomass and number – under unconstrained primary macronutrient (N-P-K) conditions (Figs. 1-4). In most of the measured parameters, values were highest in Fe(II) treatments, followed in decreasing order by

MgFe treatments, Mg and FC treatments, and finally UC treatments (Fig. 1, Fig. 2). We have not observed chlorosis of native or invasive *Phragmites* in wetlands in the field, presumably due to the abundance of reduced Fe(II) in anaerobic, saturated soil conditions (although Fe limitation may occur in some Eurasian *Phragmites australis* treatment wetlands; Brian Sorrell, Aarhus University, ‘Personal communication’). Under greenhouse conditions, radial oxygen leakage from roots compounded with changing water on a frequent basis and well-drained peat-sand growth medium likely resulted in oxidized conditions not normally encountered in the fine-textured, continuously saturated soils typically of most wetlands. Thus our findings indicate that Fe(II)-limitation can occur under some conditions and be alleviated by supplementing with freshly prepared reduced-iron solution.

The Mg treatment did not improve plant growth when compared to the FC or when comparing Fe-only to MgFe treatments for several measurements (Figs. 1 and 3, Table 1). Agarwala and Mehrotra (1984) observed an antagonistic relationship between Fe and Mg in radishes, where nutrient accumulation was dependent upon whether there was a higher concentration of Mg or Fe in the soil. A study of barley grown in Fe-depleted soils detected decreased amounts of Fe-induced chlorosis in plants treated with arsenic (As), which lowered the levels of several nutrients, including Mg, and increased the concentration of Fe in plant tissue (Shaibur et. al 2009). Likewise, we observed that Mg suppressed the positive effect of Fe, as reflected by the trend in decreasing biomass totals from Fe-only to MgFe to Mg treatments in lateral root biomass and leaf and total belowground biomass (Fig. 1). We speculate that these results are due to uptake competition between Fe and Mg in *Phragmites australis*.

Fe treatments significantly increased the leaf chlorophyll fluorescence parameters F_v/F_m and Yield ($p < 0.05$, Fig. 4, Table 1). The results confirmed coloration differences between

treatments with and without reduced Fe. Plants with Fe added showed no evidence of chlorosis throughout the experiment, which indicates lower amounts of stress in growth and production. Similar fluorescence results were surprisingly observed in the UC treatments, which we attribute to the lack of growth in the UC caused by primary macronutrient limitation (NPK). Because the UC plants grew little throughout the experiment, the plants did not become Fe(II)-limited, leading to fluorescence results similar to the Fe treatments containing mature plants.

Plants in the Fe treatment had more chlorophyll B than the FC. Chlorophyll B is an accessory pigment that increases the plant's visible light range for photosynthetic processes, indicating that plants with more Fe potentially had higher photosynthetic production (Lessmann et al. 2001). Pagter et al. (2005) found that water limitation had no significant effects on *Phragmites* chlorophyll B production, and Bragato et al. (2006) saw minimal changes in chlorophyll B when *Phragmites* was exposed to several heavy metals (Zn, Cu, Ni, Cr) in treatment wetlands. Both of these findings suggest that decreases in chlorophyll B are specifically linked to nutrient deficiency.

Our data agree with past studies that adding a general fertilizer (like Osmocote) not surprisingly improves the overall growth of *Phragmites australis*. In our study, a comparison of fertilized and unfertilized plants in a one-way ANOVA demonstrated that fertilizer had a stronger positive effect on aboveground biomass than on belowground biomass, resulting in decreased root:shoot ratio (Figs. 1 and 4, Table 2). Previous studies found similar results in a shift from belowground to aboveground growth in invasive *Phragmites* (Rickey and Anderson 2004, League et al. 2006, Saltonstall and Stevenson 2007, Engloner 2009). Our results confirmed this explanation, with fertilized plants doubling the resources put into photosynthetic organs (shoots), while unfertilized plants put most resources into finding adequate nutrients for growth

in unfertilized treatments (roots) (Fig. 4). Thus, nutrient enrichment can result in shift from belowground nutrient competition to aboveground light competition (Wilson and Tilman 1993).

Although our study found a strong positive effect of reduced Fe addition on plant growth indicators, specifically chlorophyll B, fluorescence, and plant production (Figs. 1 and 4, Tables 1 and 4), other studies on *Phragmites* (presumably the Eurasian lineage) have shown mixed results. In a study on *Phragmites* seedlings, root growth increased at water concentrations of Fe(II) up to 1 mg/L, but then declined at 2 mg/L and higher (Batty and Younger 2003). In another study, Weisner (1996) found that young *Phragmites* clones experienced reduced shoot and root production when grown in anaerobic sediment and suggested that inhibition of nutrient uptake by reduced iron might have occurred, based on higher total Fe concentrations measured in root tissue. More recently, Reed Die-Back Syndrome (RDBS) in the Mediterranean region of Europe was linked to higher flooding rates and permanent flooding conditions in *Phragmites* stands (Gigante et al. 2011, Reale et al. 2012, Gigante et al. 2014). Specifically, Gigante et al. (2014) found that permanently flooded stands tended to have elevated concentrations of (presumably) reduced iron, which was significantly correlated to stand clumping, an important indicator for RDBS. In a study that screened seedlings of 44 fen species for Fe(II) toxicity, *Phragmites* ranked 26th overall for Fe(II) tolerance based on Relative Growth Rate (and 18th out of the 22 monocot species tested), and was classified as “semi-tolerant” of Fe(II) (Snowden and Wheeler 1993). Taken together, these studies suggest that Fe(II) may promote growth of *Phragmites* up to a threshold, above which toxicity may occur. It is possible that higher concentrations of Fe(II) than we used would result in toxicity to native *Phragmites*, or that native *Phragmites* is more Fe(II)-tolerant than Eurasian *Phragmites*.

Variation in reduced and oxidized iron may shift competitive outcomes between native and invasive lineages. For example, the 300% greater rhizosphere ventilation efficiency of invasive compared to native *Phragmites* (Tulbure et al. 2012) may create oxidizing conditions that decrease Fe(II), limiting growth in the native. In other aquatic species, sulfate may result in precipitation of iron as iron sulfides, leading to less available reduced iron and iron deficiency, shifting competitive outcomes between species (van der Welle et al. 2007a, b).

In conclusion, our study highlights the importance of Fe(II) in wetland plant growth, and raises the possibility that reduced iron limitation may occur in wetlands subject to variable hydroperiods, seasonal desiccation, or rhizosphere oxidation. Changes in reduced iron abundance may play a role in plant species distribution and competitive interactions in wetlands. Given evidence of reduced growth in native *Phragmites*, supplementation may be needed for large-scale propagation of this species for restoration projects and wastewater treatment wetlands. Alleviation of iron limitation in native *Phragmites* in controlled greenhouse settings will facilitate ecological research on this lineage, a plant that may provide some of the same ecosystem services for which *Phragmites* is valued throughout the world.

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631

632 **Table 1.** Results of two-way ANOVA of Fe and Mg treatments and interactions (in fertilized plants only) on final measurements of
 633 dry biomass, morphological, and resource capture and allocation parameters. Abbreviations: ndf = numerator degrees of freedom, ddf
 634 = denominator degrees of freedom, F = F-value, P = P-value. P-values <0.05 indicated in boldface type.

Variable Group	Fe			Mg			Mg x Fe			Block		
Variable	ndf, ddf	F	P	ndf, ddf	F	P	ndf, ddf	F	P	ndf, ddf	F	P
Biomass Fractions												
Aboveground Biomass	1, 10	24.38	0.0006	1, 10	2.16	0.1724	1, 10	2.90	0.1191	4, 10	2.95	0.0751
Belowground Biomass	1, 9	16.66	0.0028	1, 9	2.80	0.1288	1, 9	3.91	0.0792	4, 9	3.04	0.0766
Lateral Root Biomass	1, 10	19.72	0.0013	1, 10	4.82	0.0529	1, 10	7.18	0.0231	4, 10	2.84	0.0825
Leaf Biomass	1, 10	21.05	0.0010	1, 10	1.94	0.1941	1, 10	4.18	0.0680	4, 10	2.84	0.0822
Rhizome Biomass	1, 9	13.29	0.0054	1, 9	1.62	0.2345	1, 9	1.87	0.2048	4, 9	2.68	0.1013
Shoot Biomass	1, 10	24.51	0.0006	1, 10	1.92	0.1964	1, 10	2.72	0.1299	4, 10	2.73	0.0899
Total Dry Biomass	1, 9	19.77	0.0016	1, 9	2.04	0.1874	1, 9	2.97	0.1191	4, 9	2.80	0.0920
Morphological Variables												
Lateral Root Count	1, 10	19.49	0.0013	1, 10	0.13	0.7294	1, 10	4.22	0.0671	4, 10	1.76	0.2130
Lateral Root/Rhizome Length	1, 10	0.34	0.5726	1, 10	0.31	0.5919	1, 10	0.12	0.7405	4, 10	0.34	0.8462
Leaf Area	1, 10	21.04	0.0010	1, 10	1.19	0.3010	1, 10	4.32	0.0643	4, 10	1.63	0.2422
Leaf Count	1, 10	0.82	0.3862	1, 10	0.02	0.8958	1, 10	5.82	0.0365	4, 10	1.96	0.1766
Percent Leaf Water	1, 10	0.03	0.8746	1, 10	0.23	0.6449	1, 10	0.03	0.8734	4, 10	0.98	0.4625
Percent Shoot Water	1, 10	0.54	0.4798	1, 10	0.29	0.5996	1, 10	0.12	0.7346	4, 10	1.23	0.3568
Rhizome Length	1, 10	29.26	0.0003	1, 10	0.72	0.4155	1, 10	5.33	0.0435	4, 10	3.17	0.0632
Shoot Basal Diameter	1, 10	4.16	0.0687	1, 10	1.02	0.3373	1, 10	1.42	0.2608	4, 10	3.61	0.0452
Shoot Count	1, 10	0.54	0.4790	1, 10	3.85	0.0783	1, 10	6.01	0.0342	4, 10	0.68	0.6200
Total Percent Water	1, 9	0.33	0.5814	1, 9	0.46	0.5137	1, 9	0.04	0.8479	4, 9	1.24	0.3594
Resource Capture & Allocation												
Chlorophyll A	1, 11	2.28	0.1595	1, 11	0.11	0.7439	1, 11	0.08	0.7771	4, 11	0.81	0.5468
Chlorophyll B	1, 11	8.30	0.0150	1, 11	0.01	0.9055	1, 11	0.01	0.9238	4, 11	0.28	0.8857
Leaf Area Ratio	1, 9	2.38	0.1571	1, 9	3.24	0.1052	1, 9	0.25	0.6320	4, 9	3.54	0.0532
Root:Shoot Ratio	1, 9	0.61	0.4539	1, 9	0.53	0.4846	1, 9	0.00	0.9806	4, 9	0.45	0.7713
Specific Leaf Area	1, 9	0.51	0.4947	1, 9	4.00	0.0766	1, 9	0.00	0.9976	4, 9	8.25	0.0044
Total Chlorophyll Mass	1, 11	0.01	0.9081	1, 11	0.12	0.7319	1, 11	0.04	0.8412	4, 11	0.91	0.4936

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Table 2. Results of one-way ANOVA exhibiting differences in biomass, morphology, and resource capture/allocation between fertilized and unfertilized treatments. Abbreviations: ndf = numerator degrees of freedom, ddf = denominator degrees of freedom, F = F-value, P = P-value. P-values <0.05 indicated in boldface type.

<u>Variable Group</u>	<u>Treatment</u>				<u>Block</u>			
Variable	ndf	ddf	F	P	ndf	ddf	F	P
<u>Biomass Fractions</u>								
Aboveground Biomass	4	12	14.71	0.0001	4	12	2.82	0.0736
Belowground Biomass	4	12.8	9.25	0.0001	4	12.8	2.53	0.0918
Lateral Root Biomass	4	13	12.68	0.0002	4	13	2.30	0.1143
Leaf Biomass	4	12	14.73	0.0001	4	12	2.64	0.0866
Rhizome Biomass	4	12.8	7.32	0.0027	4	12.8	2.35	0.1096
Shoot Biomass	4	13	15.70	< 0.0001	4	13	2.45	0.0979
<u>Morphological Variables</u>								
Lateral Root Count	4	13	16.51	< 0.0001	4	13	1.78	0.1930
Lateral Root/Rhizome Length	4	13	3.54	0.0365	4	13	0.09	0.9825
Leaf Area	4	13	18.10	< 0.0001	4	13	1.53	0.2500
Leaf Count	4	13	11.51	0.0003	4	13	2.15	0.1317
Percent Leaf Water	4	12	0.08	0.9882	4	12	0.78	0.5570
Percent Shoot Water	4	13	3.18	0.0497	4	13	1.18	0.3649
Rhizome Length	4	13	19.58	< 0.0001	4	13	2.93	0.0626
Shoot Basal Diameter	4	13	12.45	0.0002	4	13	5.45	0.0084
Shoot Count	4	13	8.84	0.0011	4	13	0.50	0.7378
Total Percent Water	4	11	0.58	0.6810	4	11	1.22	0.3569
<u>Resource Capture & Allocation</u>								
Chlorophyll A	4	15	1.47	0.2613	4	15	0.00	1.0000
Chlorophyll B	4	15	2.74	0.0679	4	15	0.00	1.0000
Leaf Area Ratio	4	11	5.03	0.0150	4	11	2.53	0.1003
Root:Shoot Ratio	4	11	78.18	< 0.0001	4	11	0.64	0.6425
Specific Leaf Area	4	11	5.68	0.0099	4	11	8.45	0.0023
Total Chlorophyll Mass	4	15	0.24	0.9110	4	15	0.00	1.0000

Table 3. Results of one-way ANOVA of all treatments from repeatedly measured morphological and fluorescence data collected throughout the experiment. Abbreviations: ndf = numerator degrees of freedom, ddf = denominator degrees of freedom, F = F-value, P = P-value. P-values <0.05 indicated in boldface type.

Variable	Effect	ndf	ddf	F	P
Cumulative Stem Height	Treatment	4	372	94.58	< 0.0001
	Day of Experiment	19	372	75.48	< 0.0001
	Treatment x Day of Experiment	76	372	8.05	< 0.0001
	Block	4	372	14.04	< 0.0001
F_v/F_m	Treatment	4	32	9.01	< 0.0001
	Date	1	32	3.33	0.0772
	Treatment x Date	4	32	0.93	0.4608
	Block	4	32	0.87	0.4937
Stem Count	Treatment	4	374	62.24	< 0.0001
	Day of Experiment	19	371	55.15	< 0.0001
	Treatment x Day of Experiment	76	371	4.33	< 0.0001
	Block	4	371	1.00	< 0.0001
Yield	Treatment	4	31	15.11	< 0.0001
	Date	1	31	0.05	0.8220
	Treatment x Date	4	31	1.01	0.4186
	Block	4	31	1.15	0.3504

Table 4. Results of two-way ANOVA of all treatments from repeatedly measured morphological data collected throughout the experiment. Abbreviations: ndf = numerator degrees of freedom, ddf = denominator degrees of freedom, F = F-value, P = P-value. P-values <0.05 indicated in boldface type.

Variable	Effect	ndf	ddf	F	P
Cumulative Stem Height	Fe	1	261	96.67	< 0.0001
	Mg	1	293	12.24	0.0005
	Mg x Fe	1	293	12.45	0.0005
	Day of Experiment	19	288	69.81	< 0.0001
	Fe x Day of Experiment	19	288	11.68	< 0.0001
	Mg x Day of Experiment	19	288	1.99	0.0089
	Mg x Fe x Day of Experiment	19	288	0.56	0.9346
	Block	4	245	20.70	< 0.0001
F_v/F_m	Fe	1	24	21.88	< 0.0001
	Mg	1	24	0.65	0.4266
	Mg x Fe	1	24	0.51	0.4826
	Day of Experiment	1	24	2.81	0.1064
	Fe x Day of Experiment	1	24	2.20	0.1513
	Mg x Day of Experiment	1	24	0.32	0.5787
	Mg x Fe x Day of Experiment	1	24	0.15	0.7031
	Block	4	24	1.41	0.2617
Stem Count	Fe	1	284	39.08	< 0.0001
	Mg	1	296	0.32	0.5729
	Mg x Fe	1	296	31.03	< 0.0001
	Day of Experiment	19	291	54.87	< 0.0001
	Fe x Day of Experiment	19	291	4.88	< 0.0001
	Mg x Day of Experiment	19	291	1.18	0.2692
	Mg x Fe x Day of Experiment	19	291	0.41	0.9875
	Block	4	276	25.98	< 0.0001
Yield	Fe	1	23	34.18	< 0.0001
	Mg	1	23	0.15	0.7024
	Mg x Fe	1	23	0.01	0.9427
	Day of Experiment	1	23	0.05	0.8208
	Fe x Day of Experiment	1	23	0.19	0.6692
	Mg x Day of Experiment	1	23	2.72	0.1129
	Mg x Fe x Day of Experiment	1	23	0.06	0.8126
	Block	4	23	1.06	0.3980

Figure captions:

Figure 1. Effects of Fe, Mg, and fertilizer on biomass of *Phragmites australis* subsp. *americanus*. Treatments are abbreviated as: Fe (only Fe(II) added); Mg (only Mg(II) added); MgFe (both Mg(II), and Fe(II) added); FC (fertilized control; only fertilizer added); and UC (unfertilized control; no fertilizer, Mg(II) or Fe(II) added). Plotted values are arithmetic mean + 1 SE of above and belowground mass (1st column, panels A and D), shoot and rhizome growth (2nd column, panels B and E), and leaf and lateral root fraction biomass (3rd column, panels C and F). Within each panel, different letters between means indicate significant differences (Tukey test, $P < 0.05$).

Figure 2. Variation in A. cumulative growth of stems per pot per treatment and B. number of stems per pot per treatment over the 91-day experiment in response to Fe, Mg, and fertilization treatments. Plotted values are arithmetic means of weekly measurements and plotted using straight lines between points.

Figure 3. Response of lateral root count (A), rhizome length (B), and total leaf area per pot (C) to Fe, Mg, and fertilization treatments, plotted as arithmetic mean + 1 SE. Means with different letters differ significantly according to Tukey means separation, $P < 0.05$.

Figure 4. The influence of Fe, Mg, and fertilization treatments on fluorescence (A and C), leaf area (B and D), and root:shoot (E) parameters. Means (+1 SE) with the same letters within a panel do not differ significantly (Tukey test, $\alpha = 0.05$).

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