Utilization of dissolved organic nitrogen by the macrophytes
Spartina alterniflora and *Phragmites australis*

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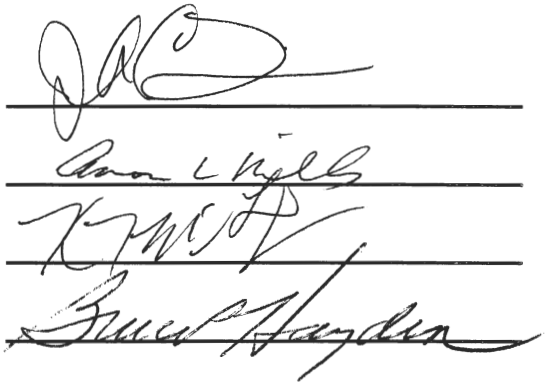

ABSTRACT

Dissolved organic nitrogen (DON) has been demonstrated as a significant pool of bioavailable nitrogen in many systems ranging from arctic tundra to temperate coastal lagoons. Current understanding is that temperate tidal plants solely utilize the inorganic form of nitrogen. Since DON has been demonstrated to be an important source of nitrogen in other systems, I investigated whether the dominant salt marsh macrophyte *Spartina alterniflora*, or if the common reed, *Phragmites australis*, have the ability to assimilate DON directly.

To test this hypothesis, short-term laboratory uptake experiments were performed using dual labelled nutrients, ^{15}N and ^{13}C, on two haplotypes of *P. australis* (native and non-native) and *S. alterniflora*. Assimilation rates were calculated on intact plants whose root systems were exposed to three nutrient solutions (glycine, glutamic acid, and urea) and a ^{15}N control (NH_4^+). My experiment indicated that the amino acids glycine and glutamic acid were assimilated intact, and that assimilation rates, 3.8 to 1.9 μmols ^{15}N g^{-1} dw hr^{-1}**,** were as high as 20% and 8%, respectively, of NH_4^+ rates.

To validate laboratory measurements, a five-day field experiment was also conducted on both *P. australis* and *S. alterniflora*. Assimilation rates of NH_4^+ and combined amino acids in *P. australis* were similar to those in the laboratory experiment. *S. alterniflora* assimilation rates were significantly lower, and this result may be a product of different field variables due to plant location in the salt marsh, and subsequent differences in tidal flushing and hydrology between the two plants. This research provides the first evidence for direct assimilation of DON by temperate marsh plants. My

results suggest that DON is potentially an important bioavailable pool of N that should be considered in temperate tidal marsh systems.

In addition to the investigation of DON, I also investigated if photosynthetic differences were observed between native and non-native *P. australis* haplotypes due to visible differences in plant morphology. Photosynthetic parameters were measured on greenhouse grown plants using infrared gas analysis (IRGA) and pulse amplitude modified (PAM) flourometry. Significant differences were observed between native and non-native *P. australis* haplotypes. Non-native *P. australis* had significantly greater concentrations of chlorophyll a, chlorophyll, b, and total carotenoids than native *P. australis* plants. Following this, CO_2 assimilation rates, and the photosynthetic parameters of A_{max}, and ETR_{max}, were all significantly greater in non-native *P. australis* haplotypes. These results demonstrate that native *P. australis* plants are physiologically different from the non-native invader, and suggest that the expansion of non-native *P. australis* into previously unoccupied habitats may be facilitated by higher photosynthetic rates.

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Chapter 1 OVERALL INTRODUCTION

Tidal marshes are a vital component of estuarine ecosystems. They serve as a nursery ground for commercially important fisheries, in addition to providing a nutrient and physical buffer between oceans and the terrestrial environment. Salt marshes of the North American Atlantic coast are dominated by the halophytic smooth cordgrass, *Spartina alterniflora* (Mitsch and Gosselink 1993). However, throughout the past 100 years, the common reed, *Phragmites australis* has become pervasive in Atlantic coast tidal marshes. This well documented expansion into tidal marshes has been of major concern to wetland ecologists in both tidal and non-tidal habitats (Chambers et al. 1999, Windham 1999, Findlay et al. 2002, Windham and Meyerson 2003). Studies investigating the impacts of the *P. australis* expansion have been inconclusive. *P. australis* expansion has been linked to a five-fold decrease in plant species richness in New England salt marshes (Bertness et al. 2002). Posey et al. (2003) and Meyer et al. (2001), however, found that change in dominant species to *P. australis* did not strongly affect the benthic infaunal or the nekton communities in mid-Atlantic salt marshes. On a smaller scale, the influence of *P. australis* on microbial activity has been shown to vary from having no apparent influence to a three-fold increase in microbial rates (Otto et al. 1999, Windham and Ehrenfeld 2003, Windham and Meyerson 2003).

P. australis, a monocot grass, is thought by some to be the world's most widely distributed flowering plant (Holm et al. 1977). It has a geographic range of 70º N latitude to 70º S latitude, and is found on every continent except Antarctica (Haslam 1972, Holm et al. 1977). *P. australis* has been a native component of North American

tidal marshes for at least 3,000 years (Niering et al. 1977). In addition, remains of *P. australis* found in Arizona were up to 40,000 years old (Hansen 1978 *in* Kiviat and Hamilton 2001). Recent genetic research has demonstrated that a non-native haplotype (genotype) was most likely introduced to the North American Atlantic coast during the 1800's via shipping (Saltonstall 2002). This non-native haplotype has been directly linked to the extinction of several native haplotypes in New England and the expansion observed in North America (Saltonstall 2002). Eleven haplotypes of *P. australis* are endemic to North America, and are genetically distinct from the non-native haplotype M. Native populations of *P. australis* in the mid-Atlantic consists mainly of haplotype F as described by Saltonstall (2002).

There have been several explanations why *P. australis* has been so successful. Overall, *P. australis* is known to be an efficient colonizer of disturbed environments, and disturbances by marsh wrack have been attributed to the facilitation of *P. australis* expansion (Minchinton 2002). Since seed germination is low (Haslam 1971, Ishii and Kadono 2002), most of the spread of *P. australis* is thought to be asexual by clonal integration and the propagation of rhizome fragments. Once a monoculture clone has been established, a clone may persist for over 1000 years, consequently, frequent seedling survival is unnecessary (Rudescu et al. 1965 *in* Haslam 1971). However B. Blossey *(personal communication*) indicated that seed germination and survival is quite high in non-native *P. australis* haplotypes and lower in *P. australis* native haplotypes.

Prior to Saltonstall's (2002) study demonstrating the invasion of the non-native genotype of *P. australis,* studies suggested that expansion was related to eutrophication of North American coastal habitats (Chambers et al. 1999, Bertness et al. 2002).

However, eutrophication alone does not appear to explain the success of *P. australis* in such environments. In Europe, chronic eutrophication may be a physiological stress on *P. australis*, and is thought to be the reason for the well documented die-back (van der Putten 1997).

This thesis consists of four chapters. Chapter 1, the overall introduction, provides background for the objectives of this thesis, which are to determine the utilization of DON by temperate macrophytes, and determine if photosynthetic differences exist between *P. australis* haplotypes. Chapter 2 describes work that shows that the temperate salt marsh macrophyte, *S. alterniflora* or *P. australis* have the ability to directly utilize dissolved organic nitrogen, a bioavailable form of nitrogen that has been neglected by marsh ecologists. Chapter 3 discusses if physical and/or physiological differences exist between native and non-native haplotypes of *P. australis* in North America. Finally, Chapter 4 synthesizes this thesis to put this study into a broader context.

Chapter 2 UTILIZATION OF DISSOLVED ORGANIC NITROGEN

2.1 INTRODUCTION

Recent studies have shown that *P. australis* requires a greater amount of nitrogen (N) than the native species it is replacing (Windham 1999, Windham and Meyerson 2003). A recent N budget in *P. australis* invaded salt marshes demonstrated a deficit of dissolved organic N (DIN) needed for the observed productivity, with *P. australis* demanding 50% more N than either *S. alterniflora* or *S. patens* (Windham 1999). Nitrogen is generally considered to be the limiting nutrient in salt marsh environments (Valiela and Teal 1974); therefore, this pattern suggests that *P. australis* may be exploiting an overlooked pool of N, such as organic nitrogen.

Monocultures of *P. australis* are often associated with large standing stocks of dead culms than can remain intact for up to 3 years. Although photosynthetically dead, these dead culms provide a mechanism for gas flow to and from the plant rhizosphere (Armstrong and Armstrong 1991). Additionally, this accumulation of organic matter, when combined with slow decomposition rates, creates an large pool of organic material within *P. australis* marshes (Windham 2001). Although this pool of N is thought to be unavailable due to its slow decomposition rate, this process may in fact be a mechanism to retain the N among *P. australis*, making it unavailable for other species.

A potential, but grossly neglected, source of bioavailable nitrogen that exists in tidal marsh systems is dissolved organic nitrogen (DON). DON is a term used to characterize a large group of organic nitrogenous compounds that can vary significantly in size and weight. In this study, DON will refer to the small, bioavailable fraction of

low C:N compounds such as amino acids and urea only. However, it must also be noted that collectively, the term DON includes large, refractory, high C: N compounds as well. The term DON in the literature usually refers to the difference of total dissolved nitrogen minus the inorganic fractions of nitrate, nitrite, and ammonium.

The classic study of Valiela and Teal (1979) demonstrated that the bulk of N in a salt marsh was in the form of DON. Traditionally, it was widely believed that salt marsh plants used only the inorganic form of N, specifically ammonium. Availability of ammonium is directly dependent upon microorganisms and the rate-limiting step of mineralization of organic matter into inorganic forms. Because DIN was thought to be the major available form of N in salt marshes, ecologists have largely ignored the DON pool in these wetland systems.

In the 1990's, studies in arctic habitats showed that pools of available inorganic N insufficiently explained the observed productivity. It was later discovered that several arctic species preferentially exploit organic pools of N (Chapin et al. 1993). Kielland (1994) showed that up to 80% of an arctic plant's N may come from direct uptake of amino acids from the soil matrix. As a result of these studies, people began investigating the utilization of DON in other systems. Studies in the arctic tundra (Raab et al. 1996, Schimel and Chapin 1996, Lipson et al. 1999, McKane et al. 2002), boreal forests (Nasholm et al. 1998, Falkengren-Grerup et al. 2000), grasslands (Falkengren-Grerup et al. 2000, Streeter et al. 2000, Weigelt et al. 2003), and artic salt marshes (Henry and Jefferies 2002, 2003b, a) have all demonstrated the importance of DON utilization. Additionally, Schimel and Chapin (1996) confirmed that plants compete well with microorganisms for particular amino acids, and also demonstrated that amino acids are an

important source of plant N in arctic systems. As a result of these findings, scientists have revisited the possibility that plants may utilize DON in ecosystems where N mineralization rates are low when coupled with an accumulation of slowly decomposing organic matter. These characteristics described above are similar to those found in temperate salt marshes.

Meyerson et al. (2000) suggested that that the expansion *P. australis* may be facilitated by an ability to utilize DON, however, this hypothesis had yet to be tested. To date, there are no published studies investigating the utilization of DON by plants in temperate tidal marshes. If either *P. australis* or *S. alterniflora* have the ability to assimilate DON directly, this would allow plants to utilize a bioavailable N pool in addition to DIN, and as a result, circumvent the traditionally understood nitrogen cycle in tidal marshes.

Tidal marsh habitats are often characterized as systems with an accumulation of organic matter, which may result in pools of bioavailable DON, or dissolved free amino acids (DFAA). In Chesapeake Bay, DFAA concentrations range from 1 - 300 μM, with the highest values found just below the surface of the sediment (Burdige and Martens 1990). Additionally, concentrations of DON in Fjords can reach almost 3 mM, and up to 82% of the DON is in the bioavailable form of DFAA, dissolved combined amino acids, and urea (Guldberg et al. 2002). Unfortunately, few published values of DFAA concentrations in salt marsh soils exist, and there are no reported values exist of DFAA concentrations in *P. australis* marshes.

Gardner and Hanson (1979) reported that in *S. alterniflora* dominated Georgia salt marshes, concentrations of DFAA ranged from 8.85 μM in short form *S. alterniflora* to

0.372 μM in tall form *S. alterniflora* marshes in the 10 – 13 cm depth range. Hydrolysable amino acids ranged from 151 μmols g^{-1} dry weight sediment to 35.3 μmols g^{-1} dry weight sediment, with concentrations decreasing with depth.

Glycine is the most commonly studied bioavailable amino acid because it is thought to be poor substrate for microorganisms; and due to plants high affinity for this amino acid (Lipson et al. 1999). Glutamic acid, on the other hand, is the most dominant amino acid observed in temperate salt mashes (Gardner and Hanson 1979), and has also shown to be an important N substrate for the arctic salt marsh grass, *Puccinellia phryganodes* (Henry and Jefferies 2003a). Other significantly bioavailable amino acids that exist in salt marshes are alanine, serine, aspartic acid (Gardner and Hanson 1979).

In addition to amino acids, urea may be another source of DON available to marsh macrophytes. Prior research has demonstrated urea assimilation in higher plants and algae; therefore it is possible that the macrophytes *S. alterniflora* and *P. australis* may have the ability to assimilate this form of N as well (in Miettinen 1959, Anti et al. 2001, Tyler et al. 2001). Although the urea molecule can be relatively short lived in soils, these tidal environments are being flushed diurnally with concentrations of urea up to 2 μM (Tyler et al. 2003). Although the urea molecule can be easily hydrolyzed by the urease enzyme, Wilson et al. (1999) demonstrated lower urea hydrolysis activity in inter-tidal arctic salt marshes, and concluded that increasing salinity reduced rates of urea hydrolysis. Currently, no published values exist for urea concentrations in the temperate marsh soil matrix.

Fungal root symbiots, such as arbuscular mycorrhizal fungi (AMF) could potentially increase rates of DON assimilation. Since plants colonized by AMF have an

advantage in accessing pools of N and phosphorus (P), which may otherwise be unavailable to them, the status of colonization is important. *P. australis* has been known to be colonized by AMF; however, reported values of AMF colonization differ greatly. For example, Cooke and Lefor (1998) reported root colonization levels in Connecticut from 0 to 80%. Burke et al. (2002) reported that *P. australis* colonization was very low (< 3%) in a New York salt marsh , and only occurred during reproduction. In polluted soils in Portugal, Oliveira et al (2001) reported AMF colonization throughout the growing season, with peaks in spring and fall (< 5%). Therefore, we may assume that *P. australis* has the potential to be colonized by AMF at low levels. *S. alterniflora*, on the other hand, is not known to be colonized by AMF, and is generally thought to be immune from colonization (Hoefnagels et al. 1993). Since it is possible for *P. australis* to be colonized by AMF, this symbiotic colonization of AMF may give *P. australis* a competitive edge over *S. alterniflora*.

The conceptual diagram, Figure 1, illustrates the availability of the DON pool. Additionally, this figure demonstrates how plants may bypass the rate-limiting step of microbial mineralization, by tapping into this organic source of N. Not included in this diagram are the inputs of DON from tidal and atmospheric sources. These are important inputs that must be taken into consideration in future studies, since (Valiela and Teal 1979) demonstrated that the bulk of N flowing through a salt marsh is in the form of DON. This study is the first to investigate the utilization of DON by macrophytes in the temperate tidal marsh environment.

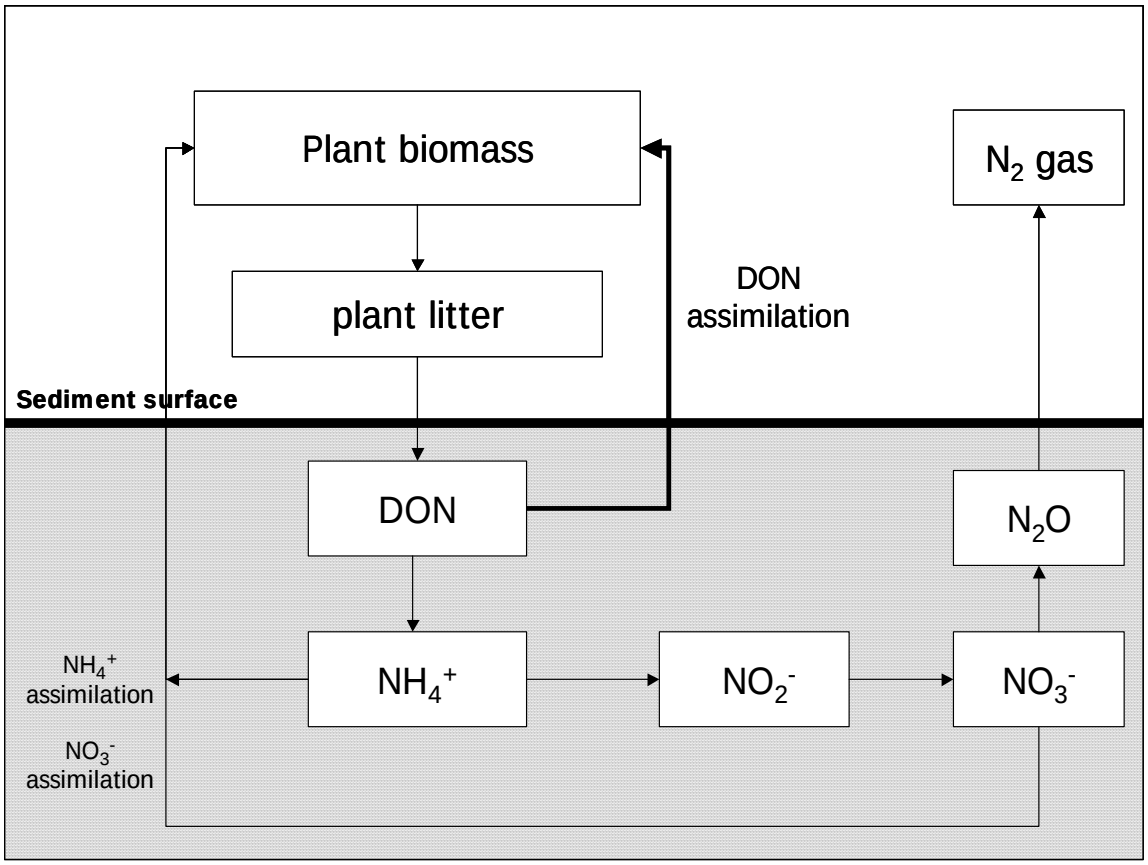

Figure 1. Conceptual diagram for the potential of plant DON utilization

2.2 OBJECTIVES AND HYPOTHESES

The objective of this study is to determine if the macrophytes *P. australis* and *S. alterniflora* have the ability to directly assimilate DON. My hypotheses are:

1. The temperate marsh plants *S. alterniflora* and *P. australis* have the ability to utilize DON, and as a result, can exploit previously neglected pools of bioavailable nitrogen.
2. The utilization of DON by *P. australis*, may be a competitive advantage facilitating the expansion of *P. australis* in N. America.

2.3 METHODS

2.3.1 Laboratory Experiment

Emergent shoots and rhizome fragments of non-native *P. australis* (type M) and *S. alterniflora* were collected from a mainland marsh, adjacent to the Cobb Island Station, Oyster, VA (37.2899013º N, 75.920079º W). Native *P. australis* (type F) was harvested from a privately owned marsh on Occupacia Creek, a tributary to Rappahannock River, near Champlain, VA (38.066944º N, 76.951389º W). Plants were collected from these locations because these populations of *P. australis* were previously genetically identified by Kristin Saltonstall (2002). Shoots and rhizome fragments were harvested in April 2004 and transported to the Biology Department greenhouse at the University of Virginia, where they were washed clean of organic matter and dead root material. Individual shoots were potted in 10 cm diameter pots in clean sand, in order to reduce mycorrhizal growth, and allow for an easy transfer into a hydroponic media (Falkengren-Grerup et al. 2000).

Twice weekly, plants were fertilized with a mixed medium of 300 ppm solution of Peters 20-20-20 (N:P:K) amended with 100 ppm glycine and glutamic acid (Sigma). Plant soils were kept at field conditions through the use of an electronic moisture control system. A mixture of inorganic and organic nitrogen fertilizers was used due to possible effects of N assimilation and due to the fact that these plants are exposed to both DON and DIN in the field. Henry and Jefferies (2003a) demonstrated that plants fed solely a medium of dissolved inorganic nitrogen (DIN) up regulated the rate of DIN uptake while down regulating DON uptake. Additionally, plants grown on a combination of DIN and

DON sources, and not starved, resulted in amino acid uptake exceeding DIN uptake (Henry and Jefferies 2003a).

Within 6 weeks, individual plants achieved a suitable root mass and a height of 60 – 100 cm. To minimize effects of diurnal fluctuations, experiments were conducted at approximately the same time in the day. Plants randomly selected for experimentation were not fertilized for approximately 1 week to deplete local excess pools of available nitrogen within the sand matrix.

Assimilation experiments were adapted from the methods of Henry and Jefferies (2003a), the main modification included the use of whole plants instead of excised roots, because treatment responses can vary greatly in excised root experiments versus those using whole plants (Falkengren-Grerup et al. 2000). Uptake rates of DON and DIN were calculated in plants exposed to 100 μM treatments, pH 6.5, for 45 minutes using uniformly, dual-labelled, ^{15}N, ^{13}C, glycine, glutamic acid, and urea (Cambridge Isotope Laboratories) and ^{15}N labelled NH_4Cl (Cambridge Isotope Laboratories). A control treatment (no nutrients) was run in order to assess natural isotopic abundances.

Intact individual plants were rinsed free of sand using tap water, then equilibrated for at least 30 minutes in a solution of 0.50 mM $CaCl_2$ at the experimental conditions of: temperature 20ºC, PAR 400 μmols s^{-1} m^{-2}, in a CONVIRON environmental chamber to eliminate any variability in experimental conditions. After equilibration, plants were transferred to a continuously aerated, well mixed 100μM media, with a volume of 500 ml for 45 min. Control treatment plants were exposed to 0.5mM $CaCl_2$ only. Preliminary uptake studies using depletion over time demonstrated a linear uptake of NH_4^+ for the initial 90 minutes. Therefore, 45 minutes should be an adequate time frame to calculate

uptake rates since it falls within this linear relationship without underestimating the rate. After 45 minutes, the roots were rinsed for 2 minutes with 1 mM KCl l^{-1} to remove any possible excess labeled substrate (Epstein et al. 1963). Whole root systems (excluding rhizomes) were then carefully blotted dry and excised. Excised roots were dried to a constant weight at 65 ºC, weighed, and ground to a fine powder using a mortar and pestle. Root samples were analyzed at the UC Davis Stable Isotope Facility for stable isotope concentrations using an Europa Integra continuous flow mass spectrometer.

Assimilation of ^{15}N and ^{13}C was calculated using a modified equation of Hauck and Bremner (1976).

$$\mu g \text{ enriched} = [m (APE_{\text{treated plants}} - APE_{\text{control plants}})] / (APE_{\text{treatment}})$$

m = mass in μg of N or C within the sample

APE = atomic percent excess = [(Atomic % ^{15}N or ^{13}C) – (natural abundance of ^{15}N or ^{13}C)]

This calculation is the difference in the atomic percent excess of the treated plants minus the atomic percent excess of the control plants, and also takes into account the composition of the tracer. The amount (μg) of ^{15}N and ^{13}C assimilated was then converted into mols of ^{13}C and ^{15}N. Assimilation rates were determined by dividing the amount assimilated by the root sample mass and time of exposure to the labelled media.

Fifteen plants of each type were sacrificed to determine if plants were colonized by AMF, using a modification of Kormanik and McGraw (1982) as described by Burke et al (2002). Entire plant root systems were preserved in formalin-aceto-alcohol. Plant roots were cleared at room temperature in 10% KOH for 6 hours, followed by bleaching of the roots in alkaline H_2O_2 for 20 minutes. Roots were then acidified in 1% HCl, and stained in 0.01% trypan blue-lactic acid staining solution for 6 hours, and destained overnight. Other comparable studies investigating AMF colonization in temperate tidal marsh plants have also used this trypan blue staining method (Cooke et al. 1993, Hoefnagels et al. 1993, Cooke and Lefor 1998, Burke et al. 2002, 2003).

2.3.2 Field Experiment

To my knowledge, no experiments investigating DON assimilation in temperate marsh plants have been conducted in tidal marshes. The effects of diurnal tides make the task of localizing a stable isotope tracer difficult without major changes in hydrology and sulfide concentrations. For this experiment, a 8.85-cm internal diameter, 30-cm length core was inserted to a depth of approximately 30 cm and left in the sediment to accomplish two goals: (1) to eliminate clonal integration, thus isolating the plant/plants in a 61.5 cm^2 area, and (2) to limit the leakage of labeled substrate. The walls of the core reduced horizontal movement of the labeled media, while allowing for vertical drainage and prevented H_2S buildup.

The field experiment was conducted on a mainland salt marsh on the Virginia National Wildlife Refuge at the southern terminus of the Delmarva Peninsula (37.12895º

N, 75.94789º W). This experiment consisted of three treatments: control, $^{15}NH_4Cl$ (Cambridge Isotope Laboratories), and a combined amino acid treatment of glycine and glutamic acid uniformly labelled with ^{15}N, ^{13}C (Cambridge Isotope Laboratories). Sample cores including plant were amended at approximately double reported pore water concentrations. The pore water volume of the core was determined to be 1.55 liters using the porosity of marsh soils (~0.85). The cores were amending with 25 μmols of combined amino acids (1:1; ^{15}N, ^{13}C glycine : ^{15}N, ^{13}C glutamic acid) and 65 μmols of $^{15}NH_4Cl$ (approximately double reported pore water concentrations (Gardner and Hanson 1979)) uniformly throughout the core with the use of a probe (Berg and McGlathery 2001). The cores were inserted on August 2, 2004, and the plant + core remained intact for five days. After five days, the entire plant + core was carefully removed for subsequent analysis.

The entire core was rinsed with 0.5 mM $CaCl_2$, as were plant roots in the laboratory experiment; and live, turgid, new root material was separated carefully from dead root material, rhizomes, and detritus. The determination of live root material was very conservative, and only a sub sample of the belowground portion that was easily identified as live. This live root tissue was dried to a constant weight at 60ºC, followed by grinding to a fine powder using a mortal and pestle. Root tissues were analyzed for isotopic concentrations at the University of California Davis Stable Isotope Facility in the same manner as those in the laboratory experiment.

Assimilation calculations were the same as described in section 2.3.1, with the following exceptions: calculations were determined only on the sub-sample of root

material determined to be live and turgid. This was done to be conservative in the determination of assimilation.

2.4 DATA ANALYSIS

All statistical analyses were performed in SAS system for Windows V9.1 (2004; SAS Institute Inc.). The experimental design of the laboratory experiment was factorial (3 plant types x 4 nutrient treatments). Post hoc Tukey tests were used to determine the significant differences demonstrated by analysis of variance (ANOVA) results within treatments in laboratory assimilation experiments. Field experiments were also factorial (2 plant types x 2 nutrient treatments).

2.5 RESULTS

2.5.1 Laboratory Experiment Results

^{15}N assimilation was observed in all nutrient treatments and in all plant types (ANOVA plant effect: df = 2, n = 180, F = 16.92, P < 0.001; ANOVA treatment effect: df = 3, n = 180, F = 119.0, P<0.001; ANOVA plant x treatment effect: df = 6, F = 11.88, P<0.001). Ammonium treated plants had the greatest observed N assimilation rates followed by urea, glycine, and glutamic acid (Figure 2). There was significant assimilation of ^{15}N in all amino acid treated plants; however, no significant differences were observed in N uptake among all plant types (P > 0.05, ANOVA). Glycine treated plants had the greatest N assimilation rates among amino acid treatments at 3.8 μmols $^{15}N\ g^{-1}dw\ hr^{-1}$ for both *P. australis* M and *S. alterniflora*, and 2.9 μmols $^{15}N\ g^{-1}dw\ hr^{-1}$ for

P. australis F, with no significant differences observed among plant types. These rates of glycine-N uptake are up to ~20% of NH_4^+ assimilation rates. Glutamic acid-N assimilation rates were lower, but not significantly different ($P > 0.05$, ANOVA) than glycine-N treated plants and ranged from 1.5 μmols $^{15}N\ g^{-1}dw\ hr^{-1}$ for *P. australis* M plants to 1.1 μmols $^{15}N\ g^{-1}dw\ hr^{-1}$ in *P. australis* F plants, and were up to ~8% of NH_4^+ assimilation rates.

Urea treated plants had significantly higher N assimilation rates than those treated with amino acids in *P. australis* plants. Additionally, both *P. australis* haplotypes exhibited similar urea-N assimilation rates of 14.4 μmols $^{15}N\ g^{-1}dw\ hr^{-1}$, however *S. alterniflora* assimilation rates (2.4 μmols $^{15}N\ g^{-1}dw\ hr^{-1}$) were significantly lower than those of *P. australis*, and similar to amino acid assimilation rates (Figure 2). Interestingly, the urea-N assimilation rate of the non-native, type M *P. australis* was not statistically different than the *P. australis* type M NH_4^+-N assimilation rates (Figure 2).

Calculated NH_4^+-N assimilation rates are similar to those demonstrated in previous studies (Chambers et al. 1998, Mozdzer unpublished data). Since NH_4^+-N was our reference treatment, assimilation rates obtained by this experiment are comparable to previous studies that have used depletion over time as a proxy for nutrient uptake. No significant differences were observed between *P. australis* M and *S. alterniflora* plants (18.9 and 20.4 μmols $^{15}N\ g^{-1}dw\ hr^{-1}$, respectively). Type F *P. australis* had the greatest N assimilation rates at 38.0 μmols $^{15}N\ g^{-1}dw\ hr^{-1}$(Figure 2).

Although the purpose of this experiment was to determine N assimilation, ^{13}C assimilation was also calculated to determine if the organic molecules were taken up intact. I found that glycine treated plants assimilated ^{13}C at the greatest rate, and rates

ranged from 5.5 to 3.6 μmols ^{13}C g^{-1}dw hr^{-1} in *S. alterniflora* and *P. australis* F plants respectively, with no significant differences observed between plant types (Figure 3). The glycine used in this experiment had two labeled ^{13}C atoms to each labelled ^{15}N atom. *S. alterniflora* plants had a 1.48:1 ratio of ^{13}C:^{15}N , followed be 1.25:1 for *P. australis* M, and 1.17:1 for *P. australis* F plants (Figure 4). Thus, we can say with confidence that at least 60%, and up to 74% glycine molecule was taken up intact based on the ^{13}C:^{15}N ratios.

Glutamic acid treated plants had significantly lower ^{13}C assimilation rates within all plant types than glycine treated plants, with no significant differences within the glutamic acid treatment ($P>0.05$). All plant types assimilated ^{13}C, and rates varied from 2.4 to 1.9 μmols ^{13}C g^{-1}dw hr^{-1} in *S. alterniflora* and *P. australis* F plants respectively. The theoretical ratio for the ^{13}C:^{15}N in the glycine molecule used in this experiment was 5:1. Observed ^{13}C:^{15}N ratios varied from 1.99:1 in *S. alterniflora*, to 3.01:1 in *P. australis* F, which are higher than the ratios observed in glycine treated plants. Although these ratios are higher, due to the amount of ^{13}C in the glutamic acid molecule, this indicates less of the intact molecule, at least 40%, and up to 60% was taken up whole. Urea treated plants did not show any assimilation of ^{13}C (Figure 3).

No significant effect of root mass was observed within treatment (ANOVA n=180, df = 3, F=0.81, P = 0.4887) or the interaction effect of plant x treatment (ANOVA n=180, df = 6, F = 0.89, P = 0.5021). A significant plant effect was observed between plant types (ANOVA, n=180, df = 2, F = 55.43, $P < 0.0001$), with *P. australis* M root masses being significantly greater than both *P. australis* F and *S. alterniflora* root mass (Figure 5).

Root structure varied considerably between plant types in both mass and morphology. *P. australis* M plants have the greatest root masses among all plant types (Figure 6), and consisted of a combination of fine feeder roots and larger root structures. *P. australis* F root masses were significantly smaller by weight than *P. australis* M plants, and morphologically consisted of mainly fine feeder roots. Whereas *S. alterniflora* roots systems were similar in mass to *P. australis* F plants, but were morphologically more similar to *P. australis* M plants.

Total aboveground plant biomass did not differ between *P. australis* haplotypes, but was significantly greater than *S. alterniflora* aboveground biomass (Figure 7). However, when the aboveground portion of plants was separated into the two main components of stems and leaves in *P. australis* haplotypes, type M *P. australis* plant leaves had significantly more mass (ANOVA n = 90, df 1, F = 5.67, P= 0.0188) than type F (Figure 8). Although differences were observed in leaf mass, no significant differences were observed between haplotypes in regards to stem mass (ANOVA n=90, df 1, F = 2.71, P = 0.1027).

AMF were not found on any plant roots from the laboratory experiment.

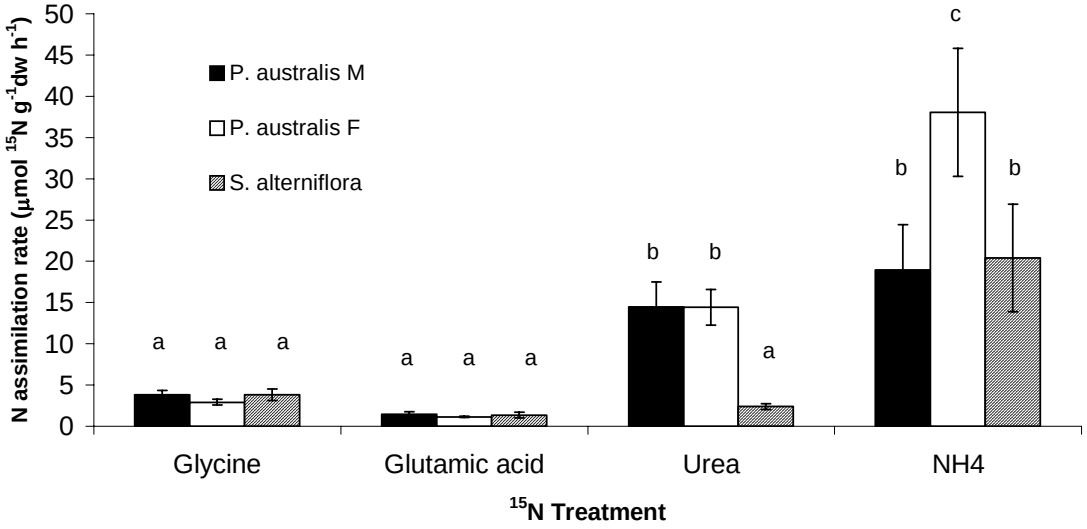

Figure 2. Laboratory ^{15}N assimilation rates

N assimilation rate presented in µmols ^{15}N gram^{-1} root dry weight hr^{-1}, n = 15 individual plants per treatment, total n = 180 plants. Error bars represent ± 2 SE. Significant differences ($P < 0.05$) are represented by different letters.

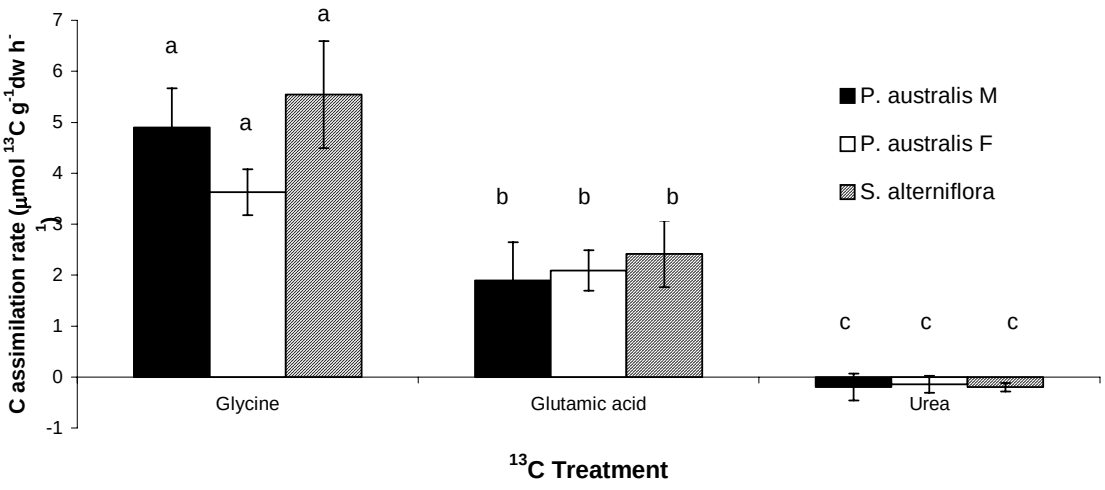

Figure 3. Laboratory ^{13}C assimilation rates

N assimilation rate presented in µmols ^{13}C gram^{-1} root dry weight hr^{-1}, n = 15 individual plants per treatment, total n = 180 plants. Error bars represent ± 2 SE. Significant differences ($P < 0.05$) are represented by different letters.

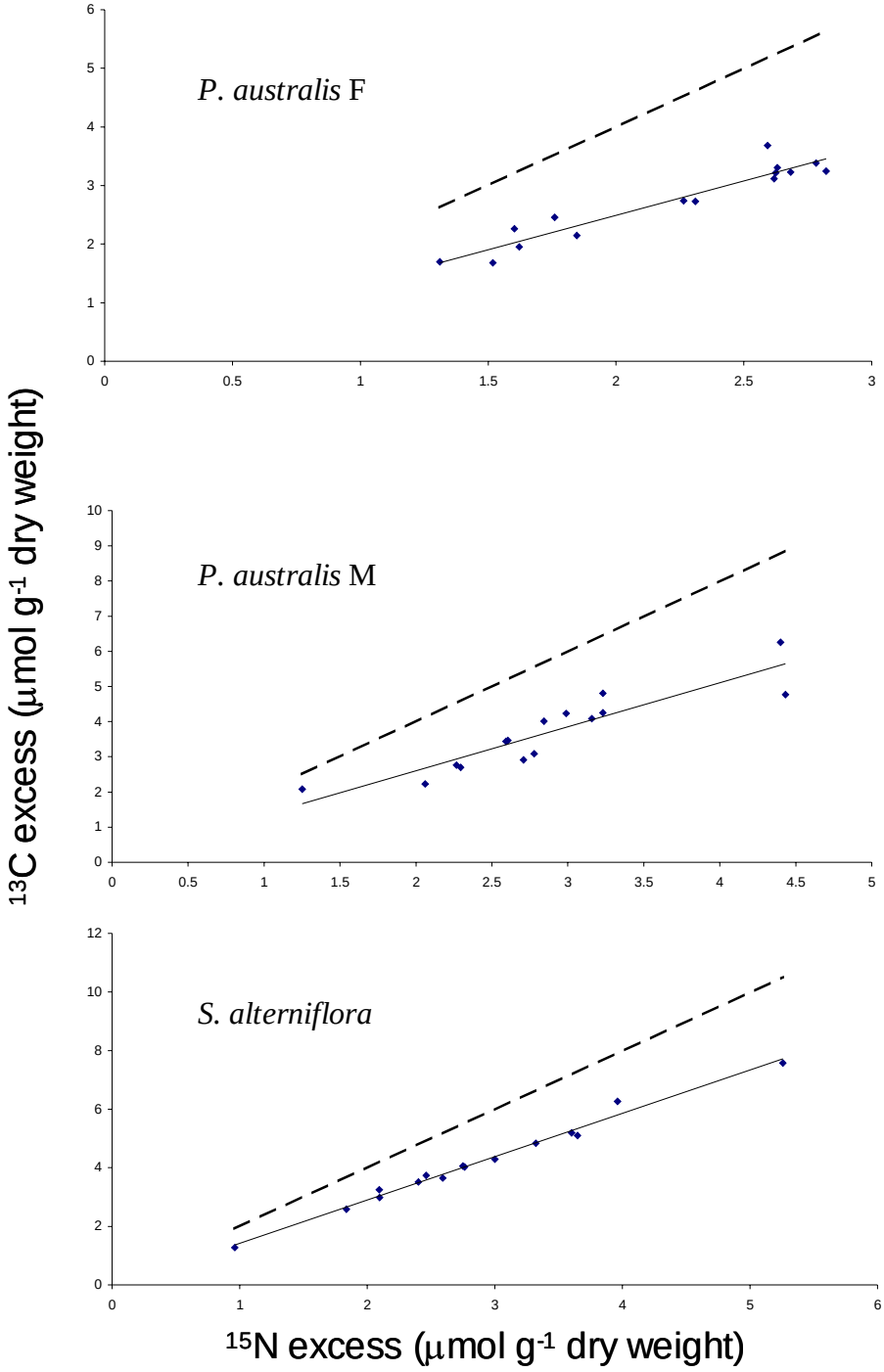

Figure 4. Relationship between ^{13}C excess and ^{15}N excess in glycine treated plats. Regression lines are the assimilated ratios of ^{13}C:^{15}N for each plant type, n = 15 individual plants: *P. australis* F (slope = 1.17, r^2 = 0.909); *P. australis* M (slope = 1.25, r^2 = 0.828); *S. alterniflora* (slope = 1.48, r^2 = 0.843). Broken lines indicate 2:1 glycine ratio.

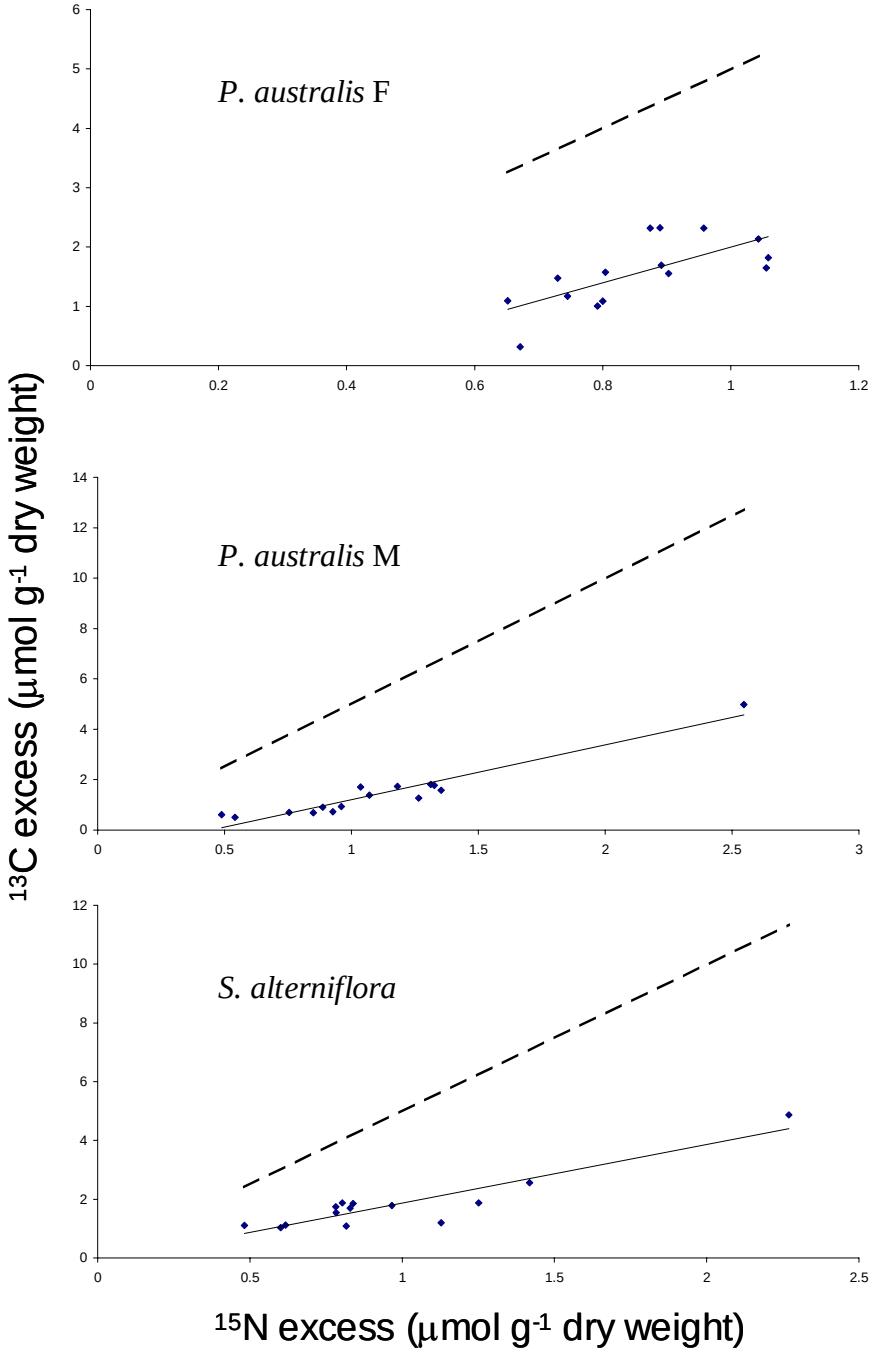

Figure 5. Relationship between ^{13}C excess and ^{15}N excess in glutamic acid treated plats. Regression lines are the assimilated ratios of ^{13}C:^{15}N for each plant type, n = 15 individual plants: *P. australis* F (slope = 3.01, r^2 = 0.482); *P. australis* M (slope = 2.17, r^2 = 0.919); *S. alterniflora* (slope = 1.99, r^2 = 0.843). Broken lines indicated 5:1 glutamic acid ratio.

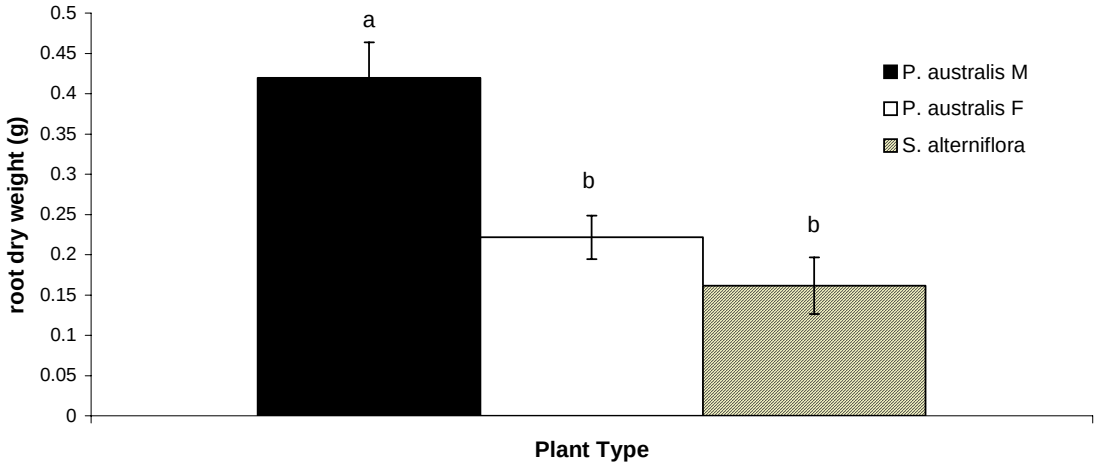

Figure 6. Plant root mass from laboratory experiment

Mean plant root mass, n= 60 per plant type, error bars represent ± 2 SE in greenhouse grown plants from nutrient uptake experiment. Significant differences ($P < 0.001$) are represented by different letters.

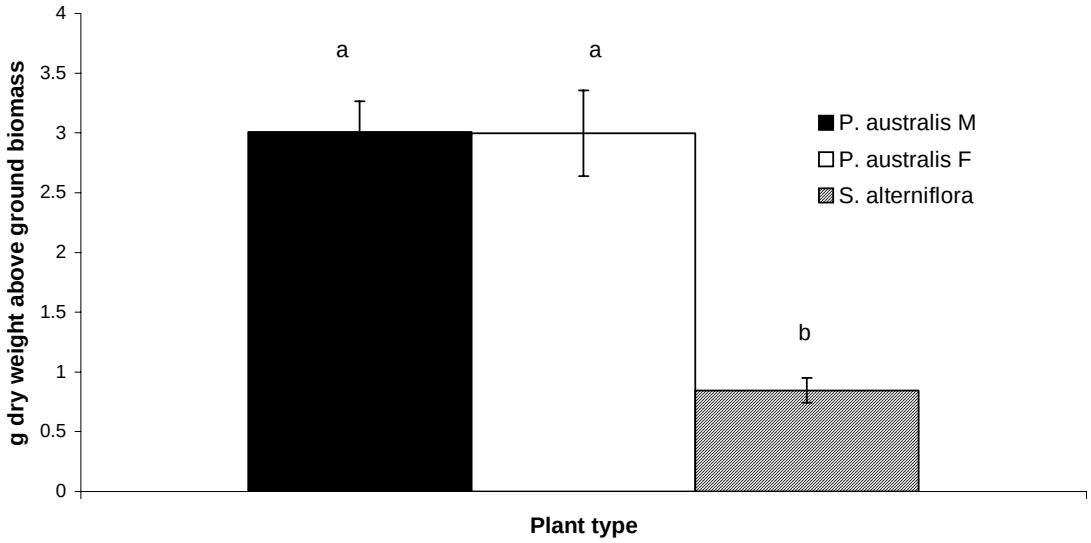

Figure 7. Above ground plant mass.

Mean plant above ground mass, n= 60 per plant type, error bars indicate ± 2 SE from greenhouse grown plants in nutrient uptake experiment. Significant differences ($P < 0.05$) are represented by different letters.

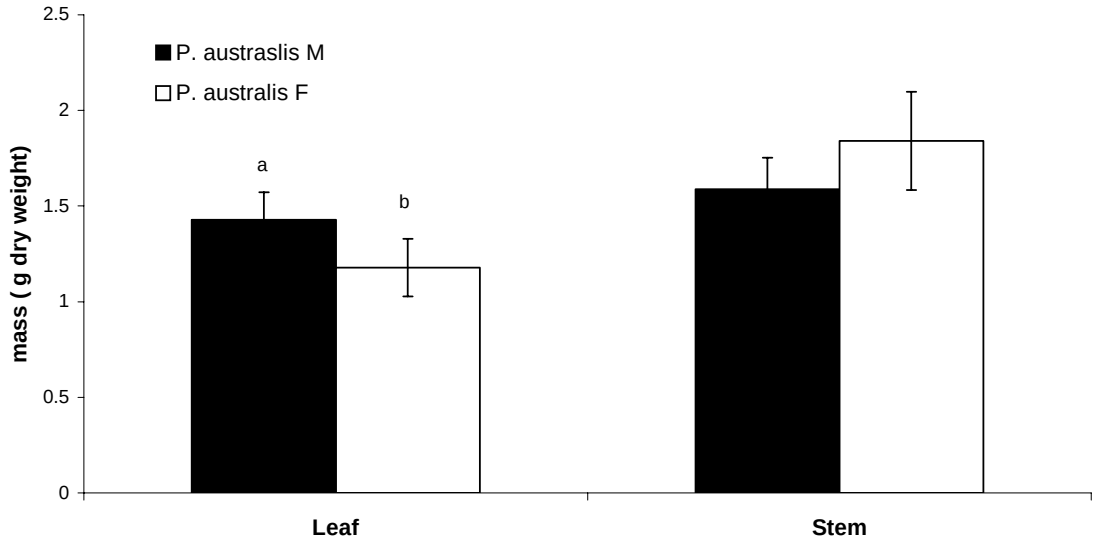

Figure 8. Above ground *P. australis* mass separated between stem and leaf portions Mean mass, n= 60 per plant type, ± 2 SE from greenhouse grown plants from nutrient uptake experiment. Significant differences between leaf mass (P = < 0.05) are represented by different letters. No significant differences were observed between stem mass in *P. australis* haplotypes (P=0.1279).

2.5.2 Field experiment

All N treated plants assimilated ^{15}N after the five-day exposure to nutrient treatments. *P. australis* M NH_4^+-N assimilation rates were calculated to be 20.5 ± 0.1 μmols ^{15}N g^{-1}dw hr^{-1}, which was significantly greater than the calculated *S. alterniflora* assimilation rate of 4.3 ± 0.7 μmols ^{15}N g^{-1}dw hr^{-1} (ANOVA df = 1, F =401.46, P < 0.0001) (Figure 9) Amino acid assimilation rates were significantly greater in *P. australis* plants, with calculated N assimilation rates of 4.0 ± 0.3 μmols ^{15}N g^{-1}dw hr^{-1} (Figure 9). *S. alterniflora* plants also assimilated ^{15}N, but at a significantly lower rate of 1.3 ± 0.6 μmols ^{15}N g^{-1}dw hr^{-1} (ANOVA df = 1, F = 18.88, P < 0.012). Interestingly, P. *australis* ^{15}N assimilation rates of NH_4^+-N and amino acid nitrogen were similar in both laboratory and field replicates (Figures 2 and 9). ^{13}C assimilation was not detectable in *S. alterniflora* plants, and assimilation of ^{13}C was determined to be 1.7 ± 0.3 μmols ^{13}C g^{-1}dw hr^{-1} in *P. australis* M plants. Since this treatment was a mix of both glycine and glutamic acid amino acids, the expected ^{13}C:^{15}N ratio is 3.5:1. As a result, at least 22% of the ^{15}N assimilated came from intact amino acids. Such a measurement cannot be determined in *S. alterniflora* plants since ^{13}C assimilation was not significantly different from the control plants.

Although *S. alterniflora* had lower assimilation of NH_4^+-^{15}N in plant roots, ^{15}N assimilation rates to aboveground plant tissue were significantly greater than in *P. australis* M (ANOVA df = 1, F = 206.08, P < 0.0001) (Figure 10). Additionally, S. *alterniflora* plants had almost double the ^{15}N assimilation rates to aboveground tissue in amino acid treated plants; however, these differences were not significant (ANOVA df =

1, F = 1.37, P >0.3073) (Figure 10). This discrepancy may be attributed to low sample size was low and high variability. These findings suggest that *S. alterniflora* plants translocate assimilated NH_4^+ to aboveground tissue more quickly than *P. australis* plants, whereas *P. australis* seems to be keeping the N belowground.

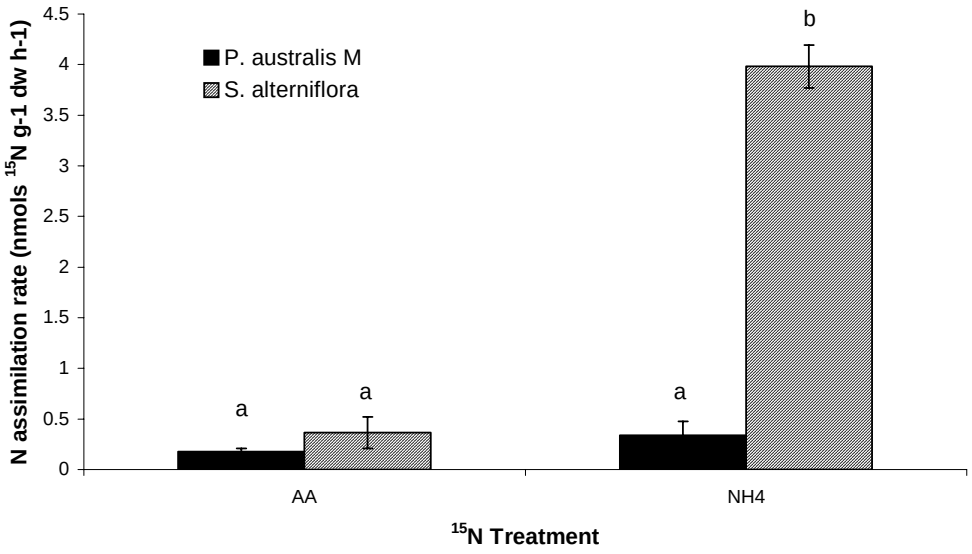

Figure 9. Calculated *in situ* ^{15}N root assimilation rates.

Mean N assimilation rate ± 2 SE in plant roots calculated from field experiment, n = 3 cores per plant treatment. Significant differences ($P < 0.05$) are represented by different letters.

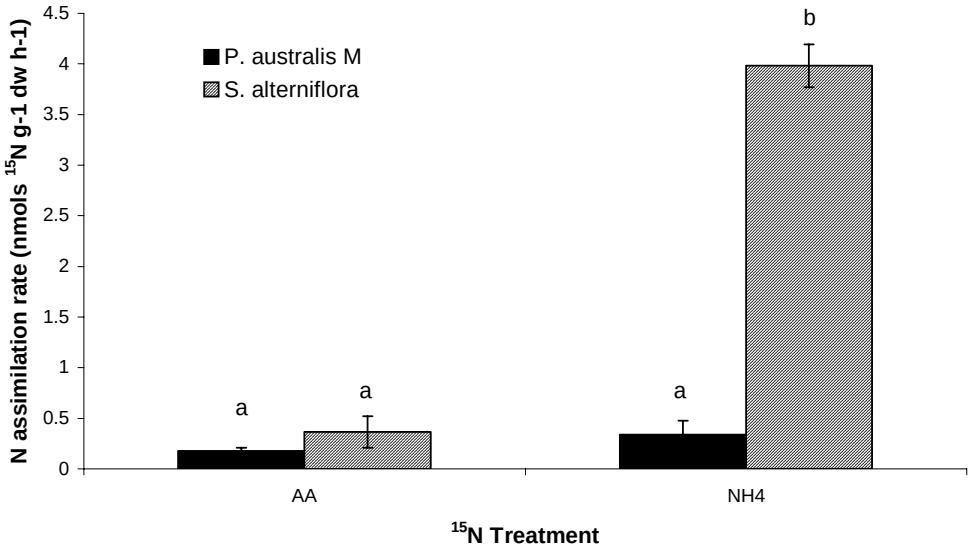

Figure 10. Calculated *in situ* ^{15}N above ground assimilation rates

Mean N assimilation rate ± 1 SE into aboveground biomass calculated from field experiment, n = 3 cores per plant treatment. Significant differences ($P < 0.05$) are represented by different letters.

2.6 DISCUSSION

2.6.1 Laboratory experiment

Amino acid treated plants assimilated both ^{13}C and ^{15}N in dual labelled amino acid treatments, demonstrating that temperate tidal marsh plants do utilize DON in laboratory experiments, and take up a portion of the DON as whole molecules (Figures 2 and 3). Assimilation ratios of the labelled organic amino acid glycine were closest to theoretical assimilation ratios of 2:1 in glycine treated plants, and this suggests that up to 74% of the glycine assimilated was intact. Glutamic acid assimilation ratios were higher, up to 3.01:1, but due the greater numbers of labelled C in the glutamic acid molecule, up to 60% of the N assimilation was intact.

Incomplete recovery of ^{13}C can be related to physiological processes after the assimilation of the molecule. Once the amino acids were assimilated into the root, many things could happen physiologically. Schmidt and Stewart (1999) reported that glycine is metabolized quickly within roots into serine and other amino compounds through the use of an aminotransferase. This metabolism of glycine is mainly done via serine synthesis from which 2 mols of glycine yield 1 mol of serine + 1 mole of NH_3 + 1 mol of CO_2 (Oliver 1994, Nasholm et al. 1998). Since plant roots are not photosynthetic, the CO_2 from the metabolism of glycine is most likely respired, resulting in the lower levels of ^{13}C.

Due do the rapid metabolism of the glycine amino acid in plant roots, our estimates of 60 to 74% of intact glycine molecules are very conservative. Previous studies that have also demonstrated the intact assimilation of amino acids also reported

similar results with regards to ^{13}C uptake (Nasholm et al. 1998, Streeter et al. 2000, Nasholm et al. 2001, Henry and Jefferies 2002, 2003b, a, Weigelt et al. 2003).

Glutamic acid is a large molecule when to compared to glycine (5-C vs. 2-C). No studies, to my knowledge, have been published regarding glutamic acid metabolism in plant roots. The lower assimilation ratio of 3:1 observed instead of the theoretical 5:1 in the labelled glutamic acid may be explained by the same reasons described above. It is likely that an aminotransferase, as used in glycine metabolism, can be used to harvest the amino group for N metabolism. Although these ratios only suggest up to 60% intact molecules of glutamic acid were assimilated, I believe that due to the large size of this molecule, these results are extremely conservative, and intact assimilation can be much higher than reported.

Additionally, incomplete recovery of ^{13}C may also be related to the background concentrations of the ^{13}C isotope. Plant roots alone are approximately 40% C by weight, and over 1% of this is ^{13}C; therefore the enrichment signal could be masked by this high background of ^{13}C. In contrast, background levels of ^{15}N are relatively small (2% N by weight, and natural abundance of ^{15}N in plants is less than 0.36%), as a result, the ^{15}N signal is much more sensitive to changes in enrichment.

Urea treated plants yielded much different results than amino acid treated plants. Both haplotypes of *P. australis* had significantly greater urea-N assimilation rates than *S. alterniflora,* and urea-N assimilation rates did not differ significantly from NH_4^+ treated *P. australis* M plants (Figure 2). However, since ^{13}C was not assimilated in urea treated plants, the assimilation of this molecule may not be complete. Previous studies investigating urea assimilation in rice plants did not use ^{13}C labelled urea (Anti et al.

2001), since they assumed that any assimilation in short term experiments would only include the intact organic molecule.

Since ^{13}C assimilation was not detected in urea treated plants, three main possibilities exist to explain this phenomenon. 1. The urea molecule may have been assimilated intact, and subsequently, the amino groups were hydrolyzed within the cells, and the resultant CO_2 was released from the plant roots. 2. Free living bacteria living on the roots of the plants may have hydrolyzed the urea molecule, thus leaving the NH_4^+ readily available for plant uptake. 3. The plants themselves may have excreted an urease like enzyme to break down the urea molecule into the inorganic form.

Testing the hydroponic solution after experimentation for changes in pH, as well as testing for the presence of both urea and NH_4^+ would allow one to understand the mechanisms of urea-N assimilation. Furthermore, the addition of a urease inhibitor to the hydroponic solution may prevent the hydrolysis of the molecule by external enzymes, and any assimilation could be then regarding as plant mediated.

Free-living bacteria on plant roots may also explain the lack of ^{13}C assimilation if they hydrolyzed the urea molecule within the hydroponic solution. AMF did not mediate any of these processes since their presence was not detected within the plants assayed. Bacteria use extra cellular enzymes, such as urease, to break down organic molecules, thus making NH_4^+ and CO_2 readily available to both plants and root surface bacteria. If one assumes that plant bacterial associations are responsible for urea assimilation in these plants, this may suggest that *P. australis* plants support different bacterial populations than *S. alterniflora.* Since the two haplotypes of *P. australis* were collected from two very different locations, this may suggest that these bacteria are not location specific, but

plant specific. Evidence of plant specific microbial populations is limited, but Burke et al. (2002) demonstrated different microbial populations between *P. australis* and *S. patens* communities in a New York salt marsh. Finally, the lack of ^{13}C observed in urea treated plants may be explained by the fact higher plants can utilize urease enzymes, which may give them a competitive advantage to access the urea molecule(Hogan et al. 1983).

Regardless of the method used to assimilate urea-N, the results of this study indicate that *P. australis* plants have a distinct advantage in accessing pools of urea-N over *S. alterniflora plants.* Whether this process is important in the field is still to be determined. Additionally, further studies are needed to elucidate the mechanisms involved in plant urea assimilation.

2.6.2 Field Experiment

The assimilation rates of amino acid discussed previously were based on laboratory uptake experiments under controlled conditions of no salinity, aerobic rhizosphere conditions, and no competition. The purpose of the laboratory experiment was to determine if the mechanism to assimilate DON existed in these plants. Since field conditions vary significantly compared to those of the laboratory experiment, my field experiment was set up to validate the laboratory result under *in situ* conditions.

The results of the field study verified that *P. australis* plants utilized ^{15}N from amino acid treated cores at similar rates to those observed in the laboratory experiment (Figures 2 & 9). *S. alterniflora* amino acid ^{15}N assimilation rates were significantly

lower, however, these results may be confounded by the more frequent flooding and flushing of these plants based on their location in the lower marsh, relative to *P. australis* plants that were located at a higher marsh elevation. Since *S. alterniflora* plants were at a lower marsh elevation, it is possible the enrichment media had a shorter residence time, and was flushed out from the core at higher rates than *P. australis* cores. Due to confounding factors of naturally occurring gradients in this marsh, it was impossible to treat all plants in the same way.

Additionally, since ^{13}C assimilation was not detected in *S. alterniflora* plants, the assimilation of the ^{15}N may not be from the amino acid molecules, but from microbial processes. Therefore, more studies are needed to validate these results. However, one must also consider that the ^{13}C may have been respired during the duration of the field experiment.

P. australis plants, on the other hand, did assimilate amino acids intact. Although their assimilation ratios of ^{13}C:^{15}N were low, the ratio accounts for at least 22% assimilation of intact amino acids as an extremely conservative estimate. As described above, once the amino acids are assimilated into plant roots, they are quickly metabolized and the subsequent ^{13}C's may have been respired in the plant roots. Additionally, since the experiment was in progress for 5 days, this provides adequate time for the plant to respire the C from the plant roots.

Although plant assimilation rates of N were significantly lower in plant roots, *S. alterniflora* translocated ^{15}N to above ground biomass approximately 10 times faster per unit mass when compared to *P. australis.* Additionally, up to 2% of the amended ^{15}N tracer was recovered aboveground in the *S. alterniflora* plants, compared to less than

0.08% in *P. australis* plants (Figure 10). Conceptually, it seems as if *P. australis* plants maybe storing more of the assimilated ^{15}N belowground, whereas *S. alterniflora* plants are translocating the nutrients to the aboveground fraction. To my knowledge, no studies are published that compare N metabolism and translocation between these two species. These differences in translocations between these two species may have many implications. Pioneer species usually allocate more resources aboveground, in an attempt to settle into an environment. This trend is would be expected in the *P. australis* plants, since they are expanding into other habitats. *P. australis* was not translocating the assimilated ^{15}N into the aboveground portion of the plant, and was instead keeping this N belowground. On the other hand, these trends may be explained by metabolic differences between plants. More studies are needed to confirm these results, but I would be interested to see how plants differ in allocation of assimilated nutrients.

Additionally, more field replicates are needed to verify the results of this field experiment. Variance was much higher than expected, and the sample size was small. As a result, more studies are needed to determine the importance of DON utilization in the field. Since amino acid metabolism can be quite high, the duration of the future field experiments should be much shorter, and on the order of hours (during a low tide) to days. Five days may have been too long, especially in tidal habitats where the enriched label could have been flushed out within the next high tide. Different methods to better retain the label, while being careful not to make such changes in hydrology or anaerobic conditions, should be investigated.

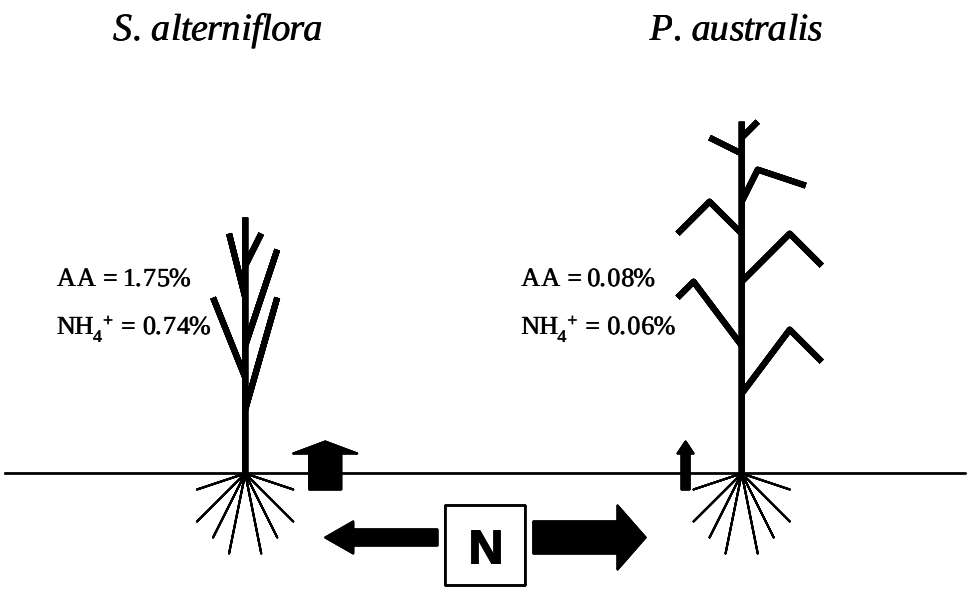

Figure 11. Conceptual flow of ^{15}N observed in field experiment.

Although P. australis assimilation rates were significantly greater in root tissue, assimilation rates to the aboveground portion of the plant were up to ten times greater in S. alterniflora plants per unit mass. Values indicate % of added label incorporated into aboveground plant biomass. AA = amino acid treated plants, NH_4^+ = NH_4^+ treated plants. This suggests different patterns of N allocation between these plants.

Chapter 3 PHOTOSYNTHETIC DIFFERENCES BETWEEN *P. AUSTRALIS* HAPLOTYPES

3.1 INTRODUCTION

The expansion of *P. australis* has been related to the introduction of a non-native genotype that seems to be more competitive than other genotypes (Saltonstall 2002). Therefore, it is likely that physiological differences exist between the native and non-native populations. Although subtle morphological differences are present, the different haplotypes of *P. australis* are very difficult to differentiate in the field. In addition, the ability to identify different haplotypes is compounded when environmental variables, which change plant morphology, are taken into account. To date, there is no published literature that describes the morphological differences between native and non-native *P. australis* haplotypes.

Differences in morphology are based on personal communication with Kristin Saltonstall and Bernd Blossey who have worked extensively in this area. Since no key morphological distinctions are currently known, RFLP (Restriction Fragment Length Polymorphism) analysis (Saltonstall 2003) is the only way to positively identify a population as native or non-native in North America.

In general, native populations of *P. australis* type F on the North American Atlantic coast tend to be more yellow-green in color, whereas, non-native populations tend to be more blue-green (K. Saltonstall *personal communication* and T.J. Mozdzer *personal observation*). These variations in leaf color are indicative of differences in plant

pigment concentrations within the leaves. Since the non-native type M is darker in color, this type may have more pigments than native haplotypes. Leaves of native type F *P. australis* are removed easily from the leaf sheath throughout the year, and most leaves fall off the culm at the end of the growing season. Conversely, the leaves of the non-native haplotype M remain attached throughout winter, and are relatively difficult to remove. Additionally, the leaf sheath remains intact on the plant culm. Fungal stem spots are supposed to be a definitive identification of native *P. australis* populations (B. Blossey *personal communication*), however, these have been observed on genetically identified non-native populations on the VCR property (T.J. Mozdzer *Personal observation*). These populations on the Virginia Coast Reserve were genetically identified as non-native type M by Kristin Saltonstall using RFLP analysis using chloroplast DNA (Saltonstall 2003)

The effects of climate, latitude, salinity, and hydrological inundation on *P australis* physiology have been studied extensively on European populations/ecotypes (Lissner et al. 1997, 1999a, b, Lessmann et al. 2001, Antonielli et al. 2002). However, since the discovery of genetically distinct haplotypes was not determined until recently in the US, no studies have tried to determine if physiological differences exist between *P. australis* haplotypes.

Infrared-gas analyzers (IRGA) have been used effectively to calculate photosynthetic rates in *P. australis* plants (Lissner et al. 1999b, Hester et al. 2001, Lessmann et al. 2001, Pezeshki 2001, Antonielli et al. 2002, Meszaros et al. 2003). In addition to the use of IRGA's, Pulse Amplitude Modulated (PAM) flourometry has been

used to determine photosynthetic stress in *P. australis* (Adams and Bate 1999, Meszaros et al. 2003). PAM flourometry allows for a non-invasive, *in-situ* examination Photo System II rates.

In addition to the physiological parameters, different haplotypes of *P. australis* may have different nutrient requirements. Ratios of elemental C, N, and P can be indicative of plant metabolic processes or nutrient allocation processes. For example, since *P. australis* M is hypothesized to contain more photosynthetic pigments, I would expect these plants to have a higher demand for nitrogen.

If differences in photosynthetic performance exist between native and non-native *P. australis* haplotypes, it may help explain why the non-native form is expanding throughout tidal wetlands in North America. Additionally, these results may also explain the success of the non-native haplotype relative to the native haplotypes.

3.2 OBJECTIVES AND HYPOTHESES

The goal of this research is to determine if physical and/or physiological differences exist between a native haplotype (Type F), and the non-native (Type M), haplotype of *P. australis*. My working hypotheses are:

(1) Photosynthetic pigment concentrations are greater in the non-native haplotype M than in the native haplotype F of *P. australis.*

(2) Photosynthetic rates in the non-native type M *P. australis* are greater due to higher concentrations of photosynthetic pigments

3.3 METHODS

3.3.1 Photosynthetic pigments

Pigment concentrations of *P. australis* leaves were determined by harvesting the third full leaf from the top of each plant immediately following the conclusion of the laboratory experiment (Chapter 2). These leaves were freeze-dried to preserve the tissue for future pigment analysis. A 5 cm length of freeze-dried tissue was pulverized under liquid nitrogen and pigments were extracted in acetone as in Lippert et al (2001). Pigment concentrations were determined using a Ocean Optics USB 200 Mini Spectrophotometer using the equations of Lichtenthaler (1987) with 100% acetone as the extractant on a minimum of 43 individual plants of each *P. australis* haplotype.

Chlorophyll a = $11.24A_{661.6} - 2.04A_{644.8}$

Chlorophyll b = $20.13A_{644.8} - 4.19A_{661.6}$

Chlorophyll a + b = $7.05A_{661.6} + 18.09A_{644.8}$

Chlorophyll x + c = $(1000A_{470} - 1.90\text{Chlorophyll}_a - 63.14\text{Chlorophyll}_b) / 214$

Using these equations and inserting the measured absorbance values yielded concentrations in the units of micrograms per milliliter plant extract solution.

3.3.2 Photosynthetic Measurements

Photosynthetic measurements were measured on the two *P. australis* haplotypes, F and M, using plants grown in the greenhouse prior to the stable isotope experiment.

Measurements were taken on July 9 and July 12, 2004 using a LI-COR 6400 portable photosynthetic system to determine if differences exist between photosynthesis versus irradiance (PI). Both CO_2 concentrations and cuvette temperature were controlled to minimize photorespiration (380 ppm CO_2, T = 25ºC). Individual plants were exposed to a Photosynthetic Photon Flux Density (PPFD) at differing light levels (1500, 1000, 700, 400, 200, 100, and 50 μmols s^{-1} m^{-2}) to generate a PI Curve (n=5 for *P. australis* F, n=6 for *P. australis* M). This curve allows for the determination of apparent quantum yield (A_{qe}), or initial slope of the PI curve, maximum photo saturated CO_2 assimilation rate (A_{max}), and the light compensation point, LCP, the x-intercept where net photosynthesis is zero. Plant photosynthetic assimilation calculations, were modeled using the Mitscherlich model equation (Peek et al. 2002):

$$A = A_{max} [1 - e^{-Aqe(PPFD - LCP)}]$$

Pulse Amplitude Modulating (PAM) Flourometry was employed to determine physiological stress (quantum yield = Y), and efficiency of photochemistry in photo system II on the same days as the IRGA experiment, July 9 and July 12, 2004 using a MiniPAM (Walz). Rapid Light Curves (RLC) and saturation pulses on dark-adapted plant leaves were performed on the third leaf from the shoot apex (n= 63 Type F *P. australis* and n=74 on type M *P. australis*). Plants were dark adapted for 25 minutes prior to the light saturation pulse as in Adams and Bate (1999). These procedures allow the determination of the apparent photosynthetic yield, F_v/F_m, of photo system II ,

maximum electron transport rate in photo system II, ETR_{max}, in addition to maximal (F_m) and minimal (F_o) chlorophyll a fluorescence in the dark adapted state.

3.3.2 Elemental C, N, and P concentrations

In order to evaluate differences in C, N, and P concentrations, individual *P. australis* plants (described in Chapter 2 from the laboratory experiment) were separated into live leaf and shoot portions, dried in an oven at 65 ºC, and ground to a homogenous powder in a coffee mill. These samples were analyzed for C and N composition using a Carlo Erba 2500 Elemental Analyzer. Two replicates of each sample were run to ensure analytical precision. Tissue P composition was determined using a hot acid extraction technique (McGlathery et al. 1994) followed by subsequent colorimetric determination using a HP 8453 UV-Visible spectrophotometer. Two replicates of each sample were run to determine analytical precision of the procedure. Recovery of P was deemed to be 97.7% ± 2.5% using peach leaves (NIST 1547).

3.4 DATA ANALYSIS

Differences in pigment concentrations between *P. australis* haplotypes were analyzed in SAS using ANOVA analysis. Post hoc TUKEY tests were employed to determine significant differences between plant types. Photosynthetic models of assimilation were analyzed using non-linear analysis in SAS (Peek et al. 2002), and significant differences between light curves were determined using the 95% confidence intervals provided by the model in SAS using proc nonlin.

3.5 RESULTS

3.5.1 Photosynthetic pigment differences between *P. australis* haplotypes

Non-native *P. australis* M had significantly higher amounts of chlorophyll a, chlorophyll b, and chlorophyll a+b (Figure 12). (ANOVA chlorophyll a: n = 175, df = 1, F = 93.96, P < 0.001; ANOVA chlorophyll b: n = 175, df = 1, F = 63.71, P < 0.001; ANOVA chlorophyll a+b: n = 175, df = 1, F = 84.99, P < 0.001). Pigment concentrations in all haplotypes followed the expected pigment gradients of chlorophyll a > chlorophyll b > total carotenoids (Figure 12) (Lippert et al. 2001). Total carotenoids, Chl x + c, were also significantly greater in *P. australis* M haplotypes (ANOVA chlorophyll x+c: n = 175, df = 1, F = 90.78, P < 0.001). No significant differences (P > 0.05) existed between *P. australis* haplotypes in any of the pigment ratios: Chl a : Chl b, Chl a: Chl x+c, Chl b: Chl x+c, Chl a+b: Chl x+c.

Additionally, pigment concentrations when normalized over leaf area demonstrated similar trends as presented in pigment concentrations per gram of sample. This is most likely due to the fact that the leaf area:mass ratio did not differ in *P. australis* haplotypes (data not presented).

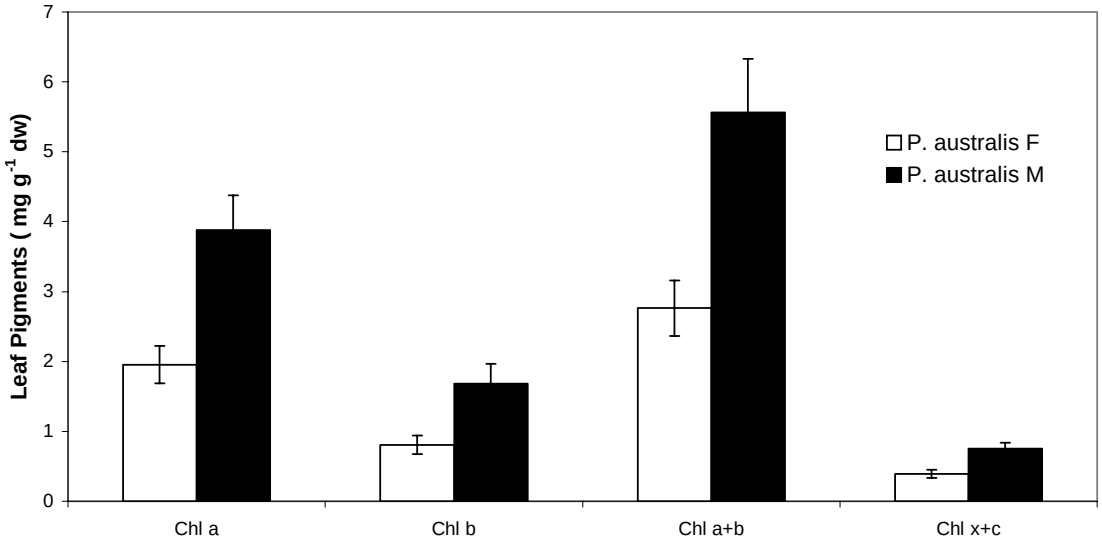

Figure 12. Plant photosynthetic pigment concentrations.
Contents of chlorophyll a, b, total chlorophyll (a + b), and total carotenoids (x + c) in greenhouse grown *P. australis* plants ± 2 SE. Pigment concentrations for all pigments types were significantly different between *P. australis* haplotypes F and M, $p < 0.0001$.

3.5.2 Photosynthetic differences between *P. australis* haplotypes

Non linear models of IRGA analysis of leaf photosynthesis revealed that non-native *P. australis* M has significantly greater CO_2 assimilation, when modeled by the Mitscherlich equation results in $A_{P.\ australis\ M} = 17.6\ [\ 1 - e^{-0.00305\ (PPFD - 3.84)}]$ than native *P. australis* F, $A_{P.\ australis} = 11.5\ [\ 1 - e^{-0.00326\ (PPFD - 0.0131)}]$. Although the sample size was low, n=5 for *P. australis* F and n=6 *P. australis* M, a significant difference was observed between plant types. Additionally, previous studies have also had similar sample sizes due to time needed to calculate a single PI curve. The results of the model

indicate that all photosynthetic variables, A_{max}, A_{qe}, LCP, determined by IRGA analysis, are significantly greater in non-native *P. australis* haplotype M (Table 3-1). These differences are clearly demonstrated in the PI curves in Figures 13 and 14. Overall, type M *P. australis* has a greater CO_2 assimilation rate, maximum photo saturated photosynthesis rate, and greater initial slope of the PI curve, which indicates that non-native *P. australis* has a greater photosynthesis rate in laboratory grown plants.

PAM flourometry data reinforced the IRGA results and demonstrated a significantly greater mean $ETR_{max,}$ a parameter similar to A_{max}, in non-native *P. australis* M plants (Tables 1 & 2). The initial slope of the P/I curve, α, was also significantly greater in non-native *P. australis* M plants when compared to native *P. australis* F plants (Table 2). It is expected that these values will be different since PAM flourometry only looks at the fluorescence of chlorophyll a in photosystem II of photosynthesis. However, the use of different methods still demonstrated similar results.

Dark adaptation revealed no significant difference between *P. australis* haplotypes with regard to minimal and maximum chlorophyll a fluorescence in the dark-adapted state (Table 3-4). Photosynthetic yield was greater than 0.7 in both haplotypes, which indicates that these plants were not photosynthetically stressed since most vascular plants have a Fv/Fm range or 0.75- 0.85 (Bjorkman and Demmig 1987). However, the photosynthetic yield in the dark-adapted state was significantly greater in *P. australis* F haplotypes. Since the 2% difference observed is relatively small, functionally there may no difference between the two haplotypes.

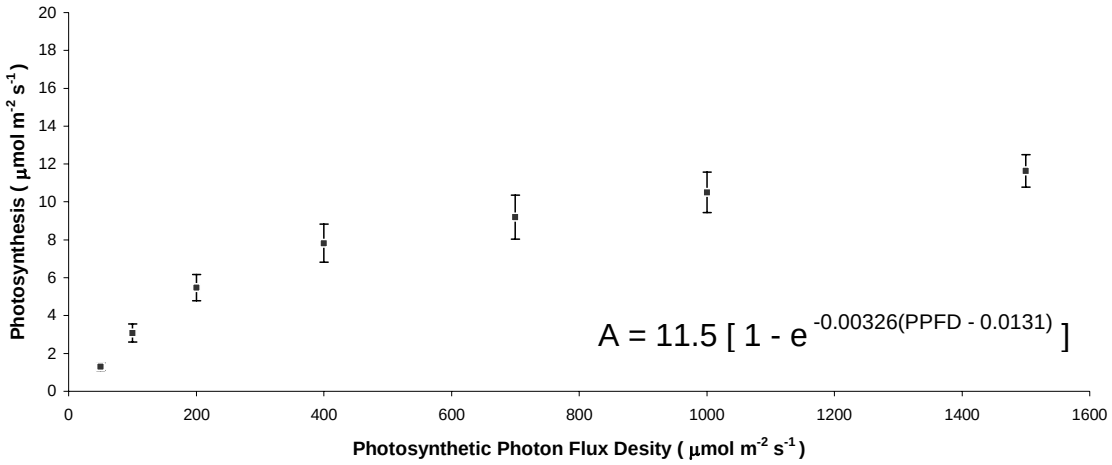

Figure 13. Native *P. australis* type F photosynthesis vs. irradiance

Mean (n=5) ± SE net CO_2 assimilation rates (Photosynthesis) vs. incident PPFD for native, type F, *P. australis* plants. Also shown is the photosynthesis model equation from parameter estimates obtained from nonlinear analysis.

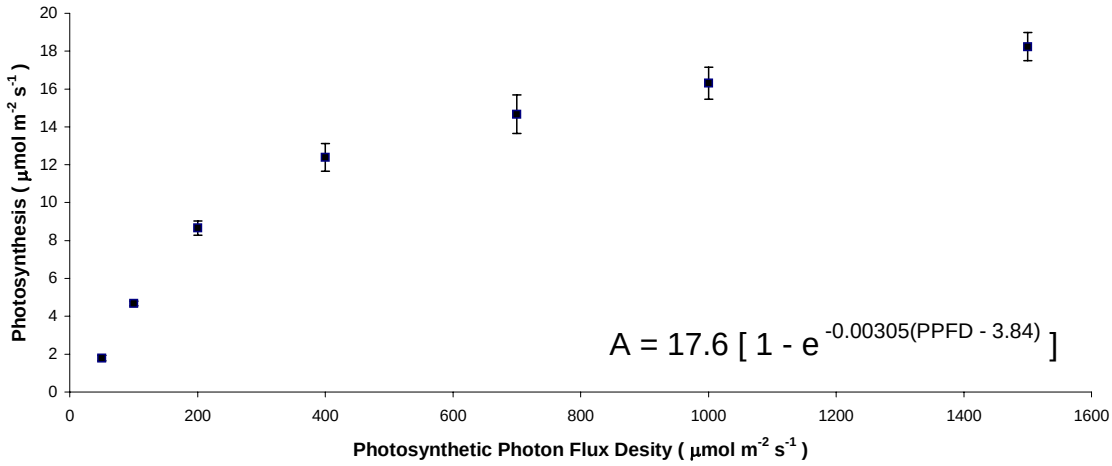

Figure 14. Non-native P. australis type M photosynthesis vs. irradiance

Mean (n=6) ± SE net CO_2 assimilation rates (Photosynthesis) vs. incident PPFD for native, type F, *P. australis* plants. Also shown is the photosynthesis model equation from parameter estimates obtained from nonlinear analysis.

Table 1. Photosynthetic parameter estimates using nonlinear models.
Mean ± SE (n=6 for *P. australis* M, n=5 for *P. australis* F) for the photosynthetic parameter estimates determined using the Mitsherlich model equation.

Plant Type	Photosaturated photosynthesis (A_{max})		Apparent quantum yield (A_{qe})		Light compensation point (LCP)	
	Mean	SE	Mean	SE	Mean	SE
P. australis F	11.53	0.7316	0.00326	0.000878	0.013	29.4411
P. australis M	17.60	0.6063	0.00305	0.000422	3.841	15.0826

Table 2. Dark-adapted plant results.

Effects of 25 min dark adaptation, n = number of individual plants, SE = standard error, F_v/F_m = maximum photochemical yield in the dark-adapted state, F_o= minimal chlorophyll a fluorescence in dark-adapted state, Fm = maximum chlorophyll a fluorescence in the dark-adapted state.

Plant	n	Variable	Mean	SE	P
P. australis F	120	F_v/F_m	0.7545	0.0043	0.013
	120	F_o	439.2	12.7	0.066
	120	Fm	1877.3	70.7	0.757
P. australis M	112	F_v/F_m	0.7394	0.0042	0.013
	112	F_o	468.6	9.4	0.066
	112	Fm	1908.0	69.7	0.757

Table 3. Rapid light curve summary

Photosynthetic response to Rapid Light Curves, n = number of individual plants, ETR_{max} = maximum electron transport rate of PSII, α = slope of P/I curve.

Plant	n	Variable	Mean	Std Error	P
P. australis F	63	ETR_{max}	14.43	0.48	0.0001
	63	α	0.646	0.101	0.0102
P. australis M	74	ETR_{max}	22.39	0.55	0.0001
	74	α	0.715	0.098	0.0102

3.5.3 Tissue C N and P composition

A significant difference in allocation of N between *P. australis* haplotypes was observed for leaf N (ANOVA: df = 1, F = 6.15, P = 0.0149) and stem N (ANOVA: df = 1, F = 5.85, P = 0.0176) (Table 3-4), with the non-native *P. australis* M having the greater N composition. Additionally, leaf C differed significantly with non-native *P. australis* M having a greater C concentration than native, type F *P. australis* (ANOVA: df = 1, F = 9.15, P = 0.0032) (Tables 3-4). No significant differences were observed between stem C, leaf P, and stem P between both *P. australis* haplotypes (ANOVA, P > 0.05) (Table 3-4).

Table 4. Elemental C, N, P composition of native and non-native *P. australis*
Elemental composition of greenhouse grown *P. australis* plants, n = 53 for *P. australis* M C &N, n = 41 for *P. australis* F C & N, n = 30 for P for both *P. australis* haplotypes. Mean values reported with 1 SE, P is the probability values given for one-way ANOVA.

	P. australis F		*P. australis* M		
	mean	SE	mean	SE	P
Leaf % C	44.72	0.37	45.85	0.16	0.003
Leaf % N	2.93	0.08	3.17	0.06	0.015
Leaf % P	0.184	0.008	0.188	0.012	0.782
Stem % C	44.50	0.35	44.57	0.21	0.854
Stem %N	1.95	0.10	1.69	0.05	0.018
Stem % P	0.182	0.008	0.195	0.011	0.353

3.6 DISCUSSION

As expected, photosynthetic differences existed between native and non-native *P. australis* haplotypes. Chlorophyll a and b, along with its accessory pigments were almost two times greater in the non-native *P. australis* M laboratory grown plants than native type F. These differences in pigment concentrations may explain the phenotypic color differences observed in nature. Although the non-native haplotype had higher pigment and accessory pigment concentrations than the native haplotype, their pigment ratios did not differ, and differences in phenotypic color are due to differences in pigment concentrations.

The higher photosynthetic pigment concentrations observed in non-native type M *P. australis* are consistent with the greater photosynthetic rates observed in this haplotype

(Tables 1 - 3). Since these plants were grown on an excess of nutrients, pigment concentrations should be at their greatest potential given that synthesis of chlorophylls are dependent upon nitrogen availability

Higher photosynthetic rates in non-native type M *P. australis* could enable it to out compete the native *P. australis* haplotypes by allowing the plants to grow more aboveground biomass, and as a result, shade out other competitors. Since genetic differences between *P. australis* haplotypes are based on mutations in chloroplast DNA (Saltonstall 2002), these chloroplasts mutations may in fact be responsible for the increased photosynthetic activity. Additional research would need to be conducted to verify this hypothesis.

Type M *P. australis* may be an opportunistic generalist that specializes in producing lots of aboveground biomass due to its greater potential photosynthetic ability. The native haplotype F, on the other hand, may be a low-nutrient specialist, which does not compete well with the non-native form of *P. australis*. Since the native form usually does not produce the large monoculture stands that the non-native haplotype does (personal observation), its growth methods are clearly different. These differences in stand morphology may be directly attributed to the native type's lower photosynthetic rates, and subsequent lower metabolism.

Notably, it seems that the greenhouse grown plants may have some characteristics of "shade-grown" plants due to the fact that the LCP was less than 5 (Taiz and Zeiger 2002). Plants grown in the greenhouse were subjected to diffuse ambient light, but were most likely never exposed to full sunlight conditions of 2000 μmols m^{-2} s^{-}

[1]. As a result, the PI curves generated are only valid for comparisons between these two plant types under these circumstances.

Results from greenhouse plants can be used to make direct comparisons to the *P. australis* haplotypes. The relatively low LCP of the plants grown in the greenhouse indicates they behave more like shade-adapted plants (Table 1). However, photosynthesis did not seem to be photosaturated at low light levels, which is typical of shade adapted plants (Figures 11 and 12). In any case, *P. australis* M had significantly greater assimilation rates of CO_2, and greater maximum photosaturated photosynthetic rates when determined by both IRGA and PAM methods in greenhouse grown plants.

Although there is a significant difference in the F_v/F_m ratio, or apparent photochemical yield of electrons between *P. australis* haplotypes, this difference was not detected in the PI curves using IRGA. These differences in photochemical yield were only 2%, which may suggest that although native *P. australis* type F plants have lower concentrations of pigments, and lower photosynthetic rates, they are more efficient at using available light. It must be noted that these differences may not have been detected due to the relatively lower number of replicates in IRGA analysis compared to PAM analysis. Due to this discrepancy, additional research is needed to verify if differences in photosynthetic yield exist between *P. australis* haplotypes.

The previously mentioned physiological differences between *P. australis* haplotypes may have resulted in differences in allocation of C, N, and P. Since *P. australis* M plants had higher photosynthetic pigment concentrations and higher photosynthetic rates; it follows logically that nutrients would be allocated differently. Since type M *P. australis* demonstrated greater concentrations of photosynthetic

pigments, this would imply that they would also be higher in leaf N than *P. australis* F plants. Although the differences were not large, 3.17% to 2.93% respectively, they were significantly different and are most likely related to pigment difference as shown in Figure 12. This small shift in N composition follows logically since photosynthetic pigments are only about 1% of the total weight of photosynthetic tissues. Nitrogen is a significant component of the chlorophyll molecule, and the increased percent composition of N may be due to increases in photosynthetic pigments.

These data demonstrated that physiological differences do exist between *P. australis* haplotypes. Essentially, non-native *P. australis* M seems to be more aggressive in the allocation of its resources to maximize photosynthetic tissue and biomass. These physiological differences then correspond to the biomass allocation of the nutrients C and N. These photosynthetic differences may be the competitive edge which is facilitating the expansion of this non-native haplotype of *P. australis* throughout North America.

Table 5. Summary of differences between greenhouse grown *P. australis* haplotypes. Ns indicates no significant difference observed between haplotypes F and M ($P > 0.05$), + or – indicate a significant difference ($P < 0.05$), and sign indicates which type demonstrated the greater or lesser variable.

Variable	***P. australis* F**	***P. australis* M**
Chlorophyll a	-	+
Chlorophyll b	-	+
Chlorophyll x+c	-	+
Chlorophyll a/b	ns	ns
Chlorophyll a/x+c	ns	ns
Chlorophyll b/x+c	ns	ns
Photosynthetic rate	-	+
A_{max}	-	+
LCP	ns	ns
A_{qe}	ns	ns
ETR_{max}	-	+
α	-	+
F_v/F_m	+	-
Leaf % C	-	+
Leaf % N	-	+
Leaf % P	ns	ns
Stem % C	ns	ns
Stem % N	-	+
Stem % P	ns	ns
Leaf mass	-	+
Stem mass	ns	ns
Root mass	-	+

Chapter 4 SUMMARY AND CONCLUSIONS

Contrary to commonly held views; DON can be a significant N source to temperate tidal marsh plants. Assimilation rates of DON are up to 20% of NH_4^+ in laboratory experiments, and conservative estimates indicated that up to 74% of the glycine molecule is assimilated intact. Additionally, field replicates demonstrated that DON assimilation rates were up to 20% of N assimilated *in situ* when compared to NH_4^+. These experiments verify the use of DON by these plants and demonstrate that it is an important, but neglected, pool of nitrogen in temperate tidal marshes.

In addition, field replicates only investigated a combined treatment of glycine and glutamic acid. Since there are many other amino acids present within marsh soils, such as alanine, serine, and aspartic acid, these results present a very conservative estimate for DON utilization. Therefore, N budgets that exclude DON are ignoring a significant pool of bioavailable nitrogen, and in turn, may underestimate available N. However, additional experiments are needed to quantify amounts of bioavailable DON since this fraction has not been studied since the 1970's in tidal marsh sediments. This study demonstrates that DON should not be ignored in temperate marsh habitats, and more research is needed to determine its availability.

Although laboratory experiments demonstrated both *P. australis* and *S. alterniflora* plants can utilize DON directly, field experiments demonstrated that only *P. australis* assimilated amino acids intact. Therefore, it is possible that DON utilization by *P. australis* may be a significant factor facilitating its expansion. However, further

research is necessary to verify these results, since *P. australis* and *S. alterniflora* were exposed to different tidal regimes and hydrology on account of plant zonation in the field experiment. Regardless, this experiment has clearly demonstrated the utilization of dissolved organic molecules by both of these dominant estuarine plants. As a result, we must reevaluate our nitrogen models to incorporate plant use of DON, and additional research is necessary to gain a more complete understanding of N cycling within temperate tidal systems.

This experiment also demonstrated the first *in situ* comparison of N assimilation between *P. australis* and *S. alterniflora* using stable isotopes. Interestingly, although uptake rates were similar in laboratory hydroponic experiments, *P. australis* demonstrated four times greater assimilation rates of NH_4^+-N in the field experiment. However, these values may have effected by location of the marsh, and the long duration of the experiment. *P. australis* was the only plant that assimilated amino acids intact, and its rates of assimilation were greater, although not significantly due to small number of replicates. These higher assimilation rates in *P. australis* may explain the mechanism by which it is getting its N for its higher N demand, since *P. australis* demands at least 50% more N when compared to *Spartina spp.* (Windham and Meyerson 2003). This greater N demand in *P. australis* was also confirmed in greenhouse grown plants (N demand = μg N in plant / # growing days: *P. australis* = 1.33 ± 0.06 μg N day^{-1}; *S. alterniflora* N demand = 0.48 μg ± 0.03 N day^{-1}). As a result, DON may be the missing pool of nitrogen needed to explain *P. australis*' high productivity.

More studies are needed to quantify the amount of bioavailable DON in the field. Current methods of quantifying DON are a subtraction of [TKN] – [nitrate + nitrite +

ammonium]. This determination does not take into account what fraction of DON is bioavailable to plants, such as amino acids, amines, and urea. Henceforth, future studies should separate DON into its respective bioavailable DON and not bioavailable fractions. Additionally, we must also reevaluate marsh nutrient budgets, to include all bioavailable pools of DON.

Future research should be directed into evaluating a better understanding of the importance of DON. Although NH_4^+ may be readily available in tidal marshes, the extent to which DON is used *in situ* relative to DIN must be studied. To gain a systematic understanding of how these plants are responding to field conditions, additional laboratory experiments identifying the effects of salinity, sulfide, and anoxia must be taken into consideration, as these variables can have significant positive and negative feedbacks on N assimilation salt marshes. While increasing salinity resulted in a decrease in NH_4^+ uptake rates (Chambers et al. 1998, Henry and Jefferies 2003a), the contrary is true for amino acids. Increasing salinity resulted in an increase in amino acid uptake rates (Henry and Jefferies 2003a).

This thesis is also the first to demonstrate differences between native and non-native *P. australis* haplotypes. Overall, the non-native type M seems to be a more aggressive variety of *P. australis* as evidenced by its greater photosynthetic rates, greater pigment concentrations, and expansion into previously unoccupied habitats. As a result, non-native type M seems to be an aggressive genotype that is more photosynthetically active than its North American counterparts.

I hypothesize that the non-native *P. australis* type M plant is a nutrient generalist, and light competitor, which is exceptional at producing large amounts of above and

belowground biomass quickly due to its higher photosynthetic rates. Since the effects of N limitation are weakened by increases N eutrophication, type M *P. australis* has been able to out compete other *P. australis* haplotypes and move into previously unoccupied habitats. On the other hand, since the native *P. australis* haplotype F is found in oligohaline tidal marshes, and does not usually form large monocultures, it is most likely a low-nutrient specialist. This hypothesis is supported by its greater assimilation rates of NH_4^+, lower photosynthetic rates, lower N content, and lower concentrations in photosynthetic pigments. These results suggest that although it can compete for nutrients effectively, it may not be best suited for aboveground competition with other plants. Addiontal ecophysiological work needs to be done to illustrate the differences observed between *P. australis* haplotypes.

In conclusion, this work has successfully demonstrated the assimilation of DON by temperate tidal marsh plants both in the lab and *in situ*. As a result, we must reevaluate our nitrogen cycle models and nutrient budgets in tidal marshes to include this neglected source of N. Since the potential for DON use is high, this may in fact be the source of the net deficit of nitrogen reported in nitrogen budgets. Additionally, the assimilation of DON by *P. australis* may be a factor facilitating its expansion into tidal marshes; however, additional field research is needed to validate this conclusion. Regardless, DON is an important pool of N that must be studied more extensively to understand primary production in this critical environment. Finally, this study is the first to demonstrate that physiological differences exist between native and non-native *P. australis* haplotypes. This greater photosynthetic potential of non-native *P. australis* may be one of the factors facilitating its expansion, as well as the reason for out-competing

native North American haplotypes. As a result, future studies regarding *P. australis* must indicate which genetic strain they are studying, because of the great physiological differences among haplotypes.

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