An isotope mass balance approach to measure variability in nitrogen fluxes, allocation and assimilation in balsam poplar (*Populus balsamifera* L.) and other plants

by

Lee Anthony Kalcsits

B.S.A. University of Saskatchewan, 2005
M.Sc. University of Saskatchewan, 2008

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Abstract

Populus species or their hybrids are being increasingly used as feedstock for the growing bioenergy industry. To optimize carbon uptake efficiencies, fertilizer inputs must be minimized while, at the same time, achieving rapid growth. The complexity of nitrogen uptake and assimilation in plants and environmental heterogeneity force simplifications that limit improvements in nitrogen-use efficiency. In particular, a lack of integration limits the applicability of many traditional nitrogen-use assays to whole plant nitrogen use. Net nitrogen isotope discrimination has potential to act as a time-integrated process indicator of nitrogen-use in plants grown under steady-state conditions. The objective of this thesis was to further develop an isotope discrimination-based integrated measure of nitrogen-use that reflects whole plant and organ level nitrogen use. Observed differences in nitrogen isotope discrimination were proposed to be a function of nitrogen isotope discrimination of the assimilatory enzymes, fluxes across the root plasma membrane and translocation of inorganic nitrogen to the shoot. As a test, nitrogen supply and demand was environmentally varied in black cottonwood (*Populus trichocarpa* (Torr & Gray)) and genetically manipulated with knockout lines of Arabidopsis (*Arabidopsis thaliana* L.). Changes in isotopic composition ($\delta^{15}N$) were interpretable within the context of the proposed model. Furthermore, efflux/influx (*E*/*I*) across the root plasma membrane calculated from the isotope mass balance approach was positively correlated with *E*/*I* measured using an established ^{15}N compartmental analysis approach indicating that the isotope mass balance approach produced a reliable measure of *E*/*I*. The isotope mass balance approach was then used to determine intraspecific variability in balsam poplar (*Populus balsamifera* L.), a species used for hybrid poplar breeding. Nitrogen use traits were calculated for 25 genotypes from five climatically dispersed provenances grown hydroponically under steady-state nitrogen conditions with either ammonium or nitrate. Genotypic variation exceeded provenance level variation in most cases and significant variation was observed in growth, nitrogen isotope composition and calculated nitrogen-use traits indicating that there is potential for breeding for nitrogen-use using balsam poplar. The isotope mass balance model

presented here provides a new approach for probing integrated nitrogen-use traits in plants, which are often technically difficult to measure.

Preface

The research chapters of this thesis were written as a series of manuscripts for publication in peer-reviewed journals. For my role, I designed the experiments and collected and analyzed the data for chapter 3, 4, 5, and 6. For chapter 2, I supplemented the work of another student with additional data and refined their interpretation. For all research chapters, I took the lead in writing the draft manuscripts, in addition to all tables and figures. My supervisor, Rob Guy, developed a preliminary isotope mass balance model, provided assistance with proposal development, experimental design, data interpretation and editing throughout all chapters.

A version of Chapter 2 will be submitted for publication as: "Buschhaus H, Kalcsits LA, Guy RD. Changes in nitrogen supply and demand impact nitrogen isotope discrimination by modifying uptake, movement and assimilation in *Populus trichocarpa*."

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A version of Chapter 6 will be submitted for publication as: "Kalcsits LA, Guy RD. Variation in nitrogen isotope discrimination indicates that genotypic variation exceeds provenance level variation in nitrogen source preference, uptake and assimilation in *Populus balsamifera* L."

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- To my wife, Jenn and my two children, Hayden and Blake

1. Introduction and research objectives

1.1. Plant nitrogen use

Nitrogen is a critical component of proteins, amino acids and DNA, and the fourth most abundant element in organic matter. Given its importance, limitations to nitrogen availability can impact plant growth and development. Nitrogen is one of the limiting plant nutrients in most natural ecosystems (Glass *et al*. 2002; LeBauer and Treseder 2008) and subsequently, nitrogen fertilization is essential for modern agricultural production. Nitrogen fertilizer use is presently seven times higher than it was at the start of the green revolution (1950's), but it is also responsible for a doubling in global food production (Hirel *et al*. 2007). However, high use of nitrogen fertilizer has come at a cost. Much of this nitrogen is not taken up by plants (Raun and Johnson, 1999) and is lost through leaching, denitrification or volatilization (Vitousek *et al*. 1997). Nitrogen fertilizer production contributes 1.2% to global energy consumption (Ahlgren *et al*. 2008). This can have negative environmental impacts as well as placing an unnecessary economic burden on producers. The potential of agricultural production of woody plant biomass to supply the growing biofuel industry (Yuan *et al*. 2008) is progressively leading to improvements in growth rates and yield (Stanton *et al*. 2010). For efficient production, high yielding biofuel crops must be able to grow with minimal fertilizer inputs on marginal lands, essentially maintaining high nitrogen-use efficiency. Agricultural nitrogen use efficiency is commonly defined two ways (Moll *et al*. 1982): 1) biomass or yield produced per unit plant nitrogen, or 2) the proportion of available nitrogen taken up by a crop. Improvements to plant nitrogen use efficiency would reduce the environmental impacts of modern agriculture in addition to reducing overall fertilizer requirements and making production feasible on marginal lands.

1.2. Nitrogen use and availability in natural ecosystems

Nitrogen is often the limiting nutrient in most natural ecosystems (Glass *et al*. 2002; LeBauer and Treseder 2008). Forest soils, in particular, are often nitrogen limited. To grow and compete under these limiting conditions, plants have adapted to acquire

nitrogen in many different forms, including ammonium, nitrate, free amino acids (Näsholm *et al.* 1998) and protein (Paungfoo-Lanhienne *et al.* 2008), as soil derived sources. Other sources include atmospheric nitrogen gas, via nitrogen-fixing plants (Deluca *et al.* 2002) and absorption of atmospheric inorganic nitrogen (Giesler *et al.* 2002). Primary nitrogen sources in temperate forest soils include ammonium, nitrate and organic nitrogen (Höberg 1997). Total inorganic and organic nitrogen in temperate forests can range from 0.05 to 1.65 kg N m^{-2} (Callesen *et al*. 2007). Nitrogen dynamics vary climatically and availability of different nitrogen forms is different between environments (Giesler *et al*. 1998). Small changes in climate can significantly alter the nitrogen cycle. For example, changes in rates of nitrification, ammonification, nitrogen fixation and denitrification are impacted by changes in temperature and precipitation patterns (Callesen *et al*. 2007; Hart 2006; Wang *et al*. 2006).

Climatic conditions can not only impact concentrations of inorganic and organic nitrogen in soil, but also modify the dominant inorganic source available to plants (Houlton *et al*. 2007). Boreal forests generally have higher ammonium and organic nitrogen concentrations in soils than nitrate (Nordin *et al*. 2001), largely a consequence of acidic soils. Prairie soils with higher pH and more developed soil structure will generally have higher concentrations of nitrate. Since woody plants are relatively long-lived species, temporal changes in nitrogen environments are likely encountered over their lifetime. Therefore, woody plants need to compete with other organisms for limited soil nitrogen that is spatially, temporally and chemically heterogeneous. Interspecific competition for nitrogen within plant communities has been extensively studied (Harrison *et al*. 2007; Weigelt *et al*. 2005). Plants have been shown to preferentially utilize either nitrate or ammonium. For example, rice (*Oryza sativa* L.) is known to prefer ammonium, or at least is able to easily metabolize ammonium without the risk of ammonium toxicity (Falkengren-Grerup 1995). This is also true for white spruce (*Picea glauca* L.) (Min *et al*. 2002; Pritchard and Guy 2005). However, intraspecific variation in nitrogen uptake and its relationship with environmental conditions is equally important (Harrison *et al*. 2004). Consequently, there needs to be a

more thorough understanding of how woody plants grow on different nitrogen sources and the degree of genotypic variability in nitrogen uptake and assimilation within climatically dispersed plant species. Competition for temporally and spatially variable pools of multiple sources of nitrogen has produced complex uptake and assimilation pathways that are variable across species and communities.

1.3. Inorganic nitrogen uptake and assimilation in plants

With the exception of organic nitrogen, uptake of nitrogen in plants directly from the soil is through uptake and assimilation of inorganic nitrogen by roots. Nitrogen uptake changes in response to variation in heterogeneous demand versus supply. Plants must exhibit some degree of plasticity to match uptake with demand for efficient growth and development. There are multiple genes controlling uptake and assimilation and each gene can have multiple isozymes (Glass *et al*. 2002). Although the specific roles of large gene families are not entirely clear the redundancy of inorganic nitrogen transporters is problematic when trying to functionally characterize precise roles of individual genes (Glass *et al*. 2002). This, combined with a complex regulatory network for nitrogen use, makes it difficult to observe distinct changes in nitrogen-use as a consequence of single gene disruption. The complexity of the genetic and regulatory network of the nitrogen uptake and assimilation pathway has been emphasized in a significant body of molecular work in the past decade (Britto *et al*. 2002; Glass *et al*. 2002; Lea and Azevedo, 2006; Miller and Cramer, 2005).

At the whole-plant level, nitrate assimilation is more complex than ammonium. After crossing the root membrane via diffusion or via nitrate transporters, nitrate is converted to nitrite in the cytoplasm by a NADPH- or NADH-catalyzed nitrate reductase (Campbell 1988). Nitrite is then immediately transported into the chloroplast where nitrite reductase converts nitrite to ammonium (Wallsgrove *et al*. 1983). Much of our functional understanding of inorganic nitrate transport has been gained from experiments using Arabidopsis (*Arabidopsis thaliana* L). Nitrate uptake has been shown to occur via two uptake pathways; a high-affinity uptake system (HATS) and a low-affinity uptake system (LATS) (Glass *et al*. 2002). HATS transporters are saturable, exist

in both constitutive and induced forms, and are active at low nitrate concentrations (~100 µM range). It is possible that constitutive forms act as sensors that, in turn, activate inducible HATS transporters. LATS transporters are unsaturable, active at higher nitrate concentrations and thought to only act constitutively. In Arabidopsis, three gene families; NRT1, NRT2 and NRT3 (NAR2), have been identified as nitrate transporters (Lea and Azevedo, 2006; Plett *et al*. 2010). NRT1 has eight genes, NRT2 has seven genes, and NRT3 has two genes in Arabidopsis (Williams and Miller, 2001; Orsel *et al*. 2002; Plett *et al*. 2002). NRT1 transporters are functional at a wide range of substrate concentrations and some NRT1 isozymes act as both HATS and LATS transporters (Guo *et al*. 2002). In contrast, inducible NRT2 genes perform at HATS concentrations (Okamoto *et al*. 2003). Different NRT1 and NRT2 transporters exist in grass species compared to Arabidopsis (Plett *et al*. 2010).

The regulation of nitrate uptake and assimilation in plants is necessary to optimize nitrogen utilization. Nitrate transport and assimilation are tightly regulated and as such, the network controlling this activity requires adaptability to take advantage of available nitrogen. Changes in environmental conditions have been shown to either induce or downregulate transporter activity. For example, the presence of nitrate increases NRT2.1 transporter activity (Nazoa *et al*. 2003) and nitrate reductase activity (Redinbaugh and Campbell 1991). However, other environmental stimuli, such as light and sucrose, do not result in significant changes in gene expression of NRT2.1 (Wirth *et al*. 2007). Vidmar *et al*. (2000) reported downregulation of NRT2.1 expression and nitrate influx in response to addition of downstream amino acids. The HATS transport system is thought to be post-transcriptionally regulated (Glass *et al*. 2002). To support this, Laugier *et al*. (2012) demonstrated that NRT2.1 protein abundance decreased under conditions where HATS decreased, demonstrating post-transcriptional regulation of HATS activity by the downregulation of NRT2.1 in Arabidopsis. Glutamine and ammonium were both found to post-translationally inhibit transporter activity (Glass *et al*. 2002). Like nitrate transporters, nitrate reductase is induced by the presence of nitrate in addition to light and oxygen (Kaiser and Huber, 2002). The balance of nitrate

supply mediated by transporter activity and demand mediated by nitrate reductase activity must be carefully regulated to ensure adequate, but still efficient supply of organic nitrogen to the plant.

Ammonium is a more direct and readily available form of inorganic nitrogen to plants (Loqué and von Wirén 2004) and is assimilated into glutamine by ATP-dependent glutamine synthetase. Similar to nitrate, there are two uptake systems; a high-affinity uptake system (HATS) active at low ammonium concentrations, and a low-affinity uptake system (LATS) active at high ammonium concentrations. Many plants have difficulty growing in ammonium-only nitrogen since cellular proton balance and consequently, pH are disrupted by rapid influx of ammonium. One hypothesis explaining this phenomenon is futile cycling of ammonium whereby cytoplasmic ammonium homeostasis is disrupted in plants susceptible to ammonium toxicity such as barley, but is maintained in tolerant plants such as rice (Britto *et al*. 2001). Essentially, susceptible plants are unable to regulate the influx of ammonium into the cell. As such, there is significant leakage from the cell that creates a proton imbalance and cellular pH becomes more acidic causing damage to the plant.

Although there is a good functional understanding of nitrogen uptake and assimilation at the molecular level, the complexity of the system limits the integration of whole-plant uptake and assimilation into molecular physiology. As knowledge of regulatory pathways and interactions, as well as the development of a complete nitrogen uptake and assimilation model become more complete, there will be a demand to integrate this valuable molecular knowledge into the whole-plant context (Hirel *et al*. 2001, Glass *et al*. 2002). There is also evidence that there is significant species variation in ammonium transporters (Lea and Azevedo, 2006) and nitrate transporters (Plett *et al*. 2010). Furthermore, within species variability in inorganic nitrogen transport may reflect differences in allelic variation of genes responsible for uptake and movement of inorganic nitrogen. To date, minimal work has been done characterizing intraspecific variation in nitrogen use traits at the molecular or physiological level.

1.4. Utilization of nitrogen isotopes in plant physiology and ecology

Isotopes in biology have mainly been employed in tracer experiments to examine the movement of targeted elements within pathways, organisms or ecosystems. In addition, stable isotopes can provide process information and have been used in plant research to answer questions on allocation, assimilation and uptake of elements during plant growth and development. Stable isotopes that have been commonly used in physiological studies include ^{2}H, ^{13}C, ^{15}N and ^{18}O. Technological advances in mass spectrometry in the past two decades have allowed for rapid determination of natural abundance ratios in materials ranging from soil to plants (Handley and Raven, 1992). The use of stable isotopes in biology has increased rapidly as a result. Natural abundance measurements indicate the ratio of the less abundant, naturally occurring isotope relative to the predominant isotope for that element. For nitrogen, carbon and hydrogen, natural abundance stable isotope ratios are indicated by $^{15}N/^{14}N$, $^{13}C/^{12}C$ and $^{2}H/^{1}H$ isotope ratios, respectively. Natural abundance ratios are commonly expressed relative to an internationally accepted reference material as (Dawson *et al*. 2002):

$$\delta^{x}E\ (\delta^{15}N) = 1000 * (R_{sample}/R_{standard} - 1),\ ‰$$

Where, in the case of $\delta^{15}N$, $R_{standard}$ is the $^{15}N/^{14}N$ ratio of air dinitrogen gas (0.00365) and R_{sample} is the $^{15}N/^{14}N$ ratio of the measured sample. Substances with isotope ratios that are greater or lower than the reference abundance ratio are referred to as enriched or depleted, respectively (Peterson and Fry 1987). Stable isotope ratios at natural abundance can be used as integrators of elemental cycling and dynamics to determine net outcomes of many biogeochemical processes that often vary spatially and temporally, and are a function of the surrounding environment (Dawson *et al*. 2002; Handley and Raven, 1992, Robinson, 2001).

The most prominent example of the use of stable isotopes at natural abundance in plant physiology is the use of carbon stable isotope composition ($\delta^{13}C$) as a measure of water-use efficiency (Farquhar *et al*. 1982) and identification of photosynthetic pathways between C_3 and C_4 plants (Smith and Epstein 1971; O'Leary 1981). Analogous

to carbon assimilation, the emerging approach of using $\delta^{15}N$ to provide information on nitrogen fluxes, assimilation and allocation has gained recent attention (Handley *et al*. 1997; Robinson *et al*. 2001; Evans 2001; Comstock 2001; Houlton *et al*. 2007; Cernusak *et al*. 2009). However, poor mechanistic understanding of nitrogen isotope fractionation has limited applications of this approach. Since nitrogen isotope fractionation is thought to be a function of nitrogen assimilatory demand interacting with efflux/influx (*E*/*I*) across the root membrane (Comstock 2001), it represents a whole-plant measure of the impact of changes to overall plant nitrogen fluxes, assimilation and allocation. Currently, the use of $\delta^{15}N$ values in plant physiology has included associating ^{15}N natural abundance with water availability from a global database (Handley *et al*. 1999) and genotypic diversity in drought and salinity tolerance (Yousfi *et al*. 2009; Yousfi *et al*. 2012; Yousfi *et al*. 2013). The most prominent example is the use of $\delta^{15}N$ to examine balance between nitrogen uptake versus fixation (Broadbent et al. 1982; Rennie and Kemp 1983). Other examples of studies using ^{15}N natural abundance values as integrators of biogeochemical processes include CO_2 (Bassirirad *et al*. 2003), nitrogen source (Hietz *et al*. 2002; Kahmen *et al*. 2008), and nitrogen-phosphorus interactions (McKee *et al*. 2002). Results, particularly from the field, are often variable and can make interpretation of nitrogen-use physiology difficult. As current and future mechanistic models become more refined, the advantages of using this method for answering questions related to plant physiology and ecology should become more applicable.

Different biochemical pools of nitrogen, from small (e.g. a leaf protein) to large (e.g. atmospheric N_2) have different isotopic compositions or "signatures". Differences in signatures can be used to infer sources of nitrogen as it is mixed or moved through plants, soils or ecosystems. Often, plant-based (most often foliar-based) isotope measurements are used to infer temporal or spatial changes in nitrogen source, concentration and availability (Houlton *et al*. 2007; Miller and Bowman, 2002). Differences in isotope signatures reflect fractionation of isotopes caused by isotope discrimination. Nitrogen isotope discrimination (i.e. the change in isotope composition of the product relative to the substrate) is also emerging as a valuable tool to measure

changes in nitrogen dynamics within ecosystems (Högberg, 1997). Nitrogen isotope fractionation may affect the similarity of whole plant and organ level isotope composition to its environmental sources. Since these measurements provide integrated values of the sum of multiple biogeochemical processes, it is difficult to distinguish between isotopic fractionation at different steps within the system. Therefore, to better utilize natural abundance values collected during field experiments, a more complete understanding of nitrogen isotope discrimination during nitrogen uptake and assimilation is required. Advances in linking measured natural abundance isotope ratios with nitrogen-use physiology would address some of the assumptions made during interpretation of natural abundance values measured under field conditions. Conversely, plant and organ differences in nitrogen isotope discrimination may also provide illumination of integrated nitrogen-use physiology in plants. For example, plant $\delta^{15}N$ values may also reflect nitrogen uptake efficiency which is inversely related to the ease with which a plant is able to draw nitrogen from its surrounding environment.

Net plant discrimination, where ^{14}N is favoured over ^{15}N, is thought to occur during the irreversible step of assimilation, either in roots or shoots (Mariotti *et al*. 1982; Mariotti *et al*. 1983; Comstock, 2001; Evans, 2001). Generally, estimates in the literature are 16.5 and 15‰ for glutamine synthetase (Yoneyama *et al*. 1993) and nitrate reductase (Ledgard *et al*. 1985), respectively. However, these values are poorly constrained. Past research used partially purified enzyme from crude plant extracts that provided inconsistent estimates of maximum enzyme discrimination factors. Recent work suggests that discrimination for nitrate reductase is greater than 15‰ and more likely around 22‰ (Needoba *et al*. 2004; Tcherkez and Farquhar, 2006), or as high as 26‰ (Karsh *et al*. 2012). Although glutamine synthetase may also have a greater discrimination factor, there have been no suggestions that the estimate by Yoneyama *et al*. (1993) is incorrect.

Isotope discrimination during nitrogen uptake and assimilation in higher plants has not been extensively studied. Often, the assumption is that plant-based ^{15}N

measurements in the field are a reflection of the source isotopic signature (Houlton *et al*. 2007). However, isotope discrimination may occur, even in the field. Although it is conceivable that nitrogen isotope discrimination may yield information on the efficiency and location of nitrogen uptake and assimilation in higher plants, some of the underlying hypotheses describing the contributions of plant physiology to nitrogen natural abundance ratios in plant tissue need to be empirically tested.

Enzyme discrimination factors for nitrate reductase and glutamine synthetase can be used as coefficients for proposed models of nitrogen isotope discrimination. Comstock (2001) and Evans (2001) both developed similar models describing the mathematical basis for nitrogen isotope discrimination. In these models, nitrogen isotope discrimination is, at least in part, a reflection of the ease with which a plant is able to draw inorganic nitrogen from its surrounding environment into its roots to match assimilation demand (enzymatic assimilation). Although both models propose different approaches to measuring inorganic and organic nitrogen pools, both suggested that nitrogen isotope composition of leaves and roots are indicative of nitrogen fluxes, assimilation and allocation in plants. However, technical difficulty in the sampling required limited the application of the model proposed by Comstock (2001). Evans provided a more simplistic model that connects the location of assimilation and E/I to nitrogen isotope composition. Comstock (2001) measured cytoplasmic pools to determine fractionation within the plant. However, this requires the assumption that isotopic composition of cytoplasmic pools is consistent across diurnal cycles and plant development. Measurements of cytoplasmic inorganic nitrogen are methodologically difficult and require assumptions (such as diurnal stability) that may be incorrect. While these models provided some insight into physiological processes contributing to whole plant and organ level nitrogen isotope composition in plants, application of these models to derive nitrogen-use traits from input measurements is either technically difficult or impossible.

1.5. *Arabidopsis thaliana* as a model system

Arabidopsis thaliana L. is a herbaceous member of the mustard family (*Brassicaceae*). The Arabidopsis genome was the first plant genome to be sequenced (Arabidopsis Genome Initiative, 2000) and Arabidopsis has been used as a model plant system because of its short life cycle (seed to flower in 20 to 40 days), wide geographic range (global distribution) and easy propagation. These advantages have spurred a large body of research dedicated to Arabidopsis physiology. Additionally, it has been used as a system for functionally characterizing the role of genes and gene families. Large selections of described t-DNA insertion and gamma-ray mutant lines are available for experiments. Of relevance to studies on nitrogen uptake and assimilation, t-DNA lines were produced using an *Agrobacterium* floral dip that randomly inserted a marker gene for kanamycin resistance on the genome (Krysan et al. 1999). Then, each seed was grown to confirm the location of the insertion and then homozygous lines were selected that did not segregate for the insertion. Gamma-ray lines were produced by exposing flowers to gamma-radiation and screening for chlorate-resistance. Chlorate is an analog for nitrate and also used by nitrate reductase to produce toxic chlorite. Therefore, chlorate resistance is an indication of low nitrate reductase activity. These described and non-described mutant lines are curated by the Arabidopsis Biological Research Centre (ABRC) and available for researchers. Furthermore, there have been multiple sets of other transgenic lines developed to answer questions on aspects of molecular, cellular and whole plant physiology.

1.6. *Populus* as a model system

Black cottonwood (*Populus trichocarpa* (Torr. & Gray)) was the first tree species to have its genome fully sequenced (Tuskan *et al.* 2006). Along with *Salix*, the genus *Populus* consists of a number of woody species in the willow family (*Salicaceae*). Sequencing of the black cottonwood genome provided a genetic map from which to approach physiological questions related to function, regulation and allelic variation of specific genes. Poplar is an ideal model system for physiological studies because of relative ease of propagation, rapid growth and an established research base from which

to draw information (Brunner *et al*. 2004). Balsam poplar (*Populus balsamifera* L.) and *P. trichocarpa* (syn. *P. balsamifera* ssp. *trichocarpa*) inhabit one of the largest geographic ranges for a woody plant "species". Balsam poplar has a particularly large climatic range with the ability to survive across a broad range of climates from prairie to high-altitude to boreal forest-tundra transition as well as coastal climates. The range extends across most of Canada and northern United States (Petersen and Petersen, 1994). The AgCanBaP collection of balsam poplar, collected from 2004 to 2010 by the Agri-Environment Services Branch of Agriculture and Agri-Food Canada, consists of 15 genotypes from each of 62 provenances spanning the geographic range of the species (Soolanayakanahally, 2010). This range produces variability in ecophysiological traits to survive across different climates. Soolanayakanahally *et al*. (2009) identified differences in phenology, photosynthetic ^{13}C isotope discrimination and growth rate between northern and southern populations of balsam poplar. Other traits that may vary over this range include nitrogen uptake efficiency and source preference. Balsam poplar is one species used in hybrid poplar breeding programs for biofuel feedstock and identification of variation in nitrogen-use traits across its range may prompt efforts to consider nitrogen-use efficiency during breeding and selection.

Poplar has potential as a feedstock for the growing bioenergy sector (Yemshanov *et al*. 2008). Currently, poplar has been shown to have a negative net carbon balance for biofuel production (Stanton *et al*. 2010). However, efforts to improve biomass yields will either result in higher fertilizer inputs or the requirement to grow on less marginal lands. To improve yields but maintain low fertilizer requirements, nitrogen-use efficiency must be addressed. Nitrogen-use traits have not been explored in natural species of poplar commonly used in breeding programs and therefore would be an important contribution to poplar research.

1.7. Research objectives

For my thesis, the five research objectives were to:

1. Test and improve a proposed nitrogen isotope mass balance model explaining nitrogen isotope composition under steady-state conditions by varying environmental nitrogen supply and physiological demand in black cottonwood (chapter 2).
2. Test the isotope mass balance model by varying nitrogen supply and demand in knockout lines of Arabidopsis (chapter 3).
3. Compare estimates of *E*/*I* using the isotope mass balance model to *E*/*I* measured using the already established ^{15}N compartmental analysis in five balsam poplar genotypes grown hydroponically with nitrate or ammonium (chapter 5).
4. Quantify and account for pre-existing nitrogen remobilized from vegetative cuttings to growing tissue of balsam poplar (chapter 4).
5. Determine if variation in nitrogen fluxes, assimilation and allocation exists among genotypes of five climatically dispersed balsam poplar provenances grown with either nitrate or ammonium (chapter 6).

1.8. Impact of research

Poplar species with intraspecific variation in nitrogen fluxes, assimilation and allocation represent an important genetic resource for future improvement to maintain or improve on the ability to produce bioenergy feedstock with minimal fertilizer input. More broadly, validation and/or improvement of proposed models for nitrogen isotope discrimination and application to intraspecific variation in nitrogen uptake and assimilation patterns would have applications from molecular physiology to plant ecology. Furthermore, as has been the case with carbon isotopes (Farquhar *et al.* 1989), a workable model of nitrogen isotope discrimination and isotope mass balance in plants would help bridge the gap between ecology and physiology and draw on strategies from both disciplines to answer questions related to nitrogen uptake and assimilation in natural populations of poplar and other plants.

2. Changes in nitrogen supply and demand impact nitrogen isotope discrimination by modifying uptake, movement and assimilation in *Populus trichocarpa*

2.1. Summary

Fractionation of nitrogen isotopes between the substrate and the plant occurs during uptake and assimilation of inorganic nitrogen. Variation in isotopic composition ($\delta^{15}N$) can also occur at the organ level. Here, a mass balance model combining measurements of $\delta^{15}N$ and nitrogen content is proposed to explain whole plant and organ level variation in $\delta^{15}N$. Discrimination is suggested to be a function of nitrogen efflux back to the substrate relative to gross uptake at the root (efflux/influx), the proportion of net uptake assimilated in the roots, and the export of remaining nitrogen for assimilation in the leaves. To manipulate demand relative to supply, rooted *Populus trichocarpa* (Torr. & Gray) cuttings were grown hydroponically under 200 or 400 µM NH_4^+ and 400 or 800 µL L^{-1} CO_2. Discrimination was reduced in plants grown under 800 µL L^{-1} CO_2. Partitioning of nitrogen to the leaves was greater with 400 µM NH_4^+ (70% plant N in leaves) compared to 200 µM NH_4^+ (58% plant N in leaves). Furthermore, our analysis indicates that increased demand under high CO_2 caused decreased efflux/influx (*E*/*I*). Most assimilation occurred in the roots, but approximately 7% was in leaves, irrespective of treatment. The model presented here provides a series of time-integrated measurements of nitrogen fluxes, allocation and assimilation in plants that are difficult to obtain using traditional, time-point assays.

2.2. Introduction

Poplar has significant potential as a renewable resource for bioenergy applications (Yemshanov *et al.* 2008). Like other agricultural crops, breeding for increases in yields will correspond to an increased demand for nitrogen fertilizer. To reduce emissions offsetting net carbon uptake from growing poplar for bioenergy, selection for improved nitrogen use must occur.

Over the past three decades, there has been substantial progress made towards the understanding of the physiological processes underlying nitrogen uptake and

utilization in plants (Glass *et al.* 2002; Hirel *et al.* 2007; Masclaux-Daubresse *et al.* 2010). Nitrogen-use can be divided into three processes; uptake, assimilation, and allocation. Gains in nitrogen-use have been limited, in part, because of the complexity of these three processes. The development of integrated measures of nitrogen-use is needed to corroborate advancements in functional molecular knowledge of nitrogen fluxes to field level improvements of nitrogen-use. Time-integrated measures of different nitrogen-use traits would provide valuable information on relatively long-term nitrogen use that is difficult to measure using established, time-point assays.

Nitrogen isotopes at natural abundance have potential to provide process information related to nitrogen fluxes, assimilation and allocation (Robinson *et al.* 1998; Evans 2001; Comstock 2001). Current understanding of controls on nitrogen stable isotope ratios (expressed as $\delta^{15}N$ values) emphasizes the role of the relationship between physiology and nitrogen nutrition (Evans, 2001, Pritchard & Guy, 2005). Change in the $\delta^{15}N$ of plant tissues relative to source nitrogen (fractionation) arises through a kinetically determined process whereby during assimilation of inorganic nitrogen, the heavier isotope (^{15}N) is "discriminated" against causing a relatively greater fraction of the lighter isotope (^{14}N) to be incorporated into product (Handley & Raven 1992).

The fundamental restriction preventing application of the basic kinetic isotope fractionation model to nitrogen is that nitrogen is usually not available from an open or steady-state environment. For carbon isotope discrimination in plants, air represents an unchanging pool of carbon where isotopic composition and concentration are relatively stable. Therefore, changes in $\delta\ ^{13}C$ reflect physiology of the plant (Farquhar *et al.* 1982). In contrast, nitrogen in natural environments is spatially, temporally and chemically heterogeneous (Nordin *et al.* 2001). Localized depletion in the rhizosphere can cause variation in substrate $\delta^{15}N$ that may complicate physiological interpretation of plant $\delta^{15}N$ values (Robinson 2001). There can be no discrimination if the substrate is fully utilized. As such, to be able to identify nitrogen uptake and assimilation traits using small changes in nitrogen isotopes at natural abundance, the substrate concentration

must be homogenous around the roots and, for comparisons, plants must be exposed to the same homogenous environment. Analogous to the fractionation of carbon isotopes during photosynthesis, if these conditions are met, variability in $\delta^{15}N$ between and within individual plants will reflect differences in internal physiology rather than the $\delta^{15}N$ of the external environment.

Nitrogen isotope discrimination ($\Delta^{15}N$) is thought to occur during the assimilation of inorganic nitrogen in roots and leaves into organic nitrogen (Mariotti *et al.* 1982; Tcherkez 2011). Nitrate reductase and glutamine synthetase, the two enzymes required for assimilation of nitrate and ammonium, respectively, are presumed responsible for nitrogen isotope discrimination. The instantaneous discrimination of these enzymes determines the maximum amounts of discrimination that can occur. Although there have been large ranges in published discrimination associated with these enzymes, best estimates for discrimination by nitrate reductase and glutamine synthetase are 15.4‰ (Ledgard *et al.* 1985) and 16.8‰ (Yoneyama *et al.* 1993), respectively.

Typically, in nitrogen isotope discrimination experiments, whole-plant $\Delta^{15}N$ does not reflect the full discrimination factor of the enzyme, even under steady-state conditions (Evans *et al.* 1996; Yoneyama *et al.* 2001; Pritchard & Guy 2005), this implies that assimilation is not the only rate-limiting step. Several models (Robinson *et al.* 1998; Evans 2001; Comstock 2001) have proposed that net discrimination arises from the partial consumption of the cytoplasmic inorganic nitrogen pool. If a portion of this pool returns to the medium or is lost to the shoot, fractionation relative to the source, or between organs, will occur (Figure 2.1). At the root level, maximum discrimination occurs when the proportion of the cytoplasmic nitrogen lost outside the root, or to the shoot, approaches the amount of nitrogen imported from the substrate. When there is no loss of cytoplasmic nitrogen prior to assimilation, discrimination cannot occur because the entire nitrogen assimilatory pool will eventually be converted to organic nitrogen in the roots. At the whole plant level, net discrimination does not occur if no inorganic nitrogen is lost from the root, irrespective of the ultimate site of assimilation.

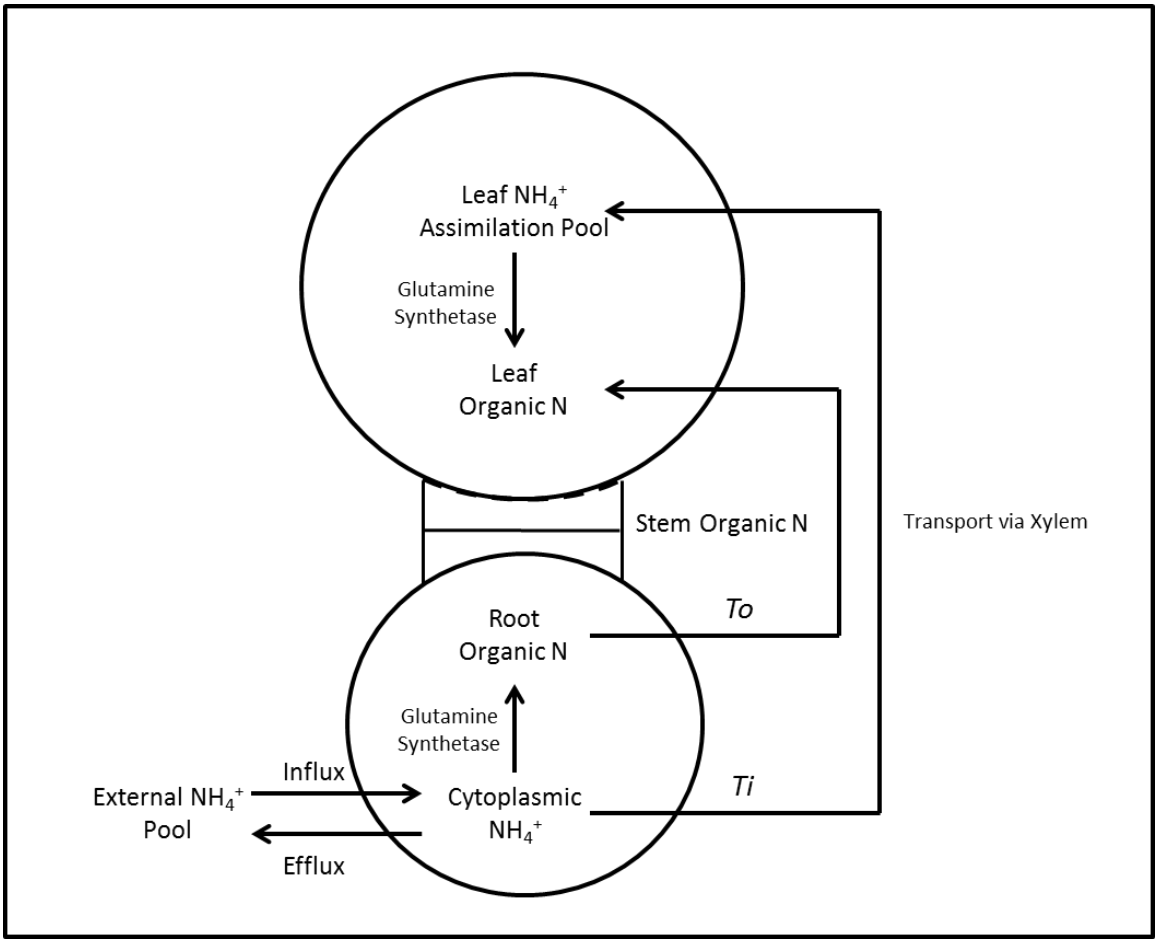

Figure 2.1. Plant nitrogen fluxes during the uptake of ammonium (NH_4^+) from the substrate pool and assimilation into either root of leaf organic sink pools. Arrows refer to unidirectional fluxes of nitrogen between pools. *To* = organic nitrogen translocated to the leaves, *Ti* = inorganic nitrogen translocated to the leaves. Stem tissue is assumed not to assimilate ammonium and receives organic nitrogen from either the roots (via xylem) or the leaves (via phloem), or a mixture of the two.

Since discrimination against ^{15}N occurs in the roots, cytoplasmic inorganic nitrogen will become enriched in ^{15}N (Evans *et al.* 1996; Yoneyama *et al.* 2001). Because unassimilated nitrogen from the cytoplasmic pool is the source of inorganic nitrogen transported from the root to the shoot, leaf nitrogen will be enriched relative to the root (Evans *et al.* 1996; Yoneyama *et al.* 2001). Cycling across the plasma membrane and partitioning of nitrogen assimilation can therefore be estimated from knowledge of dry masses, nitrogen concentration and $\delta^{15}N$ values of roots, stems and leaves. In this paper, I outline a mass balance nitrogen isotope model that uses source-to-sink differences in $\delta^{15}N$ of plant tissues to discern how changes in NH_4^+ and CO_2 supply impact nitrogen isotope discrimination in hydroponically grown black cottonwood (*Populus trichocarpa* Torr. & Gray).

2.3. Materials and methods

Common garden stock of three representative, latitudinally dispersed populations of black cottonwood from Jasper River, OR (44°N), Quesnel River, BC (52°N) and Bell-Irving River, BC (56°N) were obtained and pooled into treatments. Uniform, 5 cm cuttings (N = 4) were rooted and grown in a modified Johnson's (Johnson *et al.* 1957) hydroponic medium plus either 100 µM or 200 µM $(NH_4)_2SO_4$, buffered at pH 6.4-6.8 by adding excess powdered $CaCO_3$. Final nutrient composition, excluding $Ca(NO_3)_2$, was: 200 µM KH_2PO_4, 200 µM K_2SO_4, 100 µM $MgSO_4$, 100 µM $CaSO_4$, and micronutrients: 5 µM Cl, 2.5 µM B, 0.2 µM Mn, 0.2 µM Zn, 0.05 µM Cu, and 50 µM Fe^{2+}. Plants were grown for 6 weeks in two growth chambers (Conviron E-15, Winnipeg, MB) with either ambient (400 µL L^{-1}) or enriched (800 µL L^{-1}) CO_2 (monitored via a Vaisala GMM220) under a 16 hr photoperiod (22 °C:20 °C day: night), equivalent PPFD (450 µmol m^{-2} s^{-1}) and constant humidity. Both growth chambers shared the same nutrient solutions (each 500 L) which were drawn from common reservoirs and circulated through the media trays in each chamber. Ammonium concentration was closely monitored to prevent substrate depletion using the phenol-hypochlorite method (Solorzano 1969). As ammonium concentration approached 10% depletion, the hydroponic solution was replaced with fresh solution to avoid any significant change in its isotopic composition.

2.3.1. Isotope analysis

After 6 weeks of growth, plants were separated into leaves, stems and roots, then frozen in liquid nitrogen and stored at -20°C. Samples were freeze-dried and weighed for dry mass, before grinding to a fine powder using a ball-mill (Fritsch Laborgeratebau, Terochem Scientific). From prepared leaf and root samples, 3 ±0.1 mg was weighed into tin capsules (Elemental Microanalysis Ltd., 8x5 mm, D1008) and analyzed for nitrogen isotopes and nitrogen concentration on a Europa ANCA-SL preparation module and a Europa Hydra 20/20 isotope ratio mass spectrometer (University of California Stable Isotope Facility, Davis, CA). A subset of nine stem samples was analyzed for nitrogen isotope composition on Thermo Finnigan Delta plus and Delta plus XL stable isotope ratio mass spectrometers (University of British Columbia Stable Isotope Facility, Vancouver, BC) to confirm that stem $\delta^{15}N$ is equal to root $\delta^{15}N$. The complete set of stem samples was then analyzed using an Elementar Vario EL Cube elemental analyzer for nitrogen concentration. Isotopic composition is expressed as $\delta^{15}N$:

Equation 2.1 $$\boldsymbol{\delta^{15}N_{sample}} = \left(\frac{\boldsymbol{R_{sample}}}{\boldsymbol{R_{standard}}} - \mathbf{1}\right) \times \mathbf{1000}$$

Where, R_{sample} is the $^{15}N/^{14}N$ isotope ratio of the sample and $R_{standard}$ is the isotope ratio of a known standard ($R_{standard}$ (air) = 0.00365). Isotope discrimination is usually denoted by the equation (Farquhar *et al*. 1989):

Equation 2.2 $$\boldsymbol{\Delta^{15}N_{sample}} = \left[\frac{(\boldsymbol{\delta^{15}N_{source}} - \boldsymbol{\delta^{15}N_{sample}})}{\left(\mathbf{1} + \frac{\boldsymbol{\delta^{15}N_{sample}}}{\mathbf{1000}}\right)}\right]$$

Since $1+ \delta^{15}N_{sample}/1000$ will not deviate significantly from 1 when isotope ratios are close to 0 ‰, discrimination ($\Delta^{15}N$) is well approximated by $\delta^{15}N_{sample} - \delta^{15}N_{source}$ (Evans 2001; Pritchard and Guy 2005; Kalcsits and Guy 2013). Here, however, leaf, root and stem $\delta^{15}N$ were first converted to discrimination values as per equation 2.2 for model calculations. The $\delta^{15}N$ of the ammonium salt in the hydroponic solution was -0.96±0.12‰.

2.3.2. Statistical analysis of measured variables

Analysis of variance with unequal observations per treatment was used to compare treatment means of biomass, root:shoot ratio, $\Delta^{15}N$, nitrogen concentration and total nitrogen of leaf and root tissues. The statistical model is as follows:

Equation 2.3 $\boldsymbol{Y_{ij} = \mu + \alpha_i + \tau_j + \beta_{ij} + e_{ij}}$

where, μ is the overall mean response, α_i is the effect due to the *i*-th concentration of NH_4^+, τ_j is the effect due to the *j*-th level of CO_2 and β_{ij} is the effect due to any interaction between NH_4^+ concentration and CO_2 level. Analysis of variance procedure was carried out using Graphpad Prism 6 (La Jolla, CA, USA) to obtain estimates of the means followed by Tukey's multiple comparison tests to separate means. Where necessary, data were log transformed to meet assumptions of homogeneity of variance and normality. Differences between treatments described as significant are those where $P < 0.05$.

2.3.3. Calculation of nitrogen-use traits from biomass, nitrogen concentration and nitrogen isotope composition

Using an isotope mass balance approach, a number of time-integrated nitrogen-use traits can be calculated for plants grown under controlled hydroponic conditions (See Table 2.1 for definitions of abbreviations) assuming:

1. There is minimal loss of organic nitrogen from root or shoot.
2. There is minimal translocation of nitrogen from shoot to root.
3. The $\Delta^{15}N$ of the organic fraction in the root is equal to the bulk tissue $\Delta^{15}N$
4. Assimilation is restricted to roots and leaves.
5. Carry-over of isotopically distinct nitrogen in propagules (seeds or cuttings) is inconsequential or already corrected for.

Based on assumption 1, the assimilation-averaged net flux of inorganic nitrogen across the root is equal to the total plant nitrogen divided by the root biomass:

Table 2.1. List of abbreviations used in the text

Variables	Definitions
$\delta^{15}N$	The ratio of a sample $^{15}N/^{14}N$ ratio divided by an international standard (air N_2) $^{15}N/^{14}N$ ratio minus 1. This is expressed as per thousands (‰).
$\Delta^{15}N_i$	Isotope discrimination for a sample or pool (*i*) expressed as the difference between the source and product $\delta^{15}N$ divided by 1 minus the product divided by 1000
f_i	The fraction of nitrogen that is *i* for a given nitrogen pool.
N_i	The nitrogen content of a sample calculated from the biomass and analyzed nitrogen concentration (µmol).
$[N]_i$	The nitrogen concentration of a sample (µmol mg dw^{-1}).
Net Flux	Total plant nitrogen expressed as per unit of root biomass.
$N_{leaf\ pool}/N_{total}$	The fraction of plant nitrogen coming from the general leaf nitrogen pool. This includes a fraction of stem nitrogen and all leaf nitrogen.
Ti/*Tt*	The proportion of leaf nitrogen (*Tt*) that was transported to the leaves as inorganic nitrogen (*Ti*) (expressed as a fraction).
P_{root}	The proportion of plant assimilation occurring in the roots (expressed as a fraction).
E/*I*	Efflux/influx across the plasma membrane of the root. Efflux is equal to the net loss of inorganic nitrogen from the root. Influx is equal to the gross influx into the root from the substrate.
Δ_{enz}	The discrimination factor of the assimilatory enzyme.

Equation 2.4 $$\boldsymbol{Net\ Flux\ (\mu mol\ N\ mg\ dw^{-1}) = \frac{N_{total}}{Biomass_{root}}}$$

The mass balance equation for plant $\Delta^{15}N$ is:

Equation 2.5 $$\boldsymbol{\Delta^{15}N_{plant} = (f_{root} \times \Delta^{15}N_{root}) + (f_{stem} \times \Delta^{15}N_{stem}) + (f_{leaf} \times \Delta^{15}N_{leaf})}$$

where, $\Delta^{15}N_i$ is discrimination and f_i is equal to the fraction of tissue nitrogen contributing to overall plant nitrogen content. Although the plant was treated as having three major organs (Figure 2.1), the fourth assumption recognizes the leaves and roots as the major sites of nitrogen assimilation. Stem $\Delta^{15}N$ can be predicted by a mass balance equation using the leaf and root $\Delta^{15}N$ values as end members:

Equation 2.6 $$\boldsymbol{\Delta^{15}N_{stem} = (\Delta^{15}N_{root} \times f_{stem\ root}) + (\Delta^{15}N_{leaf} \times f_{stem\ leaf})}$$

that can be rearranged to yield the fraction of stem N that is from the roots ($f_{stem\text{-}root}$) or the leaves ($f_{stem\text{-}leaf}$). For the fraction from leaves, and noting that $f_{stem\ root} = 1 - f_{stem\ leaf}$,

Equation 2.7 $$\boldsymbol{f_{stem\ leaf} = \frac{(\Delta^{15}N_{stem} - \Delta^{15}N_{root})}{(\Delta^{15}N_{leaf} - \Delta^{15}N_{root})}}$$

To partition between leaf and root nitrogen pools and their isotopic signatures, the contributions of each to total plant nitrogen content needs to be determined. The proportion of total plant nitrogen found in the leaf pool ($N_{leaf\ pool}/N_{total}$) is then a function of plant tissue nitrogen concentration and tissue mass:

Equation 2.8 $$\boldsymbol{\frac{N_{leaf\ pool}}{N_{total}} = \frac{Biomass_{leaf} \times [N]_{leaf} + f_{stem-leaf} \times Biomass_{stem} \times [N]_{stem}}{Biomass_{plant} \times [N]_{plant}}}$$

where, $[N]_i$ is the bulk tissue nitrogen concentration. $\Delta^{15}N_{plant}$ can then be simplified as:

Equation 2.9 $$\boldsymbol{\Delta^{15}N_{plant} = \frac{N_{leaf\ pool}}{N_{total}} \times \Delta^{15}N_{leaf} + \left(1 - \frac{N_{leaf\ pool}}{N_{total}}\right) \times \Delta^{15}N_{root}}$$

Most root nitrogen will be organic ($\Delta^{15}N_{root\ organic}$), but some small fraction ($f_{root\ inorganic}$) will be inorganic ($\Delta^{15}N_{root\ inorganic}$). Consequently, $\Delta^{15}N_{root}$ can be expanded and rearranged to yield $\Delta^{15}N_{root\ organic}$:

Equation 2.10 $$\boldsymbol{\Delta^{15}N_{root\ organic} = \frac{\Delta^{15}N_{root} - \Delta^{15}N_{root\ inorganic} \times (f_{root\ inorganic})}{(1 - f_{root\ inorganic})}}$$

Relative to $\Delta^{15}N_{root\ organic}$, $\Delta^{15}N_{root\ inorganic}$ is increased by the discrimination factor associated with the assimilatory enzyme (Δ_{enz}; here taken to be 16.8‰ for glutamine synthetase). For present purposes, however, I set $f_{root\ inorganic}$ to zero as per assumption #3 but emphasize that this assumption would be unnecessary if $f_{root\ inorganic}$ were known.

To partition assimilation, it was assumed that all NH_4^+ translocated to the shoot (*Ti*; Figure 2.1) enters the xylem in isotopic equilibrium with the root cytoplasmic pool (i.e., $\Delta^{15}N_{xylem\ inorganic} = \Delta^{15}N_{root\ inorganic}$). Organic nitrogen delivered to the shoot (*To*; Figure 2.1) is assumed to not differ isotopically from root-assimilated organic nitrogen. Therefore, net leaf discrimination is the mass balance of $\Delta^{15}N_{root\ inorganic}$ and $\Delta^{15}N_{root\ organic}$ and can be calculated as:

Equation 2.11 $$\boldsymbol{\Delta^{15}N_{leaf}} = \boldsymbol{\Delta^{15}N_{xylem\ inorganic}} \times \left(\frac{Ti}{Tt}\right) - \boldsymbol{\Delta^{15}N_{root\ organic}} \times \left(1 - \frac{Ti}{Tt}\right)$$

where, *Ti*/*Tt* is equal to the proportion of total leaf pool nitrogen (*Tt*; Figure 2.1) transported as inorganic nitrogen from the roots and assimilated in the leaves.

As is the case for inorganic nitrogen in root cytoplasm, $\Delta^{15}N_{xylem\ inorganic}$ exceeds $\Delta^{15}N_{root\ organic}$ by an amount equal to Δ_{enz}. By substituting $\Delta^{15}N_{xylem\ inorganic}$ in equation 11 with $\Delta^{15}N_{root\ organic} + \Delta_{enz}$, *Ti*/*Tt* can then be calculated by rearrangement:

Equation 2.12 $$\frac{Ti}{Tt} = \frac{\left(\boldsymbol{\Delta^{15}N_{leaf}} - \boldsymbol{\Delta^{15}N_{root\ organic}}\right)}{\boldsymbol{\Delta_{enz}}}$$

According to equation 2.12, if the difference between leaf $\Delta^{15}N$ and root $\Delta^{15}N$ is equal to Δ_{enz}, all leaf nitrogen must be assimilated in the leaves and no organic nitrogen is translocated from the roots. Likewise, if the difference is 0, no inorganic nitrogen is translocated from the roots. Given assumption 2, the proportion of total plant nitrogen that was assimilated in the leaves is a product of $N_{leaf\ pool}/N_{total}$ and *Ti*/*Tt*. The remaining fraction of plant nitrogen must then be assimilated in the roots (P_{root}):

Equation 2.13 $$\mathbf{P}_{root} = \mathbf{1} - \left(\left(\frac{\mathbf{N}_{leafpool}}{\mathbf{N}_{total}} \right) \times \frac{Ti}{Tt} \right)$$

Because *Ti* removes nitrogen from the cytoplasmic pool that then becomes unavailable to root assimilation or efflux (*E*), whole plant discrimination ($\Delta^{15}N_{plant}$) is proportional to efflux over influx (*E*/*I*) and P_{root}:

Equation 2.14 $$\mathbf{\Delta^{15}N}_{plant} = \mathbf{\Delta}_{enz} \times \frac{E}{I} \times \mathbf{P}_{root}$$

In equation 2.14, as *E*/*I* and P_{root} approach 1, $\Delta^{15}N_{plant}$ approaches the discrimination factor of the enzyme and likewise, as those two variables approach 0, $\Delta^{15}N_{plant}$ approaches 0. This relationship, expressed in terms of tissue $\delta^{15}N$ values, is illustrated in the first panel of Figure 2.2 for realistic ranges of *E*/*I* and P_{root} values. Note that the partitioning of nitrogen assimilation between organs will not itself alter whole-plant or root $\delta^{15}N$ (second panel) but will affect $\delta^{15}N_{leaf}$ (third panel) and, by extension, stems (Equation 2.6), because it determines the portion of total shoot nitrogen that is root assimilated. Also note that $\delta^{15}N_{leaf}$ can be greater than the source if P_{root} and $N_{leaf\ pool}/N_{total}$ are relatively low, and especially if *E*/*I* is also low. Plant and root $\delta^{15}N$ will still be lower than the source in all cases. By rearranging equation 2.14, an estimate of *E*/*I* can be obtained from:

Equation 2.15 $$\frac{E}{I} = \frac{\mathbf{\Delta^{15}N}_{plant}}{\mathbf{\Delta}_{enz} \times \mathbf{P}_{root}}$$

Measures of root and leaf assimilation activities are obtained by calculating the amount of plant nitrogen derived from either roots or leaves and expressing it as a function of the biomass of either roots or leaves:

Equation 2.16 $$\boldsymbol{Root\ Assimilation\ Activity}\ (\boldsymbol{\mu mol\ N\ mg\ dw^{-1}}) = \frac{\mathbf{N}_{total} \times \mathbf{P}_{root}}{\boldsymbol{Biomass}_{root}}$$

Equation 2.17 $$\boldsymbol{Leaf\ Assimilation\ Activity}\ (\boldsymbol{\mu mol\ N\ mg\ dw^{-1}}) = \frac{\mathbf{N}_{total} \times (\mathbf{1} - \mathbf{P}_{root})}{\boldsymbol{Biomass}_{leaf}}$$

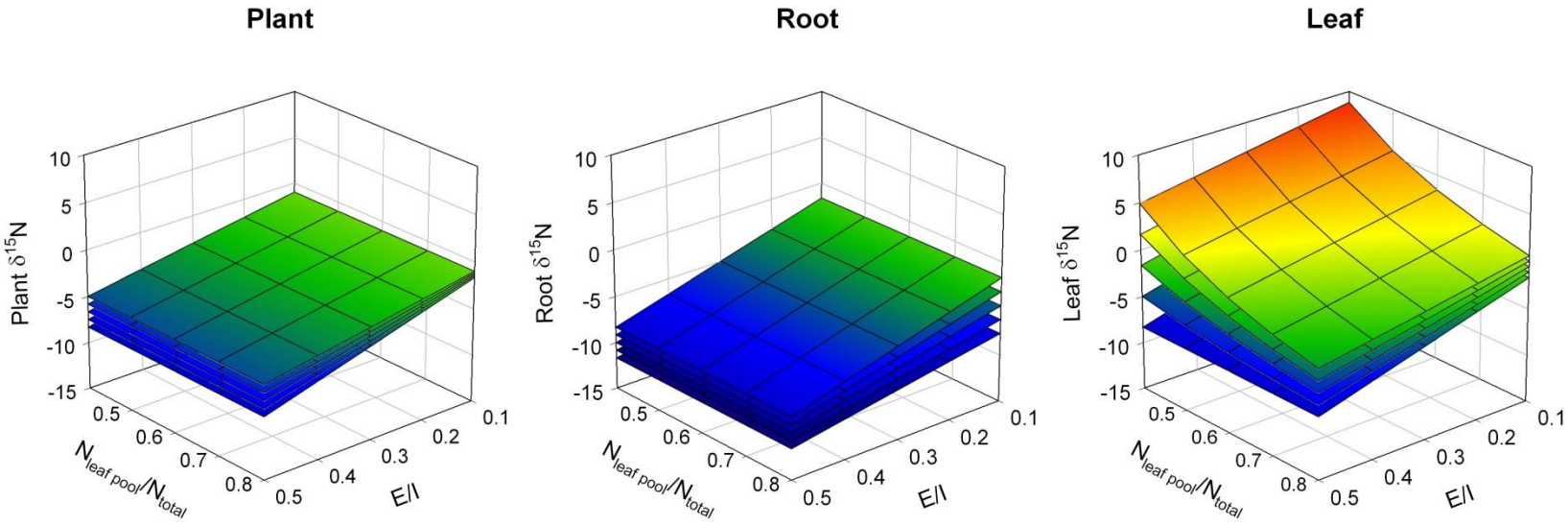

Figure 2.2. Predicted Plant (A), Root (B) and Leaf (C) $\delta^{15}N$ relative to source $\delta^{15}N$ (0‰) as a function of efflux/influx (*E*/*I*), the proportion of nitrogen assimilated in the root (P_{root}), and the partitioning of total plant nitrogen to the leaves ($N_{leaf\ pool}/N_{total}$). Each plane represents a P_{root} value held constant, while $N_{leaf\ pool}/N_{total}$ and *E*/*I* vary (P_{root} is 0.6, 0.7, 0.8, 0.9 and 1 from top to bottom for A & C, and from bottom to top for B).

2.4. Results

2.4.1. Plant growth

Total biomass of black cottonwood was almost two times greater when grown with elevated CO_2 compared with ambient CO_2 (Table 2.2) ($P<0.05$). Mean total biomass was 6.25 g for plants grown under enriched CO_2 conditions and only 3.77 g when grown under ambient CO_2 conditions. Root biomass was significantly lower in low NH_4^+ and ambient CO_2 ($P<0.05$). Root biomass for plants grown under enriched CO_2 conditions was greater than for those grown under ambient conditions. For plants grown under low NH_4^+, leaf biomass at elevated CO_2 was more than two times greater than ambient CO_2 ($P<0.05$). In contrast, the difference in leaf biomass between plants in ambient and enriched CO_2 conditions under high NH_4^+ was not significant. Although NH_4^+ and CO_2 supply affected biomass accumulation, root:shoot ratio was not significantly different between treatments.

2.4.2. Tissue nitrogen concentration

Mean nitrogen concentration decreased in plants grown under elevated CO_2 (Table 2.2; $P<0.05$). Plant nitrogen concentration was 2.4 µmol mg dw^{-1} and 2.76 µmol mg dw^{-1} in elevated and ambient CO_2, respectively. Plant nitrogen concentration was also affected by nitrogen supply, being 2.5 and 2.66 µmol mg dw^{-1} for low and high NH_4, respectively. Root nitrogen concentration was greater when grown with low NH_4^+. Plants grown with elevated CO_2 had a lower root nitrogen concentration than under ambient CO_2 when grown with high NH_4^+ supply ($P<0.05$). Interestingly, root nitrogen concentration was greater than leaf nitrogen concentration for plants grown with low NH_4^+, but the opposite was true for plants grown with high NH_4^+ supply. Plants grown in elevated CO_2 had lower leaf nitrogen concentration ($P<0.05$). Leaf nitrogen concentration was greater when grown at high NH_4^+ supply ($P<0.05$), being highest in plants grown with high NH_4^+ and ambient CO_2, and lowest in low NH_4^+ and elevated CO_2 ($P<0.05$). Stem nitrogen concentration was considerably less than either root or leaf tissue. Stem nitrogen concentration was approximately 20% greater in plants grown

Table 2.2. Biomass, nitrogen concentration and $\Delta\delta^{15}N$ of roots, leaves and stems from hydroponically grown *Populus trichocarpa* with 200 or 400 µM NH_4^+ and ambient or enriched CO_2 levels. Letters denote significant differences between treatment means determined by Tukey's Test. n/s = not significant.

* Stem $\delta^{15}N$ averaged -4.38 ±0.54‰ (±SE, N=9)

CO_2 Level	Ambient (400 ppm)		Elevated (800 ppm)	
$[NH_4^+]$(µM)	200	400	200	400
Plant Biomass (g)	3.39 ±0.541 a	4.16 ±0.720 a	7.16 ±1.128 b	5.33 ±0.812 ab
Plant [N] (µmol mg dw^{-1})	2.69 ±0.045 b	2.84 ±0.024 a	2.31 ±0.083 d	2.49 ±0.034 c
Plant $\delta^{15}N$ (‰)	-3.94 ±0.165 a	-3.61 ±0.186 ab	-3.45 ±0.099 b	-3.48 ±0.133 b
(Plant $\Delta^{15}N$)	(2.99)	(2.66)	(2.50)	(2.53)
Root Biomass (g)	0.47 ±0.088 a	0.60 ±0.100 ab	1.00 ±0.214 b	0.69 ±0.128 b
Root [N] (µmol mg dw^{-1})	2.92 ±0.026 a	2.63 ±0.038 b	2.92 ±0.051 a	2.44 ±0.033 c
Root $\delta^{15}N$ (‰)	-4.75 ±0.144 a	-4.57 ±0.176 ab	-4.21 ±0.109 b	-4.30 ±0.121 b
(Root $\Delta^{15}N$)	(3.81)	(3.63)	(3.26)	(3.35)
Stem Biomass (g)	1.21 ±0.208 a	1.09 ±0.184 a	1.83 ±0.290 b	1.39 ±0.203 ab
Stem [N] (µmol mg dw^{-1})	1.01 ±0.038a	1.37 ±0.036b	1.17 ±0.042a	1.37 ±0.037b
Leaf Biomass (g)	1.65 ±0.278 a	2.47 ±0.447 ab	4.23 ±0.798 c	3.25 ±0.503 bc
Leaf [N] (µmol mg dw^{-1})	2.41 ±0.108 b	3.02 ±0.058 a	1.78 ±0.124 c	2.56 ±0.063 b
Leaf $\delta^{15}N$ (‰)	-2.69 ±0.144 b	-2.97 ±0.225 ab	-2.31 ±0.130 bc	-2.72 ±0.188 b
(Leaf $\Delta^{15}N$)	(1.73)	(2.02)	(1.35)	(1.76)
Root N (% total nitrogen)	21.3±0.93 a	14.8±1.73 b	24.2±0.88 a	15.1±0.86 b
Stem N (% total nitrogen)	20.6±2.63 a	13.6±3.67 b	16.7±1.55 ab	15.8±1.67 ab
Leaf N (% total nitrogen)	58.1±1.99 a	71.6±2.53 b	59.1±1.07 a	69.1±1.51 b
Root: Shoot Ratio	0.144 ±0.013 n/s	0.157 ±0.009 n/s	0.151 ±0.016 n/s	0.126 ±0.011 n/s

with high NH_4^+ where nitrogen concentrations were 1.08 and 1.37 µmol mg dw^{-1} for low and high NH_4^+, respectively.

Leaf nitrogen content (Table 2.2) accounted for approximately 58% of total plant nitrogen content in plants grown in low NH_4^+ conditions and approximately 70% in high NH_4^+ conditions ($P<0.05$). Leaf nitrogen content did not vary between ambient or elevated CO_2 treatments. Root nitrogen content was greater under low NH_4^+ and not significantly different between CO_2 conditions. Similar to root nitrogen content, stem nitrogen content was lower under high NH_4^+.

2.4.3. Tissue $\delta^{15}N$

Plant $\delta^{15}N$ (Table 2.2) was more negative under ambient CO_2 than elevated CO_2 (-3.78 to -3.46‰, $P<0.05$). Leaf $\delta^{15}N$ was significantly greater than root $\delta^{15}N$ in all treatments ($P<0.05$). Similar to plant $\delta^{15}N$, leaf $\delta^{15}N$ was more negative in plants grown under ambient CO_2 conditions (-2.89 and -2.52‰ for ambient and elevated CO_2 treatments, respectively, $P<0.10$). Root $\delta^{15}N$ was more negative in plants grown at ambient CO_2 (-4.66 and -4.26‰ for ambient and elevated CO_2 treatments, respectively, $P< 0.05$). NH_4^+ concentration did not significantly affect mean plant, root, or leaf $\delta^{15}N$. Not all stems were analyzed for $\delta^{15}N$ in this experiment, but a subset of nine showed that they did not differ from their respective roots.

2.4.4. Model-derived nitrogen traits

Trait data based on model outputs are presented in Table 2.3. Because roots and stems had the same isotopic composition, $N_{leaf\ pool}/N_{total}$ was equivalent to the per cent total nitrogen found only in leaves. By comparing the difference between leaf and root $\Delta^{15}N$, a measure of the proportion of leaf nitrogen that was assimilated in the root and transferred to the leaf pool (Ti/Tt; Equation 2.12) was obtained. A slightly greater proportion of the total leaf nitrogen pool was root-assimilated in plants grown in high NH_4^+ ($P<0.05$). Root assimilation accounted for 90% and 88% of leaf nitrogen at low and high NH_4^+ supply, respectively. CO_2 level did not affect the proportion of leaf nitrogen assimilated in the root. Partitioning of assimilation (P_{root}; Equation 2.13) was

Table 2.3. Model outputs for hydroponically grown *Populus trichocarpa* with 200 or 400 µM NH_4^+ and ambient or enriched CO_2 levels. Letters denote significant differences between treatment means determined by Tukey's Test. n/s = not significant.

CO_2 Level	Ambient (400 ppm)		Elevated (800 ppm)	
$[NH_4^+]$(µM)	200	400	200	400
$N_{leaf\ pool}/N_{total}$	0.58 ±0.026 a	0.72 ±0.012 b	0.59 ±0.030 a	0.69 ±0.023 b
Ti/*Tt*	0.123 ±0.006 a	0.096 ±0.006 b	0.114 ±0.011 ab	0.095 ±0.010 b
P_{root}	0.931 ±0.004 n/s	0.934 ±0.003 n/s	0.939 ±0.004 n/s	0.939 ±0.005 n/s
E/*I*	0.247 ±0.010 a	0.233 ±0.012 ab	0.204 ±0.006 c	0.225 ±0.008 bc
Net Root Uptake (µmol N uptake mg root dw^{-1})	28.57 ±3.814 n/s	23.01 ±1.421 n/s	27.35 ±6.342 n/s	28.02 ±2.415 n/s
Leaf Assimilation Rate (µmol leaf assimilated N mg leaf dw^{-1})	0.297 ±0.0159 a	0.293 ±0.0184 a	0.225 ±0.0263 b	0.258 ±0.022 b
Root Assimilation Rate (µmol root assimilated N mg root dw^{-1})	15.76 ±1.827 a	18.66 ±1.371 ab	15.91 ±2.296 a	20.31 ±1.702 b

not significantly affected by either NH_4 or CO_2. Approximately 93% of total nitrogen assimilation occurred in roots. Although not quite significant (P=0.077), there was notably a greater proportion (93.9%) of assimilation occurring in the roots of plants grown with elevated CO_2.

I calculated efflux/influx ratio (*E*/*I*) from P_{root}, plant $\Delta^{15}N$ and the instantaneous discrimination factor for glutamine synthetase (Equation 2.9). There was a significant effect of CO_2 concentration on *E*/*I* at low NH_4^+ (P<0.05) but not at high (Table 2.3). Mean *E*/*I* was not significantly different between low and high NH_4^+ concentrations. Net flux (µmol N uptake mg root dw^{-1}; Equation 2.4) was not significantly different between treatments. However, leaf assimilation activity (µmol leaf assimilated N mg leaf dw^{-1}; Equation 2.17) was greatest in plants grown under ambient CO_2 conditions, which was 20% higher than under elevated CO_2 conditions. Since a large portion of nitrogen was assimilated in roots and roots comprise a small proportion of the overall plant, root assimilation (µmol root assimilated N mg root dw^{-1}; Equation 2.16) was more than an order or magnitude greater than leaf assimilation. Root assimilation was greater when plants were grown with high NH_4^+ and did not differ between elevated and ambient CO_2 treatments (P<0.05).

The populations of trees used in this study harbor considerable genetic and phenotypic variation. The individual root and leaf $\delta^{15}N$ values from plants in all treatments are plotted relative to $N_{leaf\ pool}/N_{total}$ in Figure 2.3A. Leaf (and thus total plant) $\delta^{15}N$ tended to be more negative at higher $N_{leaf\ pool}/N_{total}$ (r=0.233; P=0.007). This tendency was most pronounced in the low NH_4^+/high CO_2 treatment (not shown), which had the least discrimination (and therefore lowest *E*/*I*) overall, and is consistent with both $N_{leaf\ pool}/N_{total}$ and *E*/*I* increasing in the plants that were more N replete. Across all treatments there was a significant relationship (r=0.241; P=0.006) between leaf-root $\delta^{15}N$ and $N_{leaf\ pool}/N_{total}$ (Figure 2.3B). As $N_{leaf\ pool}/N_{total}$ increased, the difference between leaf and root $\delta^{15}N$ decreased, consistent with model predictions (Figure 2.2).

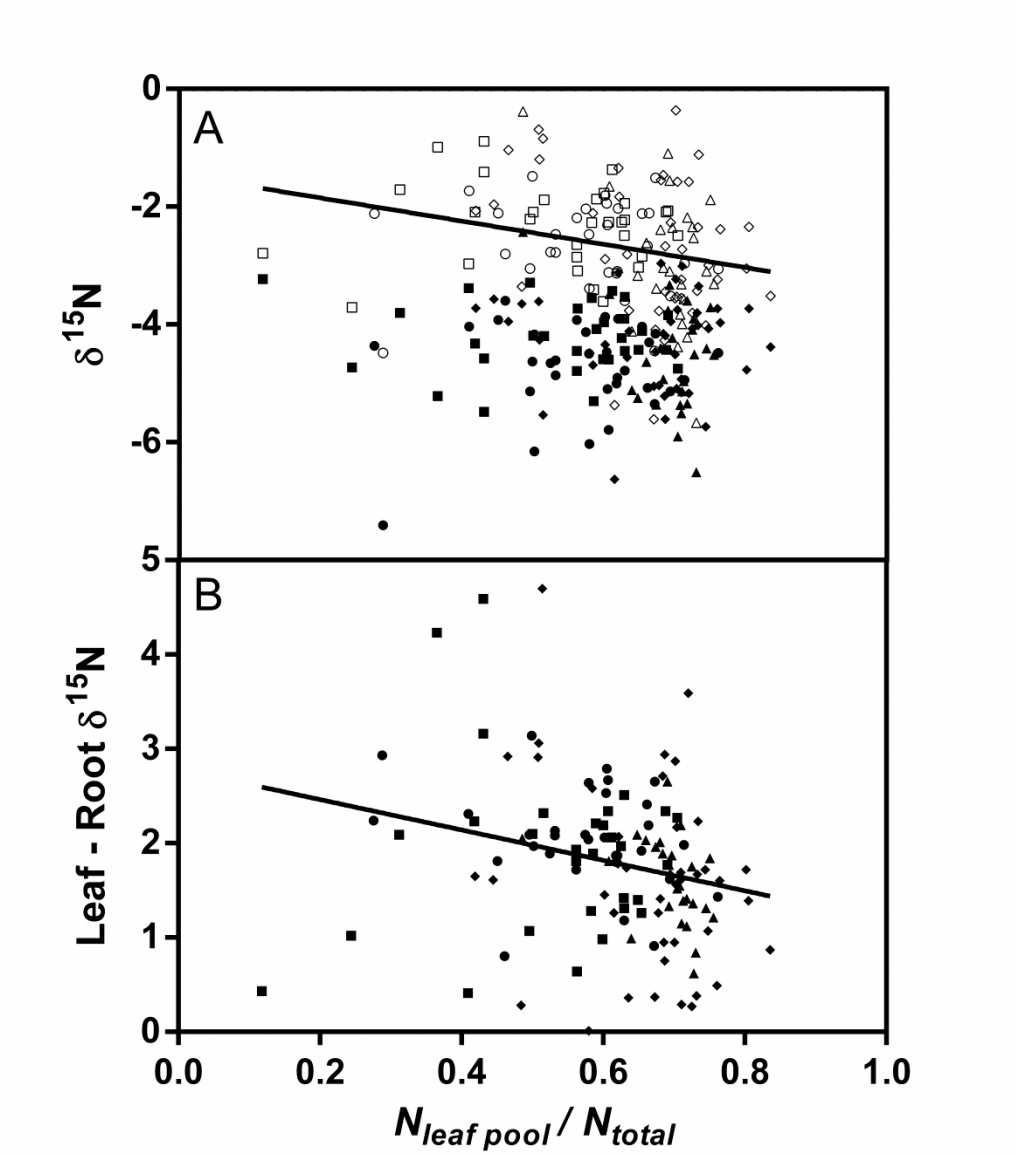

Figure 2.3. A) $\delta^{15}N$ of leaves (empty symbols) and roots (filled symbols), and B) the difference between them (Leaf – Root $\delta^{15}N$), plotted against $N_{leaf\ pool}/N_{total}$ for *Populus trichocarpa* ramets grown with 200 µM NH_4^+ and ambient CO_2 (circles), 400 µM NH_4^+ and ambient CO_2 (squares), 200 µM NH_4^+ and elevated CO_2 (triangles), and 400 µM NH_4^+ and elevated CO_2 (diamonds). Line represent a significant linear correlation between A) Pooled leaf $\delta^{15}N$ and $N_{leaf\ pool}/N_{total}$ (r=0.233; P = 0.007) and B) Pooled leaf-root $\delta^{15}N$ and $N_{leaf\ pool}/N_{total}$ (r=0.241; P=0.006)

2.5. Discussion

The objective was to determine whether changes in nitrogen and carbon supply impact nitrogen isotope composition. Here, I proposed a nitrogen isotope mass balance model explaining whole plant and organ-level nitrogen isotope composition under steady-state conditions to show that changes to nitrogen and carbon supply can alter $\delta^{15}N$ of roots and leaves, and modify nitrogen fluxes, assimilation and allocation in black cottonwood. Under carefully controlled conditions, this nitrogen mass balance approach can provide assimilation-averaged measures of nitrogen-use traits that are otherwise difficult to obtain. These estimates are subject to the value taken for Δ_{enz}, which has only rarely been measured, and the legitimacy of our other assumptions. Although these assumptions may contribute to potential error in the model, they cannot account for the magnitude of the treatment differences in model outputs observed here.

2.5.1. Model assumptions

The first assumption was that there is no loss of assimilated nitrogen from the plant during active, non-limiting growth. Similar to other nitrogen transformations, there are likely to be kinetic isotopic effects associated with either volatilization of NH_3 or nitrous oxide emissions from leaves that discriminate against ^{15}N. The reported discrimination factor for volatilization of NH_3 is approximately 40‰ (Mariotti *et al.* 1982). Although there have been observed losses of nitrogen from leaves as NH_3, it is unlikely to be large. Schjoerring & Mattson (2001) reported that 1-4% of shoot nitrogen was volatilized as NH_3 from a variety of crop plants and, importantly, that the majority of this loss occurred during senescence, which was carefully avoided.

Exudation of organic nitrogen from the root would occur primarily during root mortality or during stress events that result in loss of stability of the plasma membrane (Britto *et al.* 2001; Kronzucker *et al.* 2001). Robinson *et al.* (1998) suggested that exudation of enriched organic nitrogen causes roots to have lower $\delta^{15}N$ than leaves. In order to significantly alter isotopic mass balance in this way, organic nitrogen exudation from roots would need to be compound-specific and proportionately high enough to

impact $\delta^{15}N$. The difference in $\delta^{15}N$ between different forms of organic N rarely exceeds 10‰ (Werner & Schmidt 2002; Gauthier *et al.* 2012). Hale *et al.* (1975) provided a range of exudation rates from roots in multiple species, and in all cases, exudation was in the µmol plant^{-1} week^{-1} range. Carvalhais *et al.* (2011) reported similar amino acid exudation rates in maize (*Zea mays* L.). In the case of poplar grown here, this would amount to an inconsequential fraction of nitrogen lost from the plant.

Our second key assumption is the absence of translocation of nitrogen from the shoot to the root. In contrast, Comstock (2001) included translocation from the shoot to the root in their intra-plant discrimination model. Although some organic nitrogen may move into roots via the phloem, this flux will likely be inconsequential to the mass nitrogen budget during active growth, allowing simplification of the model and calculation of the partitioning of assimilation between roots and leaves. Although this condition may not be met under many circumstances, particularly in senescent or perennial plants, it is likely valid for young, rapidly growing poplar harvested prior to any leaf senescence. The concurrence between root and stem $\delta^{15}N$ values, in contrast to leaves (Table 2.2), is also consistent with a lack of organic nitrogen transfer to the roots.

As per our third assumption, root inorganic nitrogen was not separated from organic nitrogen, and the measured tissue $\delta^{15}N$ value was considered to be equal to the organic fraction (i.e., I set $f_{root\ inorganic}$ to zero in equation 2.10). Since cytoplasmic inorganic nitrogen in the root would be enriched relative to the root organic nitrogen pool, this would enrich root $\delta^{15}N$ and cause an underestimation of the isotopic difference between root- and leaf-assimilated organic nitrogen. In turn, the model underestimates Ti/Tt, with consequent effects on the calculation of P_{root} and E/I. Sampling for cytoplasmic nitrogen content or isotope composition at harvest is possible; however, single time-point measurements are not likely to be stable and may not coincide with time-integrated values. The fraction of inorganic nitrogen in the roots has been shown to vary from 1 to 10%, depending on growing conditions and nitrogen source (Evans *et al.* 1996; Black *et al.* 2002; Yoneyama *et al.* 2001). This fraction increases as substrate concentration increases. Yoneyama *et al.* (2001), for example,

found that the cytoplasmic inorganic nitrogen pool can account for as much as 10% of the overall nitrogen in the root when grown with either 2 or 8 mM NO_3^-. Evans *et al.* (1996) reported much lower fractions of inorganic nitrogen (approximately 1-2%) in roots of tomato (*Solanum lycopersicum* L.) grown on an ecologically relevant NH_4^+ concentration (50 µM). If also true of black cottonwood grown at 200-400 µM NH_4^+, then this could only change the root $\delta^{15}N$ by a few tenths of 1‰ which would not impact treatment ranks and would have just a small impact on model estimates.

In most scenarios, nitrogen assimilation occurs primarily in the roots and leaves of plants (assumption #4). Although assimilation might also occur in stem tissue, the assimilatory volume would be small in comparison to roots and leaves. There is no information on glutamine synthetase activity in stem tissue, but Hunter (1985) measured nitrate reductase activity in soybean (*Glycine max* (L.) Merr.) stems and found less than 3% of the activity seen in leaves. In poplar, Black *et al.* (2002) found a similarly low proportion of nitrate reductase in stems. Thus, I assigned nitrogen assimilation to either roots or leaves, and to treat stem nitrogen as originating from one or the other or both. In this particular experiment, stem $\delta^{15}N$ was not different from root $\delta^{15}N$, and therefore all stem nitrogen was presumed to have originated in the root and was assigned to that pool.

Finally, with regard to our fifth model assumption, the nitrogen in the stem cuttings used to propagate our plants had a $\delta^{15}N$ very close to that of the source (within 1‰) and was well diluted by substantial new growth. Hence, corrections for carryover of pre-existing nitrogen were unnecessary.

2.5.2. Plant growth

As expected, increased growth was observed under higher CO_2 conditions. Increased growth under elevated CO_2 has been observed in many plant systems, including poplar (Stitt & Krapp, 1999; Hymus *et al.* 2001). However, plants have been shown to "acclimate" to elevated CO_2 (Sage *et al.* 1989). When nitrogen supply cannot keep pace with increased carbon supply, photosynthesis is inhibited and the increased

rates of growth from elevated CO_2 cannot persist (Reich *et al.* 2006). For enhanced growth to be maintained under elevated CO_2, nutrient supply must match demand. In an open nutrient environment, with minimal depletion of nutrient supply, elevated CO_2 should enhance plant growth. Overall, there were no indications of nitrogen deficiency in any treatments. The high plant nitrogen concentrations observed here (Table 2.2) were sufficient when compared to previous literature on poplar (DesRochers *et al.* 2006). Leaf nitrogen concentration was lower under elevated CO_2, likely reflecting a decrease in photosynthetic protein content (e.g., Rubisco) coupled with nitrogen dilution from increases in growth and starch accumulation (Stitt & Krapp 1999).

The connection between growth and nitrogen supply is inherent in the ability to match plant growth with nitrogen supply. In this experiment, growth was reduced under low nitrogen conditions but nitrogen concentration was affected to a lesser degree. A similar buffering of nitrogen concentration has been reported in Arabidopsis where biomass increased by an order of magnitude more than tissue nitrogen concentration (Chardon *et al.* 2010). Tschoep *et al.* (2009) also observed a decrease in nitrogen concentration in Arabidopsis grown with low nitrogen compared to non-limiting nitrogen. Growth was the plastic response to low NH_4^+ rather than decreased nitrogen concentration (Tschoep *et al.* 2008). Similar to our results, Coleman *et al.* (1993) reported decreased tissue nitrogen concentration under increased CO_2 and increased nitrogen concentration with increased nitrogen availability in Amaranth (*Amaranthus sp.*), but changes in biomass were far more pronounced.

2.5.3. Interpretation of plant, leaf and root $\Delta^{15}N$

Because nitrogen assimilation is a discriminatory step, whole plant $\delta^{15}N$ should never be greater than substrate $\delta^{15}N$, as was always the case in the present study. Although theoretically expected, consistent ^{15}N depletion of the plant product has only been shown in a handful of studies (Yoneyama *et al.* 2001; Robinson *et al.* 1998, 2001; Yoneyama *et al.* 2001; Kolb & Evans 2003; Pritchard & Guy 2005). Apparently, contrary results (e.g., Evans *et al.* 1996) may be a consequence of the hydroponic design, nitrogen supply regime, mixed nitrogen sources or organ-specific sampling. Because of

restricted mixing and long diffusion distances; sand, perlite or any other structured soil medium could cause localized ^{15}N enrichment of substrate around the roots. Plants may show reduced discrimination if diffusion becomes rate limiting. In either soil or liquid media, periodic addition of nitrogen to maintain a constant supply without completely replacing residual nitrogen can result in an enrichment of the solution over time. In our work, the hydroponics solution was derived from a single source with uniform $\delta^{15}N$ values and minimal depletion of the substrate.

Since leaf pool nitrogen is considered a closed system (i.e., no loss of nitrogen), $\Delta^{15}N_{leaf}$ is the mass balance between $\Delta^{15}N_{xylem\ inorganic}$ and $\Delta^{15}N_{root\ organic}$ (Equation 2.11) because these are the only sources of leaf nitrogen. Knowledge of the isotopic composition of leaf inorganic nitrogen is inconsequential to the model because the mass balanced sum of leaf-assimilated organic nitrogen ($\Delta^{15}N_{leaf\text{-}assimilated}$) and inorganic nitrogen in the leaves ($\Delta^{15}N_{leaf\ inorganic}$) is equal to the $\Delta^{15}N_{xylem\ inorganic}$. Comstock (2001) assumed that the isotopic composition of the organic fraction assimilated in the leaves was equal to xylem inorganic nitrogen, and this must be true if all inorganic nitrogen translocated through the xylem is assimilated. However, a certain fraction will be unassimilated at any point in time and cytoplasmic inorganic nitrogen will be enriched in ^{15}N relative to leaf-assimilated organic nitrogen. In agreement, Gauthier *et al.* (2012) recently reported that nitrate $\delta^{15}N$ was greater than the organic nitrogen in leaves of rapeseed (*Brassica napus* L.).

Our treatment combinations were designed to modify isotope discrimination by manipulating nitrogen supply relative to demand at physiologically relevant concentrations. Plant $\delta^{15}N$ relative to the substrate was more negative under ambient CO_2 conditions than elevated CO_2 conditions (Table 2.2). This is in contrast to observations of Bassirirad *et al.* (2003) who showed that leaf $\delta^{15}N$ was more depleted under elevated CO_2 conditions in the field. The authors attributed this effect to changes in nitrogen cycling processes in the plant/mycorrhizal system. Here, plants were grown hydroponically without mycorrhizae or nutrient diffusion limitations as discussed above.

2.5.4. Modification of nitrogen supply and demand affects calculated nitrogen use-traits

Physiologically, demand for nitrogen would be lower under ambient CO_2 conditions because of reduced biomass and increased nitrogen concentration. Plants more replete in nitrogen should have higher efflux of unassimilated cytoplasmic inorganic NH_4^+, driven by lower demand. Accordingly, $\Delta^{15}N$ and calculated *E*/*I* were greater in plants grown under ambient CO_2 (Tables 2.2 and 2.3). Under steady-state nutrition, plants grown with elevated CO_2 have greater carbon assimilation (Reich *et al.* 2006) and should be able to supply more energy for NH_4^+ assimilation, reducing efflux. Total nitrogen content was greater in plants grown under elevated CO_2 (Table 2.2). Nitrogen concentration, however, did not affect *E*/*I*. Calculated *E*/*I* values were comparable with previous estimates of *E*/*I* in plants that did not shows signs of NH_4^+ toxicity (Kronzucker *et al.* 1995), as in the present study. Britto & Kronzucker (2002) reported high *E*/*I* associated with ammonium toxicity results in decreased growth and in some cases, mortality. These ratios were estimated at around 0.76 (Britto *et al.* 2001), well above the values reported here.

In contrast to whole plant and root $\delta^{15}N$ values, leaf $\delta^{15}N$ can be greater than the source, as explained earlier. Although most leaves were below -0.96‰, a few exceeded this value (Figure 2.3). The difference between leaf and root $\delta^{15}N$ was greater for plants grown under low NH_4^+ conditions than at high. Yoneyama *et al.* (2001) also found that leaves were enriched in ^{15}N relative to roots in NH_4^+-grown plants. Evans *et al.* (1996) observed a positive difference between leaf and root $\delta^{15}N$ when tomato plants were grown on NO_3^- but not on NH_4^+, reflecting significant nitrate assimilation, but not ammonium assimilation, in shoots of this species. Although NH_4^+ is largely considered to be assimilated in the roots of plants (Raven & Smith 1976), there is evidence suggesting that some portion of it can be translocated to the leaves. Schjoerring *et al.* (2002) reported that approximately 11% of overall nitrogen transported to the shoot was transported as inorganic NH_4^+. The ^{15}N enrichment of leaves relative to roots suggests that in poplar, some NH_4^+ assimilation does occur in the leaves. Ideally, it would have

been desirable to get an estimate of xylem ammonium content and $\delta^{15}N$. However, as for cytoplasmic ammonium, multiple single time-point samples would be required to properly reflect time-integrated values.

At the individual plant level across treatments there was a significant negative correlation between the root-to-leaf difference in $\delta^{15}N$ and $N_{leaf\ pool}/N_{total}$, most obvious within the low NH_4^+ and high CO_2 treatment (Figure 2.3). Such a pattern might emerge from variation in P_{root} (Figure 2.2). Given, however, that P_{root} was essentially invariant with treatment (Table 2.3), from Equation 2.13 it can be concluded that the correlation must reflect opposing variation in both *Ti*/*Tt* and $N_{leaf\ pool}/N_{total}$. As *Ti* would represent a constant proportion of total plant nitrogen if P_{root} is fixed, then it is only differences in the root-to-shoot export of organic nitrogen that can cause *Ti*/*Tt* and $N_{leaf\ pool}/N_{total}$ to vary inversely (Figure 2.1). A greater difference between root and leaf $\delta^{15}N$ indicates a greater proportion of leaf nitrogen is assimilated in the leaves, but in poplar these proportions are apparently not driven by variation in the relative amount of inorganic nitrogen exported from roots, but rather by variation in the relative amount of organic nitrogen that is retained. At the treatment level, *Ti*/*Tt* was unaffected by CO_2 regime but was significantly modified by the nitrogen supply. I calculate that 52.8% and 65.4% ($P<0.0001$) of root-assimilated nitrogen was exported to leaves under low versus high ammonium, respectively.

Our model implementation suggests that approximately 7% of NH_4^+ assimilation occurred in the shoot. Although there may be no active transport of NH_4^+ into the xylem like there is for NO_3^- (Lin *et al.* 2008), some passive movement of NH_4^+ might occur simply through transpirational pull into the xylem and to the shoot. Cernusak *et al.* (2009) reported a relationship between transpiration efficiency and the difference in $\delta^{15}N$ between the whole plant and root. As the difference increased between the plant and root, transpiration efficiency of nitrogen acquisition increased implying a greater fraction of leaf nitrogen assimilated in the leaf. Partitioning of NH_4^+ assimilation appears to be less plastic than traits such as *E*/*I* and leaf and root assimilation activity. At high NH_4^+, partitioning of assimilation would have been expected to increase in leaves if the

assimilatory capacity of the root did not increase proportionally. A greater increase in available plant nitrogen supply might be expected to exceed this capacity and change overall partitioning, either morphologically or biochemically. In poplar, however, increased growth likely precedes changes in allocation of assimilation. In accordance with the former, root:shoot ratio did not differ between treatments (Table 2.2).

2.6. Conclusion

Here, by changing nitrogen and carbon supply to poplar grown under steady-state hydroponics conditions, modulation of nitrogen isotope discrimination was shown at the whole-plant and intra-plant level. Although differences in discrimination were small, treatment level differences were in accordance with expectation. When appropriate simplifying assumptions are made, a tractable model for discrimination allows for the relative comparison of nitrogen-use traits that can be used to identify inter and intra-specific differences in nitrogen fluxes, assimilation and allocation under controlled nitrogen environments. This model can produce time-integrated estimates of nitrogen-use traits such as efflux/influx, partitioning of assimilation and net uptake that are otherwise difficult to assay using more traditional single-point assays.

3. Whole plant and organ level nitrogen isotope discrimination indicates modification of partitioning of assimilation, fluxes and allocation of nitrogen in knockout lines of *Arabidopsis thaliana*

3.1. Summary

The nitrogen isotope composition ($\delta^{15}N$) of plants has potential to provide time-integrated information on nitrogen uptake, assimilation and allocation. Here, existing T-DNA and gamma-ray mutant lines of *Arabidopsis thaliana* L. were used to evaluate the model explaining whole-plant and organ level fractionation of nitrogen isotopes proposed in chapter 2. Nitrate Reductase 2 (*nia2*), Nitrate Reductase 1 (*nia1*) and Nitrate Transporter (*nrt2*) mutant lines and the Col-0 wild type were grown hydroponically under steady-state NO_3^- conditions at either 100 or 1000 µM NO_3^- for 35 days. There were no significant effects on whole-plant discrimination and growth in the assimilatory mutants (*nia2* and *nia1*). Pronounced root versus leaf differences in $\delta^{15}N$, however, indicated that *nia2* had an increased proportion of nitrogen assimilation of NO_3^- in leaves while *nia1* had an increased proportion of assimilation in roots. These observations are consistent with reported ratios of *nia1* and *nia2* gene expression levels in leaves and roots. Greater whole-plant discrimination in *nrt2* indicated an increase in efflux of unassimilated NO_3^- back to the rooting medium. This phenotype was associated with an overall reduction in NO_3^- uptake, and decreased partitioning of NO_3^- assimilation to the leaves, presumably because of decreased symplastic intercellular movement of NO_3^- in the root. Although the results were more varied than expected, they are interpretable within the context of the model presented in chapter 2. Using Arabidopsis knockout lines demonstrates that changes to the capacity to either assimilate or acquire nitrate can modify nitrogen isotope composition of plants.

3.2. Introduction

Reducing the need for nitrogen fertilizer in global agricultural production represents one of the major challenges in the plant sciences. Nitrogen uptake and assimilation is a complex process that is a function of internal demand interacting with external supply. Although the localization and functional role of genes related to nitrogen uptake and assimilation have been well constrained, an integrated method of measuring nitrogen-use during the complete plant life cycle still needs to be developed (Hirel *et al.* 2007). Traditional measures of nitrogen uptake and assimilation are often not conducive to an integrated view of nitrogen use without intensive and careful sampling to reflect temporal and spatial variability in expression, activity and regulation. Using nitrogen isotope composition of plants and plant parts has potential to provide time-integrated information on nitrogen use at the whole plant and organ level.

Nitrogen isotope discrimination is hypothesized to be a function of nitrogen-use and provides an integrated picture of nitrogen uptake and assimilation that cannot be provided by traditional nitrogen-use assays (Evans, 2001). Abiotic stress (Yousfi *et al.* 2009; 2012), nitrogen availability (Chapter 2), source (Evans *et al.* 1996), and CO_2 enrichment (Chapter 2) can all impact whole-plant and organ level nitrogen isotope discrimination. Environmental effects on nitrogen isotope fractionation have been reported that suggests a relationship exists between nitrogen isotope discrimination and external supply and internal demand. Although intraspecific variation in nitrogen isotope discrimination has been reported (Yousfi *et al.* 2009; Robinson *et al.* 1998; Pritchard and Guy, 2005), the underlying reasons for this remain unclear. Several theoretical models have suggested physiological mechanisms responsible for plant and organ level variations in nitrogen isotope composition (Evans, 2001; Comstock, 2001; Robinson, 2001). However, to date few studies have used empirical data to test theoretical models of fractionation. As the understanding of nitrogen isotope discrimination becomes more refined, more accurately constrained parameters contributing to this variation can be estimated.

Any change to nitrogen supply or demand should affect nitrogen isotope discrimination. Changes in whole-plant $\delta^{15}N$ relative to source nitrogen $\delta^{15}N$ (fractionation) arise through isotopic discrimination (Comstock, 2001; Evans, 2001; Robinson, 2001). Discrimination is the kinetically determined process whereby the heavier isotope (^{15}N) is "discriminated" against causing a relatively greater fraction of the lighter isotope (^{14}N) to be incorporated into plant tissues (Handley and Raven 1992; Hayes, 2001). The fractionation for nitrate reductase has been best estimated at about 15‰ (Ledgard *et al.* 1985), where the product of the reaction was -15‰ relative to the substrate. However, recent work has suggested discrimination by nitrate reductase is greater than 15‰ and is closer to 22‰ (Needoba *et al.* 2004; Tcherkez and Farquhar 2006) or 26‰ (Karsh *et al.* 2012). The instantaneous discrimination factor determines the maximum fractionation that can occur if cytoplasmic inorganic nitrogen $\delta^{15}N$ is equal to substrate $\delta^{15}N$. If total consumption of the substrate pool occurs, product $\delta^{15}N$ must be equal to the source $\delta^{15}N$. Isotope fractionation is only observed when there is partial consumption of a substrate pool (Mariotti *et al.* 1981; Comstock, 2001). Organ level isotope fractionation occurs at branch points where there is either loss of substrate or two or more pathways competing for the same substrate (Macko *et al.* 1987; Hayes, 2001; Comstock, 2001; Werner and Schmidt 2002; Tcherkez 2011; Gauthier *et al.* 2012).

In Chapter 2, I proposed a model whereby whole-plant nitrogen isotope discrimination is mainly a function of one inward and three outward fluxes: 1) gross influx from the rooting medium, 2) partial assimilation by root nitrate reductase, 3) efflux of some unassimilated nitrate back to the medium, and 4) export of remaining nitrate to the leaves. The fourth flux, together with the xylem export of root-assimilated nitrogen, is the main determinant of root-shoot differences in $\delta^{15}N$.This comes about because partial assimilation enriches the root cytosolic nitrate pool (Robinson *et al.* 1998). ^{15}N enriched cytosolic nitrate is then transported to the shoot and assimilated in the leaf. No fractionation occurs at the assimilatory step in leaves since there is total

consumption of nitrate. Thus, leaf nitrogen is a mix of root-derived and leaf-derived organic nitrogen with identifiable $\delta^{15}N$ signatures.

Arabidopsis represents an ideal model to evaluate the model describing nitrogen isotope discrimination because of the accessibility to multiple homozygous knockout lines for nitrogen uptake and assimilation genes. Chapter 2 provided evidence for modification of nitrogen isotope discrimination by changing nitrogen supply and/or carbon supply. Although the model was in general agreement, further testing is required to examine the contribution of changes to nitrogen uptake and assimilation patterns on nitrogen isotope composition of plants. Here, existing Arabidopsis T-DNA and gamma-ray mutant lines were used to evaluate a model explaining whole-plant and organ level fractionation of nitrogen isotopes. By using Arabidopsis mutant lines in which genes associated with either nitrate uptake or assimilation have been disrupted, internal nitrate supply and demand may be manipulated manifesting in changes in nitrogen isotope discrimination. Differences in nitrogen isotope discrimination among lines may also provide new information on nitrate uptake and assimilation dynamics in the lines compared to the wild type.

Nitrate reductase is active in both roots and shoots and has two different genes responsible for the overall nitrate reductase activity in Arabidopsis (Wilkinson and Crawford 1993). When both genes are disrupted, Arabidopsis is unable to grow solely on NO_3^- (Wilkinson and Crawford 1993). Nitrate reductase 1 (NIA1) is responsible for 10% of total plant nitrate reductase activity (Wilkinson and Crawford 1993). NIA1 is expressed in both roots and leaves. However, it is expressed in a greater proportion in the leaves than NIA2 (Winter *et al.* 2007). NIA2 is responsible for the other 90% of nitrate reductase activity. NIA2 is expressed in both roots and leaves, but expression is proportionately higher in roots than NIA1 (Winter *et al.* 2007).

Nitrate transporters are a group of plasma membrane-bound proteins that are responsible for nitrate uptake from the substrate and movement of nitrate within the

plant (Glass *et al.* 2002). NRT2.1 and NRT2.2 are considered to be responsible for approximately 60 to 70% of nitrate inducible uptake in Arabidopsis at low to moderate nitrate conditions (Cerezo *et al.* 2001; Li *et al.* 2007). NRT2.1 and NRT2.2 are highly expressed throughout the root including the epidermis, cortex and endodermis (Winter *et al.* 2007; Feng *et al.* 2011). NRT2.1 and NRT2.2 have been shown to have a large role in nitrate uptake. However, there are other nitrate transporters that contribute to overall nitrate uptake, including the entire NRT1 transporter family and other NRT2 genes (Glass *et al.* 2002). Although the description of NRT2.1 and NRT2.2 and their role in controlling nitrate movement within the root has been well defined, the contributions of this group of nitrate transporters to cytosolic nitrate homeostasis and nitrate dynamics in roots remain mostly unresolved. Environmental, temporal, and intraspecfic variation can all impact nitrogen-use measurements limiting the ability to accurately describe the contributions of specific transport genes to complex nitrogen transport dynamics over time.

Knockout Arabiodpsis mutant lines that had modified nitrate reductase or nitrate transporter activity were used to determine whether whole plant and organ level nitrogen isotope fractionation is predictably modified by changing internal or external nitrogen supply or nitrogen demand. Here, I expect disruption to genes responsible for either nitrate reductase activity or nitrate transport to affect nitrogen isotope composition that correspond to reported localized expression within the plant and the known role of the disrupted genes on nitrate uptake and assimilation. I expect that modifications to nitrogen isotope composition will be interpretable within the context of the model and will demonstrate the model's value in studying nitrogen uptake and assimilation in plants.

3.3. Materials and methods

3.3.1. Plant material and growth conditions

Homozygous Arabidopsis lines of *nia1* (CS879617), *nia2* (CS2355), *nrt2* (SALK_035429C) and a wild type Col-0 (CS6673) were obtained from the Arabidopsis Biological Research Centre (http://abrc.osu.edu/). T-DNA insertions or gamma-ray knockouts were confirmed using PCR to detect the location of the insertion and homozygocity of the line. Stratified seeds were sown onto Rockwool placed in plastic microcentrifuge tubes (N=5) with bottoms removed. Tubes were inserted into pre-drilled lids of 70 L hydroponic containers filled with a modified 1/10th Johnson's solution containing either 100 (low NO_3^-) or 1000 µM (high NO_3^-) NO_3^-. Final nutrient composition, excluding $Ca(NO_3)_2$, was: 200 µM KH_2PO_4, 200 µM K_2SO_4, 100 µM $MgSO_4$, 100 µM $CaSO_4$, and micronutrients: 5 µM Cl, 2.5 µM B, 0.2 µM Mn, 0.2 µM Zn, 0.05 µM Cu, and 50 µM Fe^{2+}. Once seeds had germinated and roots began to emerge from the bottom of the Rockwool, plugs were thinned to one plant per plug.

Plants were grown at 21 °C (Table A.1) and ambient humidity with a 12 h photoperiod providing a PPFD of 150 µmol m^{-2} s^{-1}. Nitrate concentrations in the culture solution were assayed every second day using the perchloric acid method (Cawse 1967) (Figure A.1). When the NO_3^- concentration approached a 10% reduction from starting, the solution was completely replaced using $Ca(NO_3)_2$ from the same pre-mixed source ($\delta^{15}N$ = 2.78±0.23‰ [N=3]). Growth on a homogenous, steady-state nitrogen source is required for the expression of isotope effects that reflect plant physiology rather than the spatial and/or temporal isotopic vagaries typical of natural soils and pot experiments. Frequent measurements of NO_3^- concentration ensured that increased growth rates over time were matched by an increasing frequency of complete nutrient replacement sufficient to avoid any significant isotopic change in the substrate. Plants were harvested when they were 35 days old. Roots and shoots were placed into plastic vials, flash frozen in liquid nitrogen and stored at -80 °C until samples could be freeze-dried at -50 °C for two days. The plants had not yet bolted so the rosette shoot

consisted almost entirely of leaves and is hereafter treated as such. Once dried, roots and leaves were weighed for dry weight. Thirty-five days was identified as the end of the vegetative period from a previous experiment where plants grown under the same conditions started to flower at approximately 40 days. Plants were harvested prior to flowering to avoid significant remobilization of root and leaf nitrogen into the developing inflorescence, which would violate model assumptions outlined in chapter 2. The use of older but still vegetative plants also provided maximum dilution of the small amounts of nitrogen originating from the seed, minimized potential effects of changes in biomass partitioning that occur over the course of plant development (Gedroc *et al.* 1996), and ensured measurements would reflect the assimilation-averaged nitrogen use over the full vegetative phase of plant development.

3.3.2. Isotope analysis

Freeze-dried root and shoot tissue were ground to a fine powder using a mortar and pestle in liquid nitrogen. From ground leaf and root samples, 3 ±0.1 mg of each sample was weighed into tin capsules (Elemental Microanalysis Ltd., 8x5 mm, D1008) and analyzed for $\delta^{15}N$ and nitrogen concentration on either a Europa ANCA-SL preparation module and a Europa Hydra 20/20 isotope ratio mass spectrometer (University of California Stable Isotope Facility, Davis, CA) or an Isoprime (GV Instruments) Isotope Ratio Mass Spectrometer (IRMS) coupled with an Elementar Vario EL Cube Elemental Analyzer (EA) (UBC Faculty of Forestry Stable Isotope Facility). Isotopic composition is expressed as $\delta^{15}N$:

Equation 3.1 $$\boldsymbol{\delta^{15}N = \left(\frac{R_{sample}}{R_{standard}} - 1\right) * 1000}$$

where, R_{sample} is the $^{15}N/^{14}N$ isotope ratio of the sample and $R_{standard}$ is the isotope ratio of a known standard (air). In chapter 2, isotope values ($\delta^{15}N$) were converted to discrimination values ($\Delta^{15}N$). However, because the source differs so little from 0‰, discrimination can be accurately approximated by the difference in isotopic composition

of a plant nitrogen pool from the source salt and can be expressed as $\Delta\delta^{15}N$ (i.e., $\Delta\delta^{15}N_{plant} = \delta^{15}N_{plant} - \delta^{15}N_{source}$).

3.3.3. Mass balance calculations

An isotope mass balance model was used to obtain several nitrogen-use traits from measurements of nitrogen isotope composition, nitrogen concentration and biomass of roots and leaves. The proportion of total plant nitrogen found in the leaf pool ($N_{leaf\ pool}/N_{total}$) was calculated from plant tissue nitrogen concentration and tissue mass:

Equation 3.2 $$\frac{\boldsymbol{N_{leaf\ pool}}}{\boldsymbol{N_{total}}} = \frac{\boldsymbol{Biomass_{leaf}}\times[\boldsymbol{N}]_{\boldsymbol{leaf}}}{\boldsymbol{Biomass_{plant}}\times[\boldsymbol{N}]_{\boldsymbol{plant}}}$$

where, $[N]_i$ is the nitrogen concentration in the plant expressed as a fraction of total dry mass. The assimilation-averaged net flux of inorganic nitrogen across the root is equal to the total plant nitrogen divided by the root biomass:

Equation 3.3 $$\boldsymbol{Net\ Root\ Uptake}\ \ (\boldsymbol{\mu mol\ N\ mg\ dw^{-1}}) = \frac{\boldsymbol{N_{total}}}{\boldsymbol{Biomass_{root}}}$$

All inorganic nitrogen translocated to the shoot (*Ti*) by way of the xylem is assumed to be in isotopic equilibrium with the root cytoplasmic NO_3^- pool. Root-assimilated NO_3^- is depleted in ^{15}N relative to the cytoplasmic pool by the absolute difference in $\delta^{15}N$ caused by nitrate reductase ($\Delta\delta^{15}N_{enz}$), taken here to be 15‰. Organic nitrogen delivered to the shoot (*To*) is considered to be isotopically equal to root-assimilated organic nitrogen. The proportion of the total leaf nitrogen pool translocated to the leaves as inorganic NO_3^- (*Ti*/*Tt*) is then calculated from the difference between leaf and root $\delta^{15}N$:

Equation 3.4 $$\frac{\boldsymbol{Ti}}{\boldsymbol{Tt}} = \frac{\left(\boldsymbol{\Delta\delta^{15}N_{leaf} - \Delta\delta^{15}N_{root}}\right)}{\boldsymbol{\Delta\delta^{15}N_{enz}}}$$

The proportion of total plant nitrogen that was assimilated in the leaves is simply a product of $N_{leaf\ pool}/N_{total}$ and Ti/Tt. The remaining fraction of plant nitrogen is assimilated in the roots (P_{root}):

Equation 3.5 $$\boldsymbol{P_{root} = 1 - \left(\left(\frac{N_{leaf}}{N_{total}}\right) \times \frac{Ti}{Tt}\right)}$$

The isotopic composition of the whole-plant relative to the external source ($\delta^{15}N_{plant}$) is proportional to efflux over influx (E/I) and P_{root}:

Equation 3.6 $$\boldsymbol{\delta^{15}N_{plant} = -\Delta\delta^{15}N_{enz} \times \frac{E}{I} \times P_{root}}$$

where, the maximum depletion in $\delta^{15}N$ relative to the substrate is equal to $-\Delta\delta^{15}N_{enz}$. Rearrangement of equation 6 yields an estimate of E/I (Equation 3.7):

Equation 3.7 $$\boldsymbol{\frac{Efflux}{Influx} = \frac{\Delta\delta^{15}N_{plant}}{-\Delta\delta^{15}N_{enz} \times P_{root}}}$$

Root and leaf assimilation activities are obtained by calculating the amount of plant nitrogen derived from either roots or leaves and expressing it as a function of the biomass of either roots or leaves:

Equation 3.8 $$\boldsymbol{Root\ Assimilation\ Activity\ (\mu mol\ N\ mg\ dw^{-1}) = \frac{N_{total} \times P_{root}}{Biomass_{root}}}$$

Equation 3.9 $$\boldsymbol{Leaf\ Assimilation\ Activity\ (\mu mol\ N\ mg\ dw^{-1}) = \frac{N_{total} \times (1 - P_{root})}{Biomass_{leaf}}}$$

3.3.4. Statistical analysis

Analysis of variance with unequal observations per treatment was used to compare treatment means of biomass, root: shoot ratio, $\delta^{15}N$, nitrogen concentration and total nitrogen of leaf and root tissues. The statistical model was as follows:

Equation 3.10 $\boldsymbol{Y_{ij} = \mu + \alpha_i + \tau_j + \beta_{ij} + e_{ij}}$

where, μ is the overall mean response, α_i is the effect due to the seed line, τ_j is the effect due to the *j*-th level of NO_3^- and β_{ij} is the effect due to any interaction between the seed line concentration and NO_3^- concentration. Analysis of variance procedure was carried out using Graphpad Prism 6 (La Jolla, CA, USA) to obtain estimates of the means followed by Tukey's multiple comparison tests to separate means. Where necessary, data were log transformed to meet assumptions of homogeneity of variance and normality. Differences between treatments described as significant are those where $P < 0.05$.

3.4. Results

3.4.1. There were morphological differences between lines that were not differentially affected by nitrate availability

There were no significant differences in root, shoot or total dry mass between the wild type and *nia2*, *nia1* or *nrt2* mutants (Figure 3.1A). As expected, biomass was greater when grown with 1000 μM NO_3^- than 100 μM NO_3^-. Mean total dry mass was 12.6 mg and 25.3 mg for plants grown with 100 μM NO_3^- and 1000 μM NO_3^-, respectively. Although shoot and root dry masses were both greater on high NO_3^- than low NO_3^-, the effects on shoot mass were greater. Consequently, there was an overall decrease in root:shoot ratio in plants grown with 1000 μM NO_3^- (Figure 3.1B). Root:shoot ratios for *nia1* was unchanged relative to the wild type. However, for nia2, the root:shoot ratio was less than the wild type and for *nrt2*, the root:shoot ratio was greater than the wild type.

Leaf nitrogen concentration was lower in *nrt2* than the wild type but was not different for *nia2* and *nia1* (Figure 3.2A). Leaf nitrogen concentration was 4.17, 4.25 and 3.98 mmol g dw^{-1} for the wild type, *nia2* and *nia1,* respectively, and only 3.32 mmol g dw^{-1} for *nrt2*. Unlike leaf nitrogen concentration, root nitrogen concentration was not

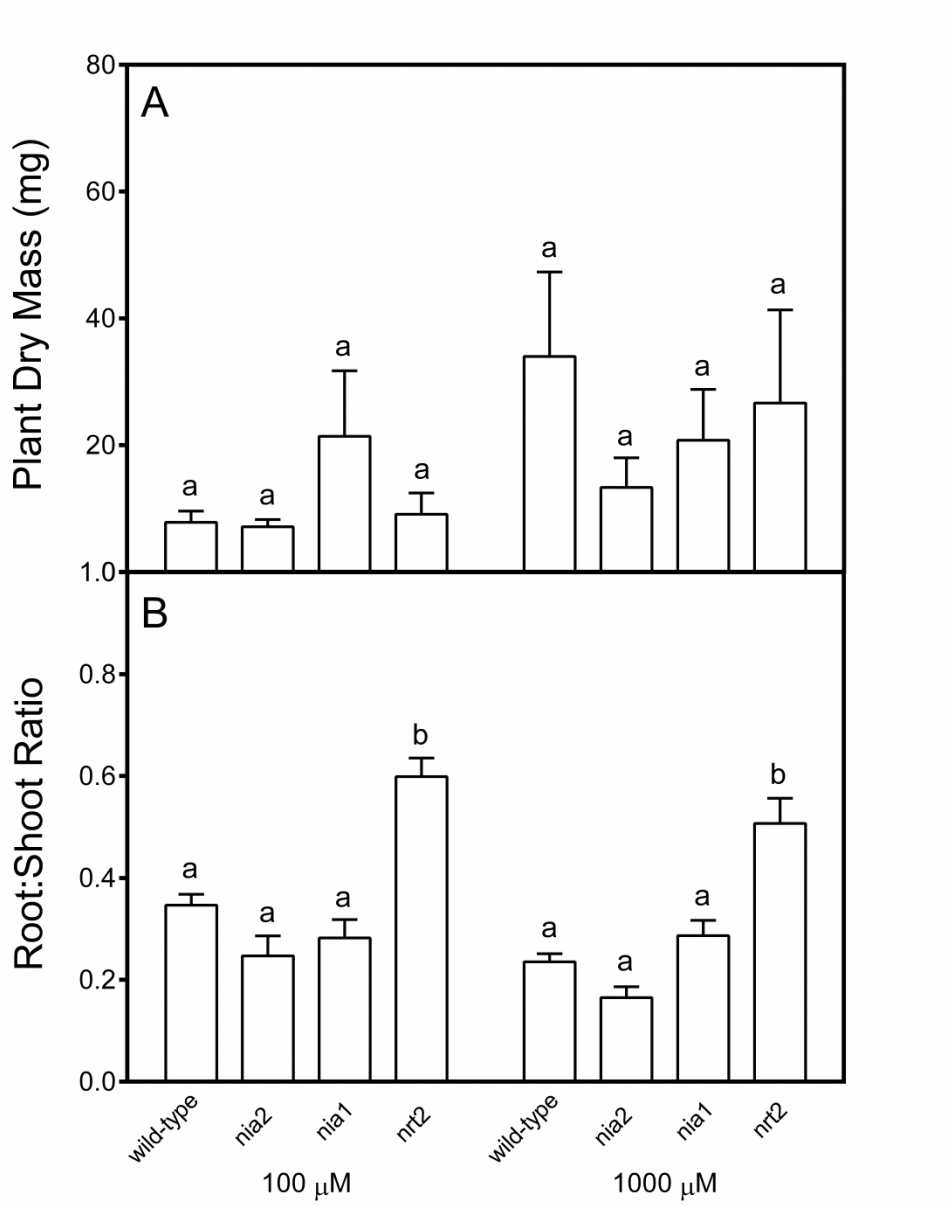

Figure 3.1. A) Total dry mass (mg) and B) root:shoot ratiofor Col-0 wild type and *nia2, nia1* or *nrt2* lines of *Arabidopsis thaliana* grown hydroponically in 100 µM or 1000 µM NO_3^-. Letters denote statistical significance determined by a Tukey's multiple comparison test ($P<0.05$).

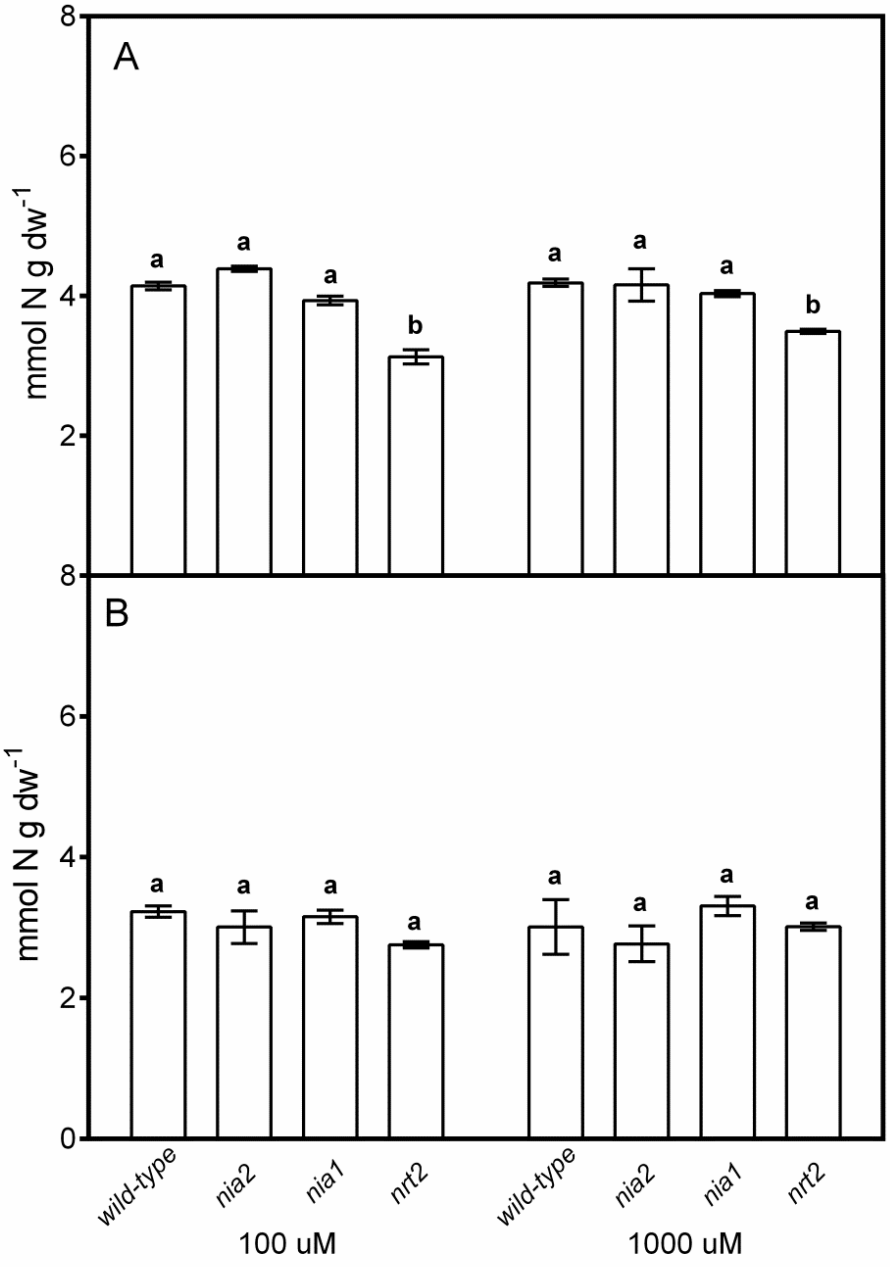

Figure 3.2. A) Leaf and B) root nitrogen concentration (mmol N g dw^{-1}) for Col-0 wild type and *nia2*, *nia1* or *nrt2* lines of *Arabidopsis thaliana* grown hydroponically in 100 µM or 1000 µM NO_3^-. Letters denote statistical significance determined by a Tukey's multiple comparison test ($P<0.05$).

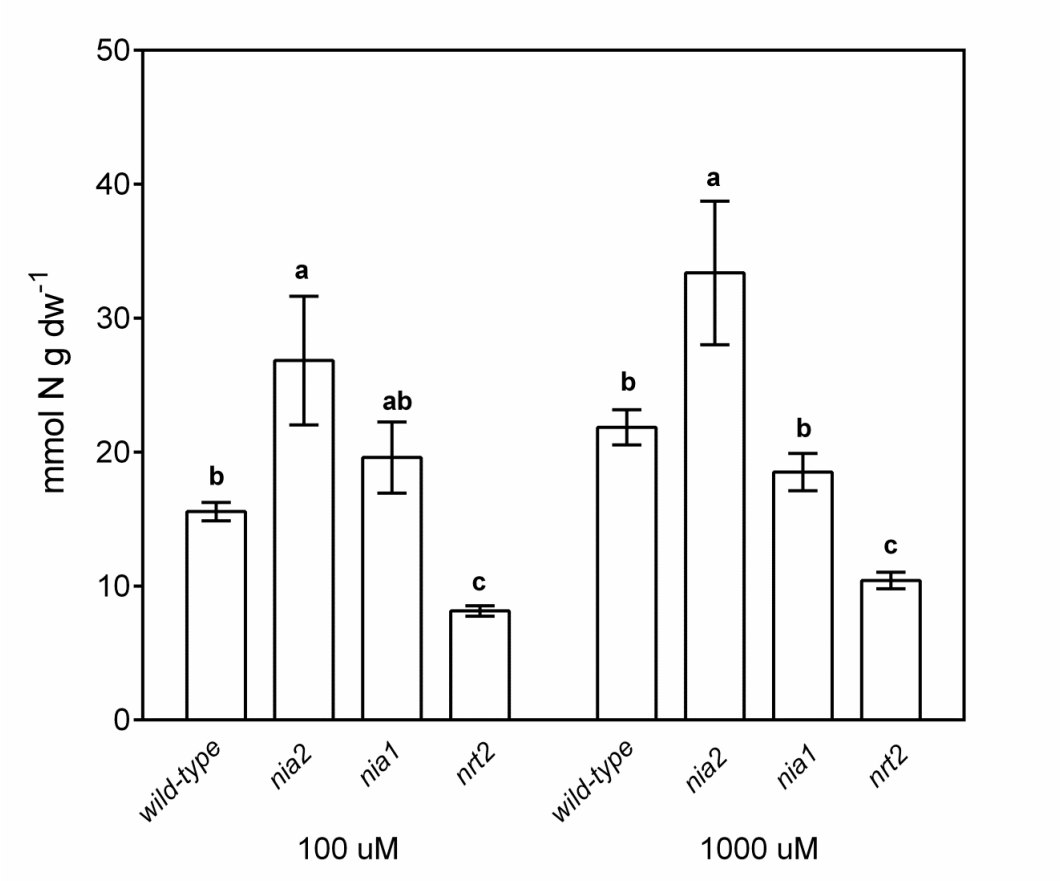

Figure 3.3. Net root uptake (mmol N g dw^{-1}) over 35 days for Col-0 wild type and *nia2*, *nia1* or *nrt2* lines of *Arabidopsis thaliana* grown hydroponically in 100 µM or 1000 µM NO_3^-. Letters denote statistical significance determined by a Tukey's multiple comparison test ($P<0.05$).

different between any of the lines, but averaged approximately 33% less than shoot nitrogen (3 mmol g dw^{-1}). Neither root nor leaf nitrogen concentration was affected by NO_3^- availability. Relative to wild-type plants, a combination of low root:shoot ratio and relatively high foliar nitrogen concentration in *nia2* resulted in a greater mass specific net uptake of NO_3^- by roots (Figure 3.3). In direct contrast, a high root:shoot ratio and lower foliar nitrogen concentration combined to yield a reduced rate of net uptake in the roots of *nrt2*. Net root uptake was not different from wild-type in *nia1*. Although not significant, net root uptake was greater in 1000 µM NO_3^- than 100 µM NO_3^- (P=0.1004).

3.4.2. *nia2* and *nia1* only show an isotopic phenotype at the organ level while *nrt2* shows an isotopic phenotype at the whole plant and the organ level

Plant $\delta^{15}N$ was more negative than source $\delta^{15}N$ in all lines growing under steady-state NO_3^- conditions (Figure 3.4). Whole plant $\Delta\delta^{15}N$ relative to the source averaged -2.74 ‰. There were differences in whole-plant and organ-level $\delta^{15}N$ between lines, but lines were similarly ranked at both 100 and 1000 µM NO_3^-. Plant $\Delta\delta^{15}N$ relative to the source $\delta^{15}N$ was more negative in *nrt2* than the wild type. Whole-plant discrimination was not different in *nia1* and *nia2* compared to the wild type. Although whole-plant $\Delta\delta^{15}N$ did not indicate differences in nitrogen isotope discrimination for *nia1* and *nia2*, differences in nitrogen isotope discrimination at the organ level were more apparent.

Leaf $\Delta\delta^{15}N$ was less than root $\Delta\delta^{15}N$ for the wild type and all mutant lines (Figure 3.3). Mean $\Delta\delta^{15}N$ was -1.70 and -7.43 ‰ for roots and leaves, respectively. Leaf $\Delta\delta^{15}N$ in *nia1* and *nia2* were not significantly different than the wild type ($P<0.05$). However, leaf $\Delta\delta^{15}N$ in the *nrt2* line was more negative than the wild type. There was no difference in leaf $\Delta\delta^{15}N$ between plants grown on either 100 or 1000 µM NO_3^-. However, there was an interaction between line and NO_3^- treatment ($P<0.05$). Plant and leaf $\Delta\delta^{15}N$ were closely related because a greater proportion of plant biomass and nitrogen was allocated to the leaves, particularly under high NO_3^- (Figure 3.3). Root $\Delta\delta^{15}N$ was more negative relative to the wild type for *nia2*, but more enriched relative to the wild type

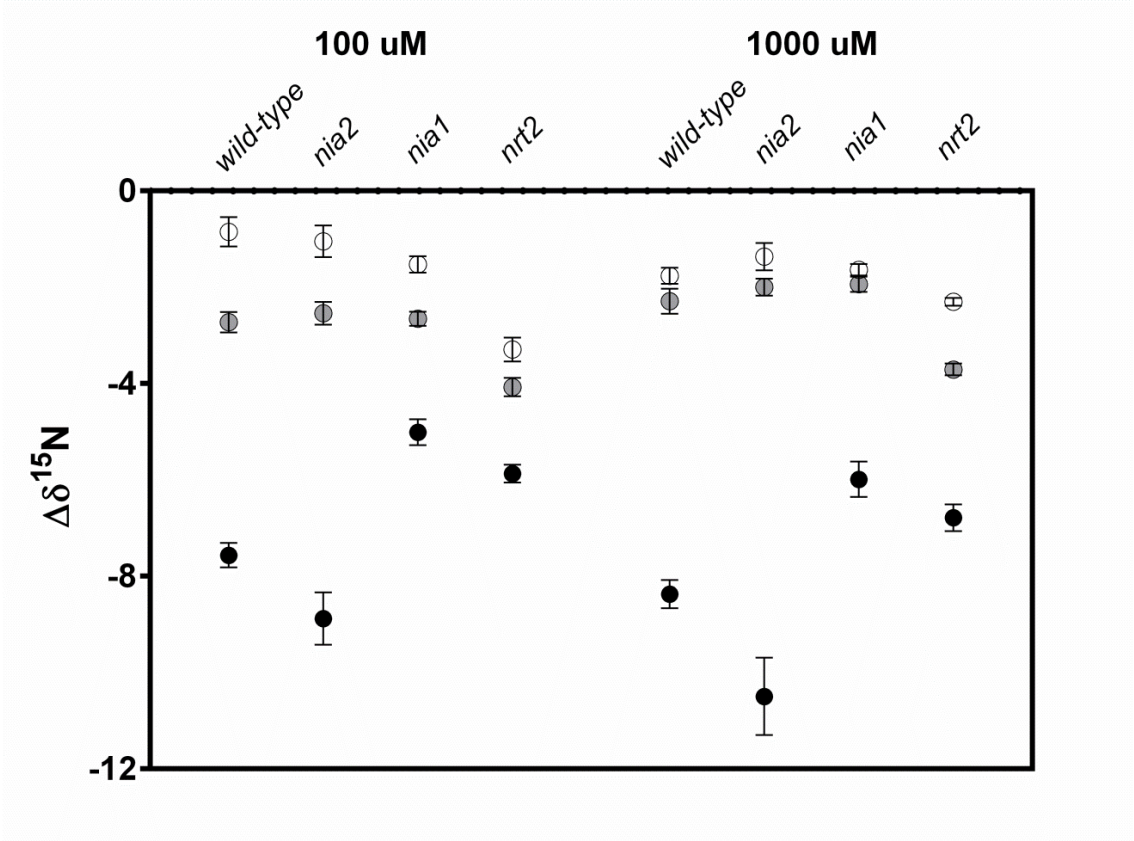

Figure 3.4. Whole plant (grey circles), leaf (clear circles) and root (filled circles) $\Delta\delta^{15}N$ for Col-0 wild type and *nia2*, *nia1* or *nrt2* lines of *Arabidopsis thaliana* grown hydroponically in 100 µM or 1000 µM NO_3^- (N=10).

for *nia1* and *nrt2*. Root $\Delta\delta^{15}N$ was more negative in plants grown at the higher NO_3^- concentration (-7.94 versus -6.92‰ for 1000 µM and 100 µM NO_3^-, respectively).

Interestingly, whole-plant $\Delta\delta^{15}N$ was more negative at 100 µM NO_3^-. Since the root:shoot ratio was lower in plants grown with high NO_3^-, leaves accounted for a greater proportion of the total nitrogen resulting in an increase in plant $\Delta\delta^{15}N$.

3.4.3. Morphological changes and partitioning of nitrogen and NO_3^- assimilation between plant organs impact whole plant and organ level isotopic composition

The partitioning of nitrogen reflected changes in biomass allocation and differences in nitrogen concentration between roots and leaves. $N_{leaf\ pool}/N_{total}$ was greater for plants grown at high NO_3^- ($P<0.05$). $N_{leaf\ pool}/N_{total}$ was not significantly different in the *nia1* and *nia2* mutant lines compared to the wild type (Table 3.2), but was lower for *nrt2*. Leaf nitrogen accounted for 82% of total nitrogen in the wild type but only 58% in *nrt2*. Although most of the difference in $N_{leaf\ pool}/N_{total}$ can be attributed to the higher root:shoot ratio of *nrt2*, a reduced leaf nitrogen concentration also contributed to its lower $N_{leaf\ pool}/N_{total}$.

Through changes in overall partitioning of nitrogen and the difference in $\Delta\delta^{15}N$ between the leaves and roots (a close approximation of $\Delta^{15}N$), the proportional distribution of assimilatory activity can be estimated (Equation 3.5). Approximately 65% of NO_3^- was assimilated in the roots of the wild type (Table 3.2). The proportion of assimilation occurring in the root was lower for *nia2* (50%), but higher for *nia1* (78%) and *nrt2* (84%). Plants grown with 1000 µM NO_3^- had a lower proportion of assimilation occurring in the root than plants grown with 100 µM NO_3^- (66% and 72% for high and low NO_3^-, respectively).

An estimate of E/I is obtained by comparing the whole-plant $\Delta\delta^{15}N$ to the instantaneous fractionation by nitrate reductase adjusted for partitioning of assimilation

Table 3.1. $N_{leaf\ pool}/N_{total}$, *Ti/Tt*, P_{root} and *E/I* for the wild type and *nia2*, *nia1* or *nrt2* lines of *Arabidopsis thaliana* grown hydroponically in 100 µM or 1000 µM NO_3^-. $N_{leaf\ pool}/N_{total}$ = proportion of total plant nitrogen that is leaf nitrogen. *Ti/Tt* = proportion of leaf nitrogen that was transported to the shoot as inorganic nitrogen. P_{root} = proportion of plant nitrogen assimilation occurring in roots. *E/I* = efflux/influx between the substrate and root. Letters denote statistical significance determined by a Tukey's multiple comparison test ($P<0.05$).

		100 µM		
Output Variable	wild type	*nia2*	*nia1*	*nrt2*
N_{leaf}/N_{total}	0.79 ±0.01 a	0.85± 0.02 a	0.82 ±0.02 a	0.66 ±0.02 b
Ti/Tt	0.44 ±0.03 a	0.55 ±0.037 a	0.24 ±0.02 b	0.17 ±0.02 b
P_{root}	0.65 ±0.02 a	0.56 ±0.04 a	0.81 ±0.02 b	0.89 ±0.01 b
E/I	0.10 ±0.01 a	0.08 ±0.01 a	0.11 ±0.01 a	0.25 ±0.01 b
		1000 µM		
	wild type	*nia2*	*nia1*	*nrt2*
N_{leaf}/N_{total}	0.84 ±0.01 ab	0.90 ±0.02 a	0.81 ±0.02 b	0.70 ±0.02 c
Ti/Tt	0.44 ±0.02 b	0.61 ±0.07 a	0.29 ±0.02 c	0.30 ±0.02 c
P_{root}	0.63 ±0.02 b	0.46 ±0.06 a	0.77 ±0.02 c	0.79 ±0.02 c
E/I	0.12 ±0.01 ab	0.08 ±0.014 a	0.13 ±0.008 b	0.19 ±0.006 c

(Equation 3.7). *E*/*I* was not different in the *nia1* and *nia2* mutant lines relative to the wild type (Table 3.2). However, in *nrt2*, *E*/*I* was greater than in the wild type at both NO_3^- concentrations. Likewise, NO_3^- concentration did not significantly affect *E*/*I* in the wild type or *nia1* and *nia2*, but in *nrt2* there was a decrease in *E*/*I* from 0.25 at 100 µM NO_3^- to 0.19 at 1000 µM NO_3^-.

The mass specific root and leaf assimilation activities were determined by accounting for total plant nitrogen and the proportional assimilation occurring in either the root or the shoot (Equations 3.8 and 3.9). Overall, mean root assimilation was 13.1 mmol g dw^{-1} (Figure 3.5). Root assimilation activity was not different compared to the wild type for *nia1* and *nia2*, but was lower in *nrt2*. However, leaf assimilation activity was greater in *nia2* (2.47 mmol g dw^{-1}) and lower in *nia1* (1.05 mmol g dw^{-1}) and *nrt2* (0.79 mmol g dw^{-1}) compared to the wild type (1.83 mmol g dw^{-1}). Overall, the mass specific leaf assimilation activity was approximately 17% greater at high NO_3^- (1.40 mmol g dw^{-1}) than at low NO_3^- (1.68 mmol g dw^{-1}). Although leaf assimilation accounted for about 40% of total nitrate assimilation, root assimilation per unit mass was approximately eight times greater than leaf assimilation because of the low amount of root biomass relative to leaf biomass.

3.5. Discussion

As a test of a proposed model of nitrogen isotope discrimination proposed in chapter 2, disrupting the gene expression of genes critical to nitrogen uptake or assimilation resulted in strong variation in nitrogen isotope composition compared to wild-type plants. Although growth phenotypes were not observed; morphological, isotopic and physiological phenotypes were apparent. It was expected that disruption of uptake or assimilatory genes, alone or in combination with changes in substrate concentration, would impact nitrate supply and demand. Variation in supply and demand would subsequently affect whole plant and organ level nitrogen isotope composition (Evans, 2001; Pritchard and Guy, 2005). At the whole-plant level, a

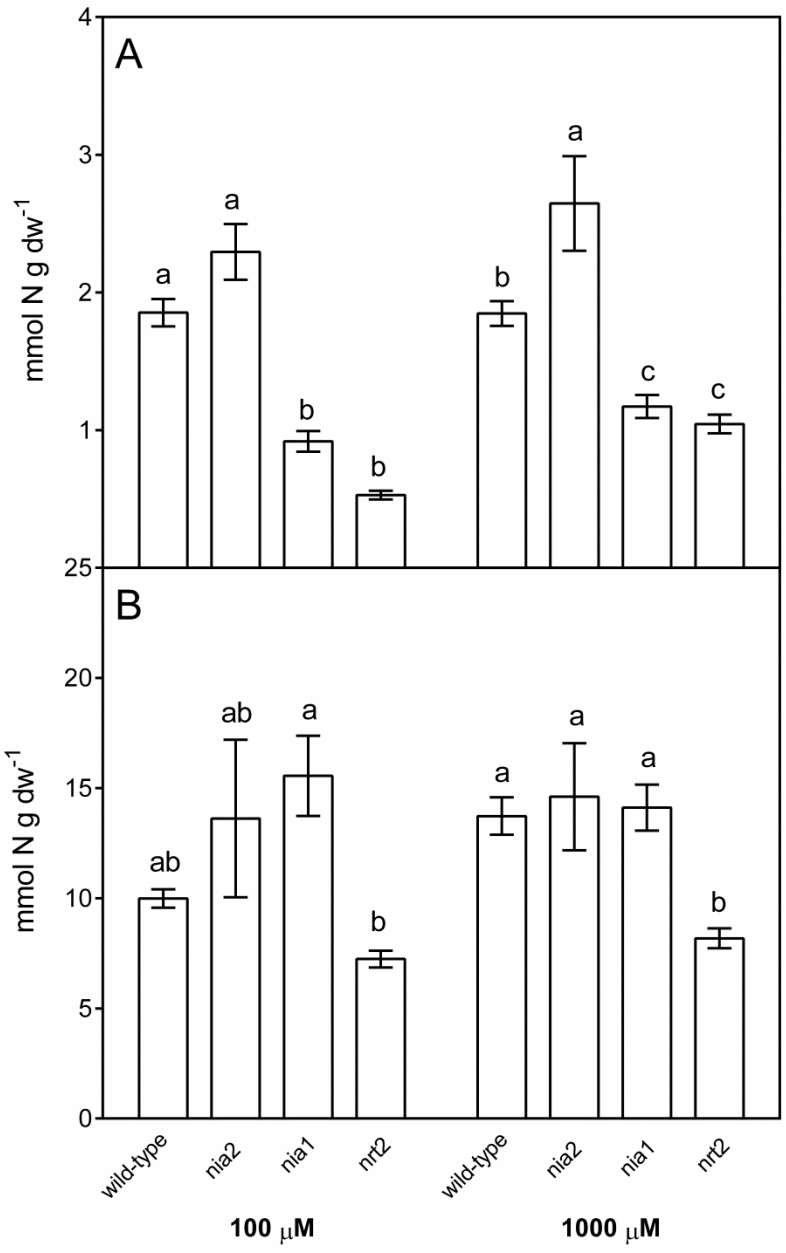

Figure 3.5. A) Leaf and B) Root assimilation (mmol N g dw^{-1}) over 35 days for Col-0 wild type and *nia2*, *nia1* or *nrt2* lines of *Arabidopsis thaliana* grown hydroponically in 100 µM or 1000 µM NO_3^-. Letters denote statistical significance determined by a Tukey's multiple comparison test ($P<0.05$).

decrease in demand relative to supply should increase *E*/*I* and thereby increase $\delta^{15}N$ discrimination if assimilation occurs partly in the root. In contrast, a decrease in *E*/*I* and a decrease in discrimination are expected if uptake is suppressed, but would be dependent on whether uptake physically precedes assimilation across all tissues. If there are restrictions in transport that are localized beyond the epidermis, or if restrictions occur generally throughout the root, *E*/*I* could instead increase.

3.5.1. Disrupting *nia2* activity decreases root:shoot ratio but does not affect nitrogen concentration, while disrupting *nrt2* activity reduces nitrogen concentration and increases root:shoot ratio

Although not significantly different, the mean biomass of *nia2* was lower than the wild type (P=0.1574). High variability in biomass among seedlings within lines contributed to the lack of confidence in the differences in mean biomass between *nia2* and the wild type. Wilkinson and Crawford (1993) reported no differences in growth for the same *nia2* line under NO_3^- nutrition compared to wild-type plants. However, Stitt and Feil (1999) observed reduced growth in single-knockout tobacco transformants with reduced nitrate reductase activity when grown at multiple NO_3^- concentrations. Although there were no significant differences in total biomass in our experiments, root growth was impacted more than shoot growth by disruption of NIA2 activity, causing the root:shoot ratio to decrease (Stitt and Feil, 1999). Conversely, there was an increase in root:shoot ratio in *nrt2* grown with either 100 or 1000 μM NO_3^-, as described by Filleur *et al.* (2001) and Li *et al.* (2007). Changes in root:shoot ratio may change the demand for nitrogen from the shoot relative to the amount of root tissue available to take up NO_3^- from the substrate. The reduction in root and leaf nitrogen concentration in *nrt2* (Figure 3.2) indicates that net uptake is restricted at either the epidermal and/or intercellular level. On a mass basis, net uptake was reduced in *nrt2*. An approximately 20% reduction in tissue nitrogen concentration in *nrt2* suggests that there is some disruption in supply relative to demand. Although plants had reduced nitrogen levels, they did not show any outward signs of stress or limitation.

Differences in $N_{leaf\ pool}/N_{total}$ in mutant lines compared to the wild type demonstrate the impact of changes in biomass partitioning on overall nitrogen allocation (Table 3.2). Since the root:shoot ratio of *nia2* was low compared to the wild type, a greater portion of overall nitrogen was allocated to the leaf. In essence, *nia2* behaved like a plant growing under a higher nitrate concentration (Gedroc *et al.* 1996) since supply exceeded demand (reduced by knocking out 90% of total NR activity). On the other hand, *nrt2* behaved like a plant that was nitrogen limited and allocated a greater proportion of overall biomass to the root (Bloom *et al.* 1985).

3.5.2. The distribution of assimilatory activity indicated by ^{15}N mass balance corresponds to the distribution of nitrate reductase expression in roots and leaves

Nitrate reductase activity is closely linked with substrate availability and assimilatory demand (Campbell 1999). The presence of nitrate has been shown to induce nitrate reductase activity in numerous plant species including Arabidopsis (Crawford *et al.* 1988) and poplar (Black *et al.* 2002). Since NIA2 would normally constitute 90% of the nitrate reductase activity, one would expect to see a stronger response in *nia2* than *nia1*. Although not significant, there was a decrease in growth in *nia2*, but not *nia1*. Interestingly, Wilkinson and Crawford (1993) observed no decrease in nitrate reductase activity after disrupting either NIA1 or NIA2. It was suggested that compensatory upregulation of NIA1 offsets the effects of disrupting NIA2 (Wilkinson and Crawford, 1993). It is therefore not surprising that both *nia1* and *nia2* showed no significant growth phenotype when grown under either nitrate concentration. In contrast, there were morphological and physiological phenotypes that indicate differential contributions of NIA1 and NIA2 to whole plant nitrate assimilation activity.

Our calculations of P_{root} indicate that root assimilation accounted for between 50 and 90% of total nitrate assimilation depending on the mutant line, which is a greater proportion than is generally expected from reports in the literature. Expressed relative

Table 3.2. Distribution of NIA1 and NIA2 expression between leaves and roots of *Arabidopsis thaliana* compared to observed leaf:root proportioning of nitrate assimilation in Col-0 wild type (NIA1 + NIA2), NIA1 only and NIA2 only. Leaf and root GfP (Green Fluorescent Protein) expression were taken as expression in whole leaf and root cortex tissue under standard conditions. Expression data taken from the Bio-Array Resource for Plant Biology Arabidopsis eFP expression browser (http://bar.utoronto.ca/welcome.htm; Winter *et al.* 2007)

Gene	**Leaf GfP expression**	**Root GfP expression**	**Leaf:root expression ratio**	**Leaf:root proportioning of assimilation**
NIA1 + NIA2	2656	1308	2.03:1	0.5:1
NIA1	478	146	3.23:1	1:1
NIA2	2178	1162	1.87:1	0.33:1

to each other at the tissue level, measurements of nitrate reductase activities have been used to provide a first approximation of how assimilatory activity is partitioned between roots and leaves. In most cases, leaf nitrate reductase activities are considerably greater than root nitrate reductase activities (Andrews 1986; Black *et al.* 2002). Similarly, according to the BAR Arabidopsis expression tool (Winter *et al.* 2007), nitrate reductase is expressed at higher levels in leaves than roots (Table 3.2). Similar to nitrate reductase assays, expression is a measure of transcript abundance, not *in vivo* enzyme activity or assimilation activity. Changes in *in vitro* activity are not always reflective of *in vivo* nitrate reductase activity. Andrews *et al.* (1992) estimated partitioning of assimilation in grasses using nitrate reductase activity, tissue nitrate content and reduced nitrogen in the xylem sap and concluded that assimilation is weighted more towards roots, particularly at concentrations below 1 mM nitrate. Nitrate reductase can undergo strong substrate (cytoplasmic NO_3^-) limitation (Kaiser and Huber 2001). Furthermore, cytosolic nitrate concentrations can fluctuate with regulation of assimilatory enzymes (Fan *et al.* 2006). Root nitrate concentrations are likely more stable than leaf nitrate concentrations given that leaf nitrate concentrations are entirely dependent on xylem transport. Under non-saturating nitrate conditions, less nitrate may reach the xylem to be transported to the leaf and consequently, a greater proportion will be assimilated by root nitrate reductase.

Despite these qualifications, our ^{15}N mass balance model predicts the same ranking for the partitioning of assimilation as would be expected based on tissue level nitrate reductase expression levels (Table 3.1). Furthermore, because *nia2* expression is proportionately higher in roots than *nia1*, when *nia2* is disrupted, nitrate reductase expression is likely to be proportionately even higher in leaves, consistent with the decrease in P_{root} indicated by the isotopic mass balance. In contrast, when *nia1* is disrupted, nitrate reductase expression is weighted more towards the roots and an increase in the partitioning of assimilation to the roots was observed.

When nitrate transport was disrupted in *nrt2*, there was an overall decrease in net uptake and assimilation activity accompanied by an increase in the root:shoot ratio. The concomitant changes in ^{15}N mass balance indicate an increase in P_{root}, suggesting that with decreased symplastic movement of nitrate within the root and decreased rate of loading into the xylem, there is less NO_3^- translocated to the leaves. Expression of NRT2.1 and NRT2.2 is localized to not just the plasma membranes of epidermal cells, but also cortical and endodermal cells (Winter *et al.* 2007; Feng *et al.* 2010). With assimilatory demand remaining high and movement of nitrate through the root tissue reduced, assimilation would become more weighted to the root since translocation to the leaf would be restricted.

3.5.3. Changes in assimilatory demand do not impact efflux/influx but restriction of nitrate transport within root tissue does

For both nitrate reductase mutant lines, our estimates of *E*/*I* remained unchanged compared to the wild type. This was contrary to our initial expectations, as predicted, reduced rates of assimilation allowed more substrate to cycle back to the medium. However, as indicated above, nitrate reductase activities was marginally impacted in *nia1*, whilst *nia2* had a much reduced root:shoot ratio, placing an increased demand on the roots for xylem transport of inorganic NO_3^- (i.e., in competition with efflux). There is also increasing evidence of homeostatic control of cytoplasmic nitrogen concentrations (Glass *et al.* 2002; Miller and Smith 2008; Huang *et al.* 2012) whereby plants can modulate demand (through modifying growth) and supply (through modulating uptake) so that cytosolic nitrogen concentrations are buffered to temporal and spatial changes in supply or demand.

In contrast, when genes associated with NO_3^- transport were disrupted the ^{15}N mass balance was consistent with an increase in *E*/*I*. This result suggests that the sites of root assimilation are morphologically internal to the sites where transport was actually disrupted, which is quite possible given that, as indicated above, NRT2 is expressed

throughout the root. Although overall uptake into the root was reduced, further movement within the cortex or to the stele may have been more greatly impacted, increasing efflux. Multiple nitrate transporter genes contribute to nitrate uptake and movement within roots (Glass *et al.* 2002). Although there is a degree of redundancy within the NRT gene family and these genes operate at different NO_3^- concentrations, *NRT2.1* and *NRT2.2* are considered to be two of the primary genes responsible for high affinity uptake in Arabidopsis (Glass *et al.* 2002; Li *et al.* 2007). The small differences between leaf and root $\delta^{15}N$ in the double mutant indicate that the leaves rely more heavily on the translocation of organic N from the roots, consistent with a reduced loading of inorganic nitrate into the xylem.

3.6. Conclusion

Arabidopsis, with its wide array of described mutant lines, provides an excellent platform to modify nitrogen isotope discrimination by restricting the expression of critical genes in the nitrogen uptake and assimilation pathway. Induced variation in nitrogen isotope composition suggests that there is variation in nitrogen uptake, or use within plants, that affects relative inorganic nitrogen fluxes between the root and substrate as well as between plant parts. Here, I demonstrated here that whole plant and organ level variation in isotopic composition can be successfully interpreted using the mass balance model proposed in chapter 2, in turn providing more detail on overall nitrogen uptake and assimilation.

4. Quantifying remobilization of pre-existing nitrogen from cuttings to developing roots, stems and leaves of woody plants using ^{15}N at natural abundance

4.1. Summary

For measurements of nitrogen isotope composition at natural abundance, pre-existing nitrogen remobilized to new plant growth can cause deviation of measured isotope composition ($\delta^{15}N$) from the $\delta^{15}N$ of newly acquired nitrogen. To account for this problem, a two-step approach was proposed to quantify and correct for remobilized nitrogen from vegetative cuttings of *Populus balsamifera* L. grown with either nitrate ($\delta^{15}N$ = 58.5‰) or ammonium ($\delta^{15}N$ = -0.96‰). First, the fraction of carry-over nitrogen remaining in the cutting was estimated by isotope mass balance. Then measured $\delta^{15}N$ values were adjusted for the fraction of pre-existing nitrogen remobilized to the plant. Mean plant $\delta^{15}N$ prior to correction was 49‰ and -5.8‰ under nitrate and ammonium, respectively. Plant $\delta^{15}N$ was non-linearly correlated to biomass (r^2 = 0.331 and 0.249 for nitrate and ammonium, respectively; $P<0.05$), where the $\delta^{15}N$ of plants with low biomass approached the $\delta^{15}N$ of the pre-existing nitrogen. Approximately 50% of cutting nitrogen was not remobilized, irrespective of size. The proportion of carry-over nitrogen in new growth was not different between sources but ranged from less than 1% to 21% and was dependent on plant biomass and, to a lesser degree, the size of the cutting. The $\delta^{15}N$ of newly acquired nitrogen averaged 52.7‰ and -6.4‰ for nitrate and ammonium-grown plants, respectively; both lower than their source values, as expected. Since there was a greater difference in $\delta^{15}N$ between the cuttings and newly assimilated nitrogen where nitrate was the source, the difference between measured and adjusted $\delta^{15}N$ was also greater. There was no significant relationship between biomass and plant $\delta^{15}N$ with either ammonium or nitrate after adjusting for carry-over nitrogen. Here, I provide evidence of remobilized pre-existing nitrogen influencing $\delta^{15}N$ of new growth of *balsam poplar*. A simple, though approximate, correction is proposed that can account for the remobilized fraction in the plant. With careful sampling to quantify pre-existing nitrogen, this method can be used to more accurately determine changes in nitrogen isotope discrimination in response to environment.

4.2. Introduction

Measurements of nitrogen isotope composition at natural abundance ($\delta^{15}N$) may be affected by remobilization of previously acquired nitrogen into new growth of plants. To precisely determine the $\delta^{15}N$ of newly acquired nitrogen, the carry-over of old nitrogen needs to be accounted for. The use of $\delta^{15}N$ at natural abundance has increased in plant physiology and ecology where small, but tractable changes in $\delta^{15}N$ of plants or plant tissues can indicate changes in soil nitrogen sources or changes to nitrogen use physiology (Comstock, 2001; Evans, 2001; Dawson *et al.* 2002; Houlton *et al.* 2007). Quantifying temporal changes in plant nitrogen sources in the field or plant nitrogen isotope discrimination ($\Delta^{15}N$) requires confidence in the measurement of the $\delta^{15}N$ of newly acquired nitrogen. When not working at the natural abundance level, as in many ^{15}N enrichment experiments, knowledge of the precise isotopic composition of pre-existing nitrogen may not be necessary because newly acquired nitrogen will have a markedly different isotopic signature (Dawson *et al.* 2002; Dong *et al.* 2004).

During establishment of sexually and vegetatively propagated plants, stored nitrogen is remobilized from the propagule (seed or cutting) to the newly growing tissue as a nitrogen supply until the roots are capable of supporting the nitrogen demands of the plant (Dong *et al.* 2004). In woody perennials, nitrogen from senescing leaves accumulates in stem tissue during autumn to be re-used by developing tissues during regrowth in the spring (Millard and Thomson 1989; Malaguti *et al.* 2001). Nitrogen transported to flushing shoots and/or root growth come from vegetative storage proteins kept over the dormant period (Cooke and Weih 2005). Vegetative storage proteins can account for a sizeable fraction of total nitrogen in dormant stem tissue (Greenwood *et al.* 1986; Gomez and Faurobert 2002). If the $\delta^{15}N$ of remobilized nitrogen is different from the $\delta^{15}N$ of newly acquired nitrogen, it may influence the measured $\delta^{15}N$ of new growth.

Within a bulk nitrogen pool (e.g., a cutting), there may be differences in $\delta^{15}N$ between various amino acids and other nitrogen-containing compounds (Werner and Schmidt 2002, Tcherkez 2011, Gauthier *et al.* 2012). This may impact the $\delta^{15}N$ of

remobilized stem tissue relative to the $\delta^{15}N$ of the bulk tissue if the $\delta^{15}N$ of the remobilized fraction is different from the non-remobilized fraction. However, if a substantive fraction of pre-existing nitrogen contributes to the growth of new plant tissue, the $\delta^{15}N$ of the remobilized nitrogen should resemble the $\delta^{15}N$ of the bulk source tissue because many, and certainly the most predominant, nitrogen-containing compounds will contribute to this flux. If there were any isotope discrimination associated with remobilization itself, as consumption of a nitrogen pool approaches completion, the transferred fraction (the product) approaches the $\delta^{15}N$ of the stored fraction (the source).

Here, a two-step mass balance approach is proposed to account for carry-over nitrogen remobilized from cuttings to growing tissues in plants using *Populus balsamifera* L. (balsam poplar) grown with either ammonium or nitrate. The two nitrogen sources bracketed the $\delta^{15}N$ of pre-existing N, with the nitrate being enriched in ^{15}N relative to the cutting and the ammonium being depleted relative to the cutting. Therefore, if carried-over nitrogen from pre-existing nitrogen has an appreciable effect on newly assimilated nitrogen isotope values, contrasting relationships with biomass should be evident. Poplars in general have long been used as model systems in tree biology and genetics, and *Populus trichocarpa* Torr. & Gray, (syn: *P. balsamifera* ssp. *trichocarpa*) was the first woody species to have its genome completely sequenced (Tuskan *et al.* 2006). The approach described here is applicable not just to plants grown from cuttings, but also with little adjustment to plants grown from seed.

4.3. Materials and methods

4.3.1. Plant material and experimental design

First year branches of 25 genotypes of *Populus balsamifera* L. ranging from 51 °N to 56 °N from the Agriculture Canada Balsam Poplar (AgCanBaP) collection (Soolanayakanahally *et al.* 2009) were obtained from the AAFC-AESB Agroforestry Development Centre at Indian Head, Saskatchewan, Canada and stored at 4 °C for approximately three months to fulfill chilling requirements. The five provenances

reflected a climatic gradient for the species that extends from a prairie ecosystem to the boreal forest of the Canadian Shield; namely Outlook (OUT; 51.1 °N, 106.2 °W), Saskatoon (SKN; 2.2 °N, 106.4 °W), Turtleford (TUR; 53.2 °N, 108.3 °W), Cold Lake (CLK; 54.2 °N, 110.1 °W) and Gillam (GIL; 56.4 °N, 94.7 °W). These same genotypes were used for analysis of genotypic variation in nitrogen isotope composition presented in chapter 6 and the comparison of two independent measures of calculating *E*/*I* in chapter 5. Two-node cuttings, approximately 6-8 cm long were weighed for fresh weight and arranged in a randomized complete block design with three blocks of two nitrogen treatments supplied as either 250 μM $Ca(NO_3)_2$ or 250 μM $(NH_4)_2SO_4$. Plants were grown for 45 days in a hydroponics solution until harvest. Complementary samples of each genotype were collected as reference samples (N = 3) for initial isotope composition and nitrogen concentration.

4.3.2. Hydroponics system

The hydroponics system was comprised of six 1000L containers lined with rubber pond liner material (Firestone, Nashville, TN, USA) constructed in a greenhouse under ambient light conditions supplemented by sodium halide lighting (600 μmol m^{-2} s^{-1} PPFD) and 18/6 photoperiod. Temperatures in the greenhouse were maintained between 20 and 24 °C. Each container was fitted with a floating Perspex "raft" that held up to 32 plants. Unused plugs in the raft and the rest of the container were covered with black polythene to prevent algal growth. The hydroponics solution was a modified $1/10^{th}$ strength Johnson's solution (Johnson *et al.* 1957) supplemented with either 250 μM $Ca(NO_3)_2$ or $(NH_4)_2(SO_4)$. Final nutrient composition, excluding nitrogen salts, was: 200 μM KH_2PO_4, 200 μM K_2SO_4, 100 μM $MgSO_4$, 100 μM $CaSO_4$, and micronutrients: 5 μM Cl, 2.5 μM B, 0.2 μM Mn, 0.2 μM Zn, 0.1 μM Mo, 0.05 μM Cu, and 50 μM Fe^{2+}. Containers were fitted with 20 L per minute centrifugal pumps, to circulate and aerate the media. Solutions were monitored periodically for oxygen levels, pH (Figure A.2) and temperature (Figure A.3). Powdered calcium carbonate ($CaCO_3$) was added to buffer pH in the range of 6-7.5. Media NH_4^+ and NO_3^- concentrations were assayed using the phenol:hypochlorite (Solorzano 1969) and perchloric acid (Cawse 1967) methods,

respectively (Figure A.4). The solution was completely replaced every 14 days to ensure that there was no substantial decrease (>10%) in concentration of nitrate or ammonium over time that could increase the solution $\delta^{15}N$.

4.3.3. Sampling and natural abundance isotope analysis

After 45 days of growth, plants were separated into leaves, stems, roots and the original cutting. Samples were flash frozen in liquid nitrogen and stored at -80 °C until samples could be freeze-dried at -50 °C for two days. Once dried, roots, leaves, stems, cuttings and the reference cuttings were weighed. Samples were then ground to a fine powder using a mortar and pestle and then ball milled (Fritsch Laborgeratebau, Terochem Scientific). Sub-samples of 3±0.1 were weighed into tin capsules (Elemental Microanalysis Ltd., 8x5 mm, D1008) and analyzed for nitrogen concentration and $\delta^{15}N$ on a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) (University of California Stable Isotope Facility, Davis, CA). Isotopic composition is expressed as $\delta^{15}N$:

Equation 4.1 $$\boldsymbol{\delta^{15}N = \left(\frac{R_{sample}}{R_{standard}} - 1\right) * 1000}$$

where, R_{sample} is the $^{15}N/^{14}N$ isotope ratio of the sample and $R_{standard}$ is the isotope ratio of a known standard (air). The $\delta^{15}N$ values of the ammonium and nitrate salts used for the growth media were -0.96 and +58.5‰, respectively.

4.3.4. Correcting for pre-existing cutting nitrogen remobilized to growing plant organs

The $\delta^{15}N$ and nitrogen concentration of the cutting after harvest was compared to the $\delta^{15}N$ and nitrogen concentration of the cutting before the start of the experiment to correct for pre-existing nitrogen remobilized to the actively growing plant. Nitrogen content of the cutting at harvest *(N_{end})* can be calculated as:

Equation 4.2 $$\boldsymbol{N_{end} = Biomass_{cutting\ end} \times [N]_{cutting\ end}}$$

where, $Biomass_{cutting\ end}$ and $[N]_{cutting\ end}$ are the biomass and nitrogen concentration of the cutting at harvest. Since the cutting was part of the growing stem, dry mass

accumulated in the cutting. The initial dry mass of the cutting ($Biomass_{cutting\ start}$) was estimated from the fresh mass of the cutting at the start of the experiment and the mean dry mass content of the reference samples (0.584). From this, nitrogen content at the start of the experiment (N_{start}) was estimated as:

Equation 4.3 $$\mathbf{N}_{start} = \mathbf{Biomass}_{cutting\ start} \times [\mathbf{N}]_{start}$$

where $[N]_{start}$ is the mean nitrogen concentration of the reference samples at the start of the experiment. Assuming that the isotopic composition of a cutting at harvest ($\delta^{15}N_{end}$) is a mixture of stem $\delta^{15}N$ ($\delta^{15}N_{stem}$) (containing a portion of remobilized nitrogen) and non-remobilized nitrogen remaining in the cutting ($\delta^{15}N_{start}$), the fraction of non-remobilized ($f_{nonremobilized}$) nitrogen can be estimated as:

Equation 4.4 $$\boldsymbol{f}_{nonremobilized} = \frac{(\boldsymbol{\delta}^{15}\mathbf{N}_{end} - \boldsymbol{\delta}^{15}\mathbf{N}_{stem})}{(\boldsymbol{\delta}^{15}\mathbf{N}_{start} - \boldsymbol{\delta}^{15}\mathbf{N}_{stem})}$$

The amount of nitrogen remobilized ($N_{remobilized}$) to new growth can then be calculated as:

Equation 4.5 $$\mathbf{N}_{remobilized} = \mathbf{N}_{start} - (\mathbf{N}_{end} \times \boldsymbol{f}_{nonremobilized})$$

The proportion of nitrogen in the general plant nitrogen pool that is carried over into new growth (C) is given by:

Equation 4.6 $$\boldsymbol{C} = \frac{\mathbf{N}_{remobilized}}{\mathbf{N}_{plant}}$$

where, N_{plant} is equal to the sum of all nitrogen contents in the roots, leaves and stems (including new and remobilized nitrogen allocated to the expanded cutting):

Equation 4.7 $$\mathbf{N}_{plant} = \mathbf{N}_{leaves} + \mathbf{N}_{roots} + \mathbf{N}_{stem} + \mathbf{N}_{end} \times (\mathbf{1} - \boldsymbol{f}_{nonremobilized})$$

Assuming that there is a proportionate distribution of remobilized nitrogen throughout the plant (i.e., C is the same for all plant organs), the mass balance equation showing the contributions of new and carry-over nitrogen to measured $\delta^{15}N$ for roots, stems and leaves is:

Equation 4.8 $\boldsymbol{\delta^{15}N_{measured}} = \boldsymbol{\delta^{15}N_{carry-over}} \times \boldsymbol{C} + \boldsymbol{\delta^{15}N_{new}} \times (\boldsymbol{1} - \boldsymbol{C})$

where, $\delta^{15}N_{uncorrected}$, $\delta^{15}N_{carry\text{-}over}$ and $\delta^{15}N_{new}$ are the measured isotopic compositions of each plant organ, the isotopic composition of remobilized nitrogen (equal to $\delta^{15}N_{start}$), and the isotopic composition of newly assimilated nitrogen, respectively. Equation 4.8 can be rearranged to yield:

Equation 4.9 $\boldsymbol{\delta^{15}N_{new}} = \frac{\boldsymbol{\delta^{15}N_{measured}} - \boldsymbol{\delta^{15}N_{carry-over}} \times \boldsymbol{C}}{(\boldsymbol{1}-\boldsymbol{C})}$

This equation was used to obtain root, stem and leaf $\delta^{15}N$ values adjusted for the contribution of carry-over nitrogen from the cuttings..

4.3.5. Statistical analysis

Two-way ANOVA was used to test for the fixed effects of nitrogen source and genotype on 1) biomass, 2) measured $\delta^{15}N$ values, 3) the proportion of cutting nitrogen remobilized to the growing plant, 4) the proportion of nitrogen in new growth that is carry-over nitrogen, and 5) the adjusted $\delta^{15}N$ values of plant, root and leaf tissues. The statistical model was as follows:

Equation 4.10 $\boldsymbol{Y_{ij}} = \boldsymbol{\mu} + \boldsymbol{\alpha_i} + \boldsymbol{\tau_j} + \boldsymbol{\beta_{ij}} + \boldsymbol{e_{ij}}$

where, μ is the overall mean response, α_i is the effect due to the genotype, τ_j is the effect due to the nitrogen source and β_{ij} is the effect due to any interaction between the genotype and nitrogen source. Analysis of variance procedure was carried out using Graphpad Prism 6 (La Jolla, CA, USA) to obtain estimates of the means followed by Tukey's multiple comparison tests to separate means. Differences between treatments described as significant are those where $P < 0.05$. Whole plant $\delta^{15}N$ was plotted against biomass and per cent carry-over nitrogen to examine the influence on measured $\delta^{15}N$ of genotypes grown using nitrate or ammonium. Non-linear regression was performed for the measured plant $\delta^{15}N$ versus biomass using Graphpad Prism 6 to fit an exponential model to the data. Linear regression was performed on adjusted plant $\delta^{15}N$ versus biomass and on both measured and adjusted plant $\delta^{15}N$ versus the proportion of carry-over nitrogen in new plant growth.

4.4. Results

4.4.1. $\delta^{15}N$, growth and nitrogen concentration

Both ammonium and nitrate-grown plants were depleted in ^{15}N relative to the source (Table 4.1). Under nitrate, mean root, stem and leaf $\delta^{15}N$ was 44.4, 47.1 and 50.1‰, whereas for ammonium, these values were -8.6, -6.6 and -5.1‰, respectively. The vegetative cuttings used to propagate the plants were not different between sources and had a mean $\delta^{15}N$ of 0.92‰ at the start of the experiment. During plant growth, the $\delta^{15}N$ of the cuttings moved towards and/or past the isotope signature of the source (Table 4.1). For nitrate-grown plants, cuttings became enriched (23.5‰) and for ammonium-grown plants, cuttings became depleted (-3.1‰) relative to their starting isotopic composition ($P<0.05$). Genotypic means for biomass ranged from 0.51 to 4.46 g and 0.33 to 3.48 g under nitrate and ammonium conditions, respectively (Table 4.2). There was a significant correlation between genotype means for biomass on nitrate versus ammonium ($r= 0.54$, $P<0.05$) whereby clones that grew well on one source also grew well on the other. The range in mean genotype biomass provided a range of isotopic dilutions to determine the influence of remobilized pre-existing nitrogen on whole-plant and organ level $\delta^{15}N$. When the uncorrected $\delta^{15}N$ for new growth of the whole plant was plotted against biomass, there was a significant non-linear relationship ($r^2 = 0.331$ and 0.249 for nitrate and ammonium, respectively; $P<0.05$) (Figure 4.1). The uncorrected $\delta^{15}N$ for new growth of the whole plant for genotypes with low biomass were closer to the $\delta^{15}N$ of cutting nitrogen, whereas for genotypes with high biomass they were closer to the $\delta^{15}N$ of newly acquired nitrogen (which resembles the source, but does not equal it due to isotope discrimination that occurs during uptake and assimilation). Nitrogen concentration was not different between cuttings before the start of the experiment, but was significantly greater at the end of the experiment when grown with ammonium as compared to nitrate (Table 4.3). Stem and cutting nitrogen concentrations were not significantly different from each other on either source. Root nitrogen concentration was not significantly different between sources, but stem and leaf nitrogen concentrations were greater when grown with ammonium ($P<0.05$).

Table 4.1. Mean measured and, for new tissues, adjusted root, stem, leaf and cutting $\delta^{15}N$ values (‰ ±SE; N=75) of *Populus balsamifera* L. hydroponically grown with nitrate (58.5‰) or ammonium (-0.96‰).

	Nitrate	**Ammonium**
	Measured $\delta^{15}N$	
Root	44.42±0.42	-8.59±0.15
Stem	47.08±0.40	-6.62±0.18
Leaf	50.10±0.44	-5.07±0.15
$Cutting_{start}$	0.87±0.35	0.97±0.31
$Cutting_{end}$	23.47±0.88	-3.10±0.23
	Adjusted $\delta^{15}N$	
Root	47.87±0.28	-9.45±0.19
Stem	50.76±0.26	-7.29±0.19
Leaf	54.01±0.25	-5.59±0.18

Table 4.2. Mean root, stem, leaf, cutting and whole plant biomass (g ±SE; N=75) of *Populus balsamifera* L. hydroponically grown with nitrate or ammonium.

	Biomass (g)	
	Nitrate	**Ammonium**
Root	0.18±0.02	0.20±0.01
Stem	0.40±0.03	0.29±0.02
Leaf	1.28±0.09	1.15±0.07
$Cutting_{start}$	0.49±0.03	0.54±0.04
$Cutting_{end}$	0.67±0.06	0.65±0.05
Whole Plant	1.86±0.14	1.64±0.10

Table 4.3. Mean nitrogen concentrations (µmol g dw^{-1} ±SE; N=75) of roots, stems, leaves and cuttings of *Populus balsamifera* L. hydroponically grown with nitrate or ammonium.

	Tissue nitrogen concentration (µmol g dw^{-1})	
	Nitrate	**Ammonium**
Root	2.50±0.04	2.55±0.03
Stem	0.41±0.01	0.76±0.03
Leaf	1.84±0.03	2.19±0.04
$Cutting_{start}$	0.83±0.01	0.83±0.02
$Cutting_{end}$	0.59±0.03	0.71±0.02

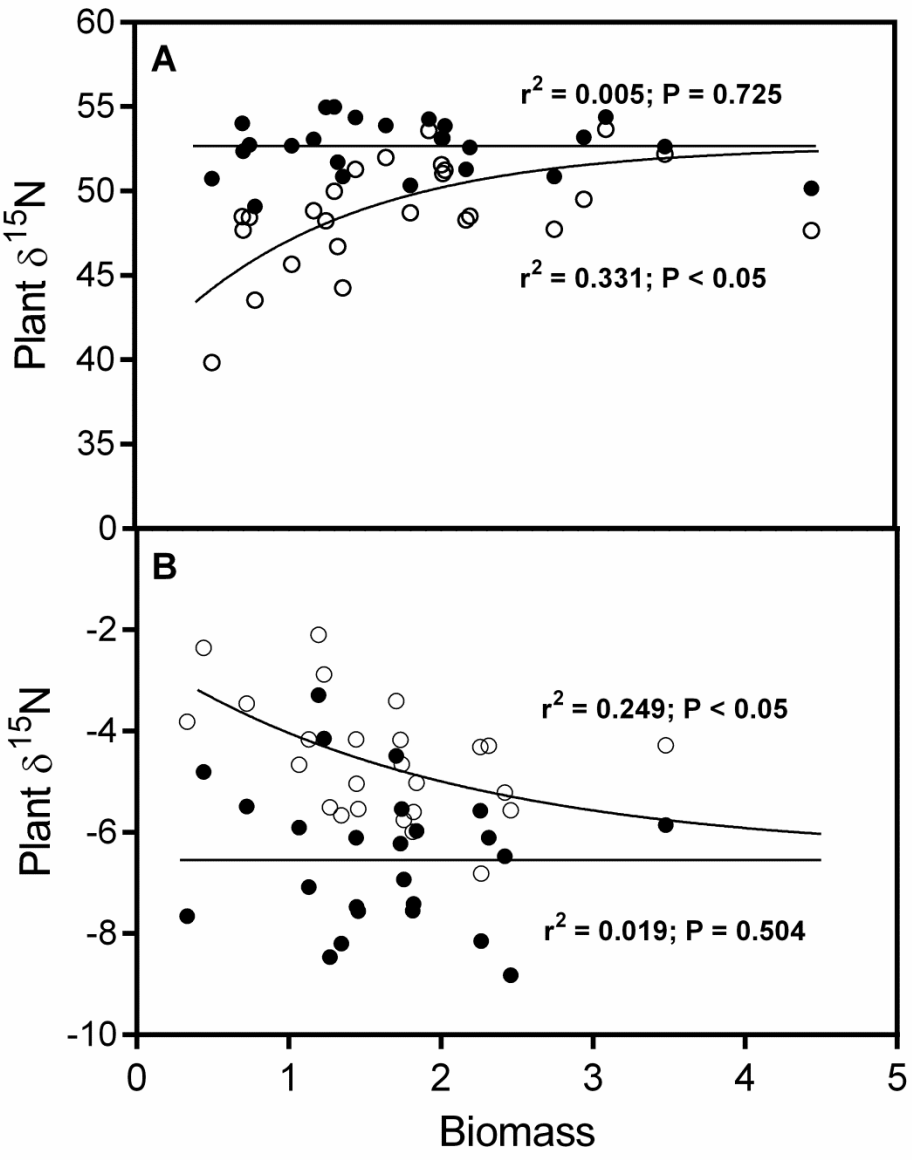

Figure 4.1. Unadjusted (open symbols) and adjusted (closed symbols) δ^{15}N for new growth of the whole plant plotted against biomass for *Populus balsamifera* L. grown with either A) nitrate or B) ammonium. Each data point represents a genotypic mean (N=3). Correlation coefficients and P-values are placed near each line.

4.4.2. Carry-over nitrogen in new plant growth is dependent on the size of the nitrogen pool in the cutting relative to the nitrogen pool in the new growth

The $\delta^{15}N$ of vegetative cuttings at the end of the experiment fell close to the middle between the starting cutting $\delta^{15}N$ and the $\delta^{15}N$ of newly formed organs, indicating that some considerable portion of the original nitrogen was retained within the cuttings. From equation 4.4, the nonremobilized fraction of the nitrogen contained within the cuttings at the end of the experiment averaged 50% and 48% for nitrate and ammonium, respectively. The two nitrogen sources were not significantly different from each other and, using equation 4.5, this translated into approximately half of the starting nitrogen being remobilized for new growth (Figure 4.2). The mean quantity of nitrogen remobilized was 3.2 mg but was dependent on the size of the cutting ($r=0.057$; $P<0.05$). There was no source effect on the proportion of carry-over, which averaged 8% but decreased non-linearly from 21% to 1% with the biomass of new growth (not shown). The difference between source $\delta^{15}N$ and the old cutting $\delta^{15}N$ was greater for nitrate than ammonium (Table 4.1), causing carry-over nitrogen to have a greater influence on measured plant $\delta^{15}N$ under nitrate (Figure 4.3A) than ammonium (Figure 4.3B).

4.4.3. Relationships between plant $\delta^{15}N$ and biomass were not significant after accounting for carry-over of pre-existing nitrogen

There were significant linear relationships between the contribution of nitrogen carried-over from the cutting and the uncorrected $\delta^{15}N$ for new growth of the whole plant ($P<0.05$) (Figure 4.3). Relative to the measured $\delta^{15}N$ of each corresponding organ, newly assimilated nitrogen was more depleted in ^{15}N under ammonium and more ^{15}N enriched under nitrate. After accounting for the presence of remobilized nitrogen, the adjusted root, stem and leaf $\delta^{15}N$ values were 47.87, 50.76 and 54.01‰ and -9.45, -7.29 and -5.59‰ for nitrate and ammonium, respectively. This represented an approximate shift of 3.5 to 4‰ and 0.5 to 0.8‰ for nitrate and ammonium grown plants, respectively. There was no residual relationship between biomass and newly acquired

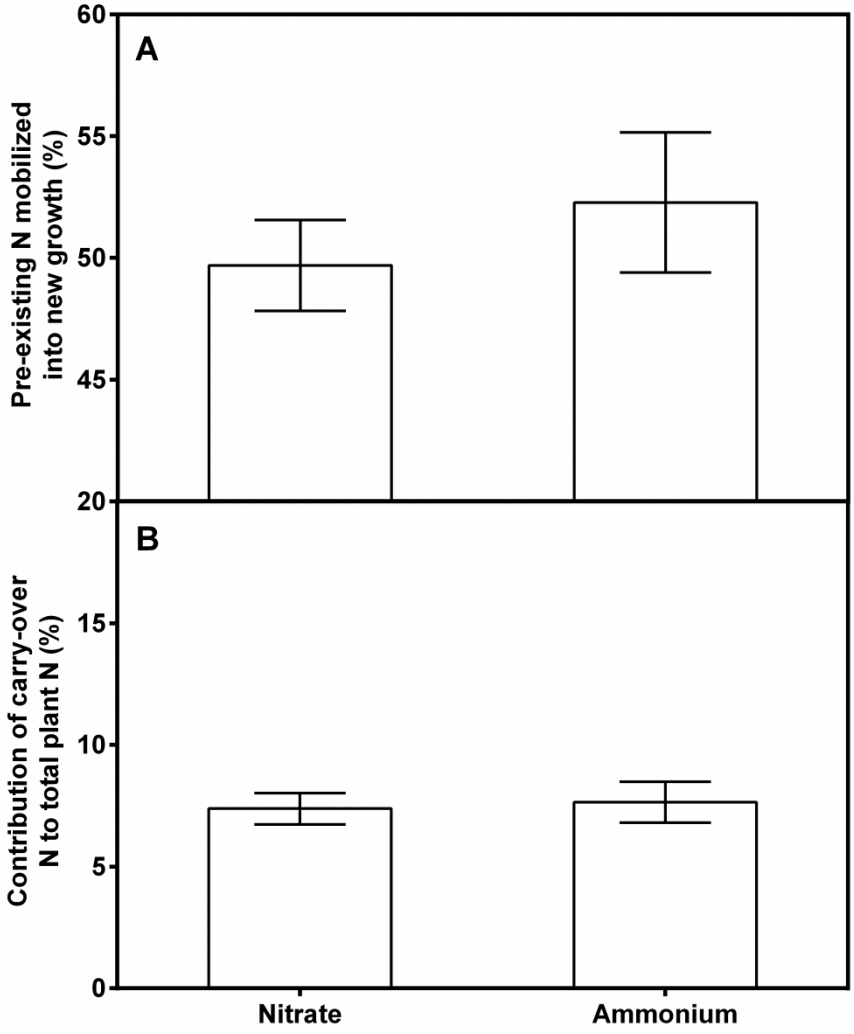

Figure 4.2. Percentage of A) pre-existing nitrogen remobilized into new growth and B) percentage contribution of pre-existing cutting nitrogen to total plant nitrogen in *Populus balsamifera* L. grown with either nitrate or ammonium. Bars are means ±SE (N=75).

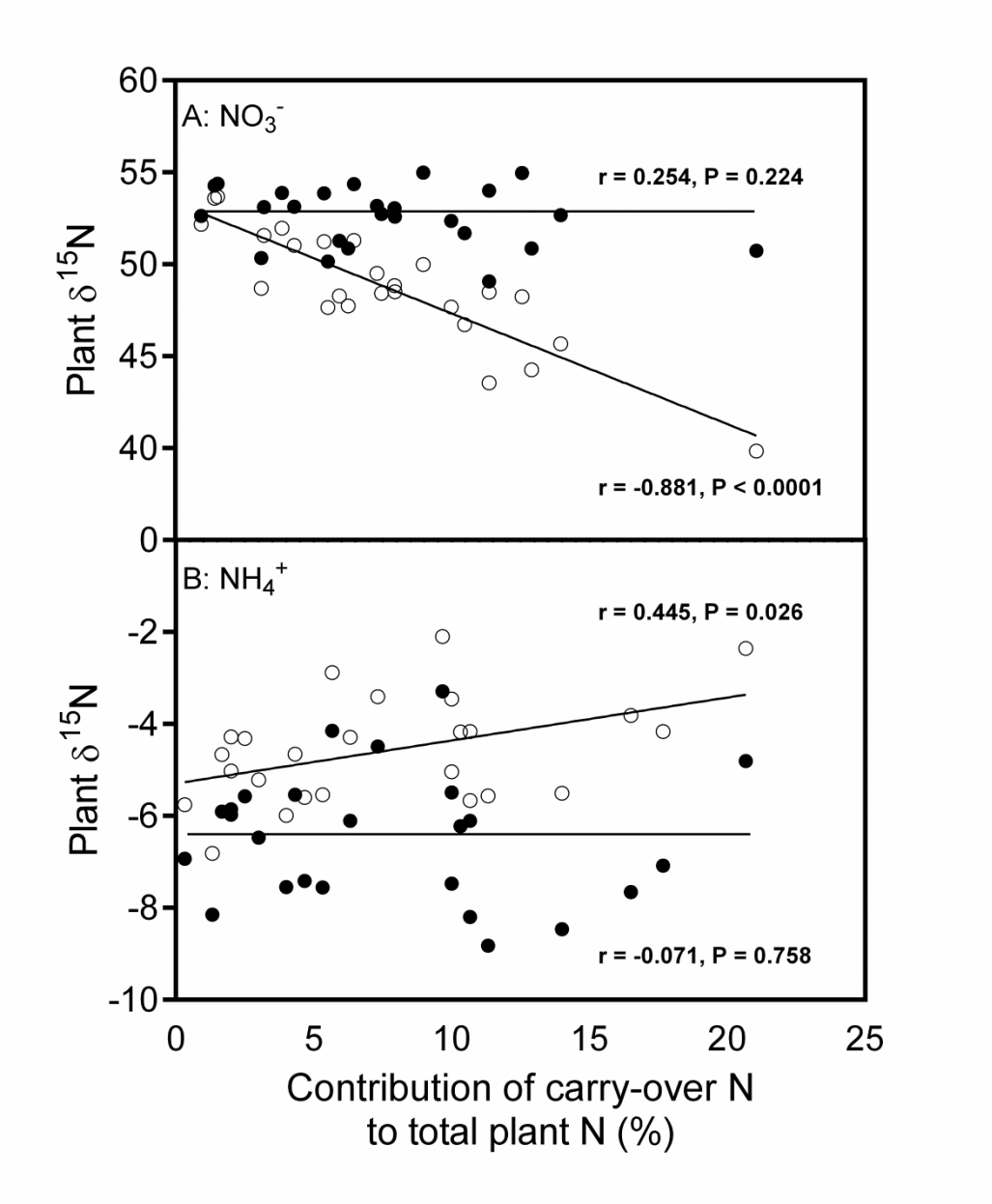

Figure 4.3 Unadjusted (open symbols) and adjusted (closed symbols) $\delta^{15}N$ for new growth of the whole plant plotted against the percentage contribution of carry-over nitrogen to new growth of *Populus balsamifera* L. supplied with either A) nitrate or B) ammonium. Each data point represents a genotypic mean (N=3). Correlation coefficients and P-values are placed near each line.

plant $\delta^{15}N$ (i.e., non-significant regressions in Figure 4.3) indicating that the carry-over nitrogen was well accounted for.

4.5. Discussion

The objective was to quantify and correct for carry-over nitrogen from cuttings in new growth of balsam poplar hydroponically grown under steady-state conditions. Here, it was shown that nitrogen remobilized from cuttings influences plant $\delta^{15}N$. Carry-over would be inconsequential for plants grown from relatively small seeds or in longer-term experiments, where the total accumulated nitrogen pool represents almost all of the nitrogen within the plant. However, in cases where biomass accumulation is low relative to the size of the propagule, without correction, deviation of measured $\delta^{15}N$ from the $\delta^{15}N$ of newly acquired nitrogen can lead to misinterpretations in physiological and ecological studies. It has been suggested that there are carry-over effects of interannual variability in carbon isotope signatures ($\delta^{13}C$) in woody plants (Vaganov *et al.* 2009; Offerman *et al.* 2011), but, aside from Kolb and Evans (2003), this is the first attempt to quantify the carry-over effect on nitrogen isotope composition of new growth in woody plants.

Accounting for remobilized nitrogen from the cutting to the root, stems and leaves required a two-step approach. Since vegetative cuttings are a part of the stem, nitrogen contained within the cutting should be combined with the general stem nitrogen pool. However, there is a certain fraction of nitrogen in the vegetative cutting that was not mobilized and therefore, needs to be quantified to determine the corresponding amount of cutting nitrogen that was remobilized into the general nitrogen pool. In the present study, and noting that some isotope discrimination is expected during nitrogen uptake and assimilation, the presence of non-remobilized nitrogen in the cutting was indicated by a difference between stem and cutting $\delta^{15}N$ that was weighted towards the source $\delta^{15}N$ (Table 4.1). Previous work has demonstrated that there is often a fraction that is not remobilized to sink tissue (Millard and Thomson 1989). Once non-remobilized nitrogen and the proportion of cutting nitrogen that is part of the stem nitrogen pool had been partitioned, the amount of

nitrogen mobilized into the plant nitrogen pool was quantified. To make this partitioning calculation, it was assumed that new nitrogen in the cutting was mixed with remobilized (contaminating) nitrogen from the cutting similar to that of stem tissue. Since there was influx of newly assimilated nitrogen into the vegetative cutting, as indicated by a growth dependent movement of cutting $\delta^{15}N$ towards the source $\delta^{15}N$, it would be reasonable to assume there is a certain degree of mixing of nitrogen within the mobile stem nitrogen pool.

Here, it was assumed that remobilization was proportional to organ biomass and otherwise equal among plant organs. Variation in the difference in $\delta^{15}N$ between measured and newly acquired nitrogen may, in part, be a consequence of assuming that the distribution of carry-over nitrogen was proportionate to the nitrogen allocation between roots, stems and leaves. Dong *et al.* (2004) reported that approximately 30% of remobilized nitrogen was allocated to the root and 70% was allocated to the shoot in vegetatively propagated poplar and although the proportion allocated to the root was slightly higher than our results, the proportion was similar enough to validate the assumption of proportional remobilization. Even though biomass allocation was not reported in Dong *et al.* (2004), if the root:shoot ratios were similar to those reported here, this would indicate proportional allocation of remobilized nitrogen to roots, stems and leaves in our experiments.

Another assumption made was to take the $\delta^{15}N$ of remobilized nitrogen as being equal to the $\delta^{15}N$ of the cutting at the start of experiment. Compound specific variation in $\delta^{15}N$ (Werner and Schmidt 2002, Tcherkez 2011, Gauthier *et al.* 2012) may result in divergence of remobilized $\delta^{15}N$. However, Kolb and Evans (2003) reported that there was no difference in remobilized $\delta^{15}N$ and stored $\delta^{15}N$, and that no fractionation occurred during remobilization. Additionally, as a greater proportion of nitrogen is remobilized, it becomes less likely that compound-specific variation in $\delta^{15}N$ will contribute to divergence of remobilized $\delta^{15}N$ from the reference $\delta^{15}N$ unless there are large differences between structural and mobile nitrogen $\delta^{15}N$. Although biochemical

variation in $\delta^{15}N$ may add error to our approach, it would seem to be small relative to the correction applied.

The proportion of soluble nitrogen in cuttings may limit nitrogen remobilization which may provide an explanation for why approximately half of pre-existing nitrogen was not remobilized. During the dormant period, nitrogen storage proteins accumulate in stem tissue and the proportion of soluble, stored nitrogen increases (Marmann *et al.* 1997, Bollmark *et al.* 1999, Coleman 2004, Millar and Grelet 2010). Estimates of soluble nitrogen in stems of *Fraxinus excelsior* were between 60 and 70% during the dormant period (Marmann *et al.* 1997). If the proportion of soluble nitrogen in balsam poplar is similar, then only a small fraction of soluble nitrogen was not remobilized to new growth. Our natural abundance nitrogen isotope mass balance approach indicates that approximately 50% of cutting nitrogen was remobilized to sink tissues (leaves, roots and stems). In hybrid poplar, between 60 and 70% of nitrogen in cuttings was remobilized into actively growing tissue (Dong *et al.* 2004). This proportion was comparable with estimates of remobilization of nitrogen from source tissue (leaves) of *Brassica napus* to sinks (developing siliques) (Rosatto *et al.* 2001).

Using two separate nitrogen sources with $\delta^{15}N$ values both greater and lower than reference $\delta^{15}N$ provided some validation for the correction. Previously, enriched nitrogen applied as ^{15}N allowed for the quantification of remobilized nitrogen into growing plant tissue (Dong *et al.* 2004). In the present study, newly acquired $\delta^{15}N$ under ammonium ($\delta^{15}N$ = -0.96 ‰) was on average 5 to 10‰ depleted relative to the cutting $\delta^{15}N$, while newly acquired $\delta^{15}N$ for nitrate ($\delta^{15}N$ = 58.5 ‰) was approximately 50 to 55‰ enriched relative to the cutting. This contrast in the isotopic composition of two sources of newly acquired nitrogen, relative to nitrogen that previously existed in the cutting, gave negative and positive non-linear relationships between biomass and the unadjusted plant $\delta^{15}N$. Here, after correction, biomass had no impact on corrected plant $\delta^{15}N$ for either source and any observed relationship was primarily a result of nitrogen carried-over from the cutting. Our approach worked well either way, which should not

be the case if the $\delta^{15}N$ of remobilized nitrogen were consistently offset in one direction from the $\delta^{15}N$ of bulk nitrogen.

4.6. Conclusion

Here, evidence was provided that remobilized nitrogen carried-over from stem cuttings can affect measured $\delta^{15}N$ of new growth in poplar, a perennial model plant. Carry-over nitrogen from pre-existing nitrogen pools needs to be considered when measuring $\delta^{15}N$ at natural abundance. By applying a two-step mass balance correction, the contributions of pre-existing nitrogen to the nitrogen content and $\delta^{15}N$ of new growth can be quantified and accounted for. This, in turn, will allow for a better assessment of isotope discrimination associated with the acquisition of new nitrogen from the rooting environment and could be helpful in physiology and ecology studies measuring nitrogen isotope composition at natural abundance to address questions relating to nitrogen use dynamics at the soil or plant level. The methods presented here are not only applicable to this species or set of conditions but, with careful sampling protocols, can be applied to other experimental systems that require the measurement of $\delta^{15}N$ at natural abundance during plant growth and development.

5. Coherence of independent isotopic measures of nitrogen efflux/influx (*E/I*) in roots of *Populus balsamifera* L.

5.1. Summary

Acquisition of mineral nitrogen from solution is often not completely efficient, there being a variable amount of leakage (efflux) relative to gross uptake (influx). The efflux/influx ratio (*E*/*I*) is inversely related to the efficiency of nutrient uptake at the roots. Radioactive label-based isotopic measurements of ion flux and *E*/*I* have been traditionally used, but are laborious and may not reflect time-integrated *E*/*I*. Alternatively, information on *E*/*I* and other nitrogen use traits may also be obtained from variation in stable isotope ratios. Five genotypes of *Populus balsamifera* L. were grown hydroponically on either 500 μmol L^{-1} NO_3^- or NH_4^+. *E*/*I* was calculated using an isotope mass balance model and compared to *E*/*I* calculated using ^{15}N compartmental analysis. Both methods indicated that plants grown on ammonium had greater *E*/*I* than plants grown on nitrate. Genotype specific means were linearly correlated (r=0.77; P=0.0065) with few rank changes, but *E*/*I* was approximately 0.15-0.2 higher using the nitrogen isotope mass balance approach. Similarly, net uptake based on isotope mass balance was greater than indicated by compartmental analysis. Discrepancies in *E*/*I* between methods may reflect uncertainties in discrimination factors for the assimilatory enzymes (nitrate reductase and glutamine synthetase), temporal differences in uptake patterns (compartmental analysis provides a single point measurement, whereas isotope mass balance is integrated over weeks), and the possibility of some discrimination associated with membrane transport. This chapter demonstrates the relationship between E/I calculated using the isotope mass balance approach presented in this thesis and E/I calculated using a previously established, independent approach providing.

5.2. Introduction

One prospect for improving nitrogen-use efficiency is thought to lie in improving the ability of plants to efficiently acquire nitrogen from their environment. To achieve

this, an integrated approach to understanding nitrogen uptake and assimilation in plants is required (Hirel *et al.* 2007). Garnett *et al.* (2009) suggested that root-based approaches are under-explored and may represent an avenue for improving plant nitrogen-use efficiency. The acquisition of inorganic nitrogen from the surrounding environment is a function of root architecture, ion preference and nitrogen demand. In essence, improving acquisition is not only dependent on uptake, but also on the ability of plants to assimilate and translocate nitrogen into sink tissues.

Nitrogen movement between the rhizosphere and roots is bidirectional. Following uptake (influx), nitrate and/or ammonium ions may be assimilated or, alternatively, return to the rooting medium (efflux) through leakage or excretion (Xu *et al.* 2012). Inorganic nitrogen may also pass directly to the xylem for transport to the shoot. These processes, in combination, result in the homeostatic balance of cytoplasmic inorganic nitrogen in roots (Lee and Clarkson, 1986). The fact there is significant efflux suggests some inefficiency in nitrogen acquisition whereby the plant is unable to expeditiously assimilate available cytosolic inorganic nitrogen or to balance intracellular pH and ionic balance (Britto and Kronzucker, 2006). Efflux of inorganic nitrogen has been reported under both changing and steady-state nitrogen conditions (Kronzucker *et al.* 1995; Hawkins and Robbins 2010), and is dependent on substrate concentration (Morgan *et al.* 1973; Kronzucker *et al.* 1995; Hawkins and Robbins, 2010), root maturity and plant nitrogen demand (Bloom *et al.* 2010; Hawkins and Robbins, 2010). Cycling of unassimilated, inorganic nitrogen between the substrate and the root is thought to reflect inorganic nitrogen source preference (Kronzucker *et al.*, 1997) and may be associated with reduced nitrogen use efficiency. High efflux/influx (*E*/*I*) can have high energetic costs (Kronzucker *et al.* 2001; Scheurwater *et al.* 1999). At high ammonium concentrations, these costs are sufficient to reduce growth, and may cause damage or even death to the plant (Britto and Kronzucker, 2006).

Though important, characterizing nitrogen fluxes can be problematic because of high spatial and temporal heterogeneity in roots of actively growing plants (Alber *et al.* 2012; Bloom *et al.* 2012). Inorganic nitrogen uptake has been shown to vary diurnally

(Delhon *et al.* 1995; Gessler *et al.* 2002). Diurnal variation in nitrogen uptake patterns may result in poor characterization of genotypic variation in nitrogen use. Fluxes within roots have previously been characterized using microelectrode measurements (Henricksen *et al.* 1990; Miller and Zhen, 1991; Hawkins *et al.* 2010) or compartmental analysis using ^{13}N radioisotope (Lee and Clarkson, 1986; Siddiqi *et al.* 1991; Kronzucker *et al.* 1995) or ^{15}N stable isotope (Macklon *et al.* 1990; Devienne *et al.* 1994) labelling, under steady state conditions. Compartmental analysis provides tissue specific information that cannot be obtained using other methods, including compartment-specific measures of half-life of exchange, nitrogen concentration and efflux (Lee and Clarkson, 1986; Min *et al.* 2002; Kronzucker *et al.* 2005). Microelectrode measurements have the benefit of being a calibrated measure of nitrogen flux that is tissue or even cell-specific (Henricksen *et al.* 1992). Although fast and accurate, these single time-point measurements need extensive replication to deal with changes in nitrogen flux over a diurnal cycle.

The dynamic nature of nitrogen flux in space and time suggests that an integrated approach to assess nitrogen flux may better capture whole plant nitrogen use. Differences in the natural abundance of stable nitrogen isotopes of plants grown under steady-state nitrogen conditions have potential to provide time and spatially-integrated information on whole plant and organ level nitrogen uptake and assimilation patterns. Chapter 3 demonstrated that whole plant and organ level variation in isotopic composition can be successfully interpreted using a version of the mass balance model proposed in Chapter 2. The model does not discern between cellular compartments at the tissue level, but can quantify whole-plant and organ-level nitrogen use traits such as partitioning of organic and inorganic nitrogen, partitioning of assimilation, and gross and net ion fluxes. For evaluating short-term responses to changes in nitrogen concentration or other treatments, compartmental analysis and/or microelectrode measurements are appropriate. However, without a comprehensive sampling protocol, only nitrogen isotope discrimination can provide reliable information on long-term nitrogen use.

Furthermore, isotope mass balance approaches can be scaled up to accommodate larger experiments or phenotypic screening.

Nitrate and ammonium transport have been extensively studied in model plants such as *Arabidopsis thaliana* (L.) Heynh. and *Nicotiana tobacum* L. (Stitt and Feil, 1999; Glass *et al.* 2002). However, to date, few studies have investigated nitrate or ammonium transport in *Populus* sp. (Min *et al.* 2002; Selle *et al.* 2005; Couturier *et al.* 2007). As interest increases for using *Populus* as a bioenergy resource (Yemshanov *et al.* 2008), identifying variability in nitrogen transport fluxes will be important for improving nitrogen-use efficiency in new cultivars. A high degree of trait variability, particularly for adaptive traits such as phenology and photosynthesis, has been observed in balsam poplar (*Populus balsamifera* L.) and black cottonwood (*Populus trichocarpa* Torr. & A. Gray) (Soolanayakanahally *et al.* 2009; McKown *et al.* 2013). However, intraspecific variability in nitrogen flux within these species, or indeed any woody plant, has not been explored. The objective of this experiment was to compare independent methods of estimating *E*/*I* and net nitrogen uptake using five genotypes of balsam poplar grown on either nitrate or ammonium. More specifically, I sought to validate the isotope mass balance approach by comparing it to compartmental analysis whereby *E*/*I* was estimated from ^{15}N isotope discrimination in the former, versus ^{15}N labelling in the latter.

5.3. Materials and methods

5.3.1. Plant material and experimental design

Five genotypes (GIL-9, GIL-7, GIL-2, CLK-4 and OUT-9) of balsam poplar from three provenances from the Agriculture Canada Balsam Poplar (AgCanBaP) collection (Soolanayakanahally *et al.* 2009) were randomly chosen from genotypes selected for evaluation of genotypic variation in nitrogen isotope discrimination presented in chapter 6. First-year branches were obtained from the AAFC- AESB Agroforestry Development Centre, Indian Head, Saskatchewan, Canada and stored at 4 °C for approximately 3 months to fulfill chilling requirements. The three provenances were:

Gillam (GIL), Manitoba (56.4 °N, 94.7 °W), Outlook (OUT), Saskatchewan (51.1 °N, 106.2 °W), and Cold Lake (CLK), Alberta (54.2 °N, 110.1 °W). Two-node vegetative cuttings, approximately 6-8 cm long, were arranged in a randomized complete block design with three blocks consisting of two nitrogen source treatments; either 500 µM nitrate or 500 µM ammonium. There were four clonal replicates in each block to provide sufficient plant material for complimentary sampling at the end of the experiment. Pre-existing nitrogen was accounted for by applying a correction (Chapter 4) that uses a mass balance model to quantify the amount of nitrogen remobilized from the cutting and proportionately adjust growing tissue $\delta^{15}N$.

5.3.2. Hydroponics system

The hydroponics system was comprised of six 1000L containers lined with black 45 mil rubber pond liner material (Firestone, Nashville, TN, USA) constructed in a greenhouse under ambient light conditions supplemented by sodium halide lighting providing a minimum PPFD of 600 µmol m^{-2} s^{-1} and 18/6h day/night photoperiod. Temperatures in the greenhouse were maintained between 20 and 24 °C. Each container was fitted with a floating Perspex "raft" that held up to 32 plants, as described in Chapter 4. The hydroponics solution was a modified $1/10^{th}$ strength Johnson's solution (Johnson *et al.* 1957) with either 250 µM $Ca(NO_3)_2$ ($\delta^{15}N$ = +60.3 ‰) or 250 µM $(NH_4)_2SO_4$ ($\delta^{15}N$ = -0.96 ‰). Final nutrient composition, excluding the nitrogen salts, was: 200 µM KH_2PO_4, 200 µM K_2S0_4, 100 µM $MgSO_4$, 100 µM $CaSO_4$, and micronutrients: 5 µM Cl, 2.5 µM B, 0.2 µM Mn, 0.2 µM Zn, 0.05 µM Cu, and 50 µM Fe^{2+}. A centrifugal pump, with a pumping capacity of approximately 20 L per minute, provided circulation and aeration of the solution for each container. The solution was monitored periodically for oxygen levels, pH (Figure A.2) and temperature (Figure A.3). Powdered calcium carbonate ($CaCO_3$) was added to buffer pH in the range of 6-7.5. Media NH_4^+ and NO_3^- concentrations were assayed using the phenol:hypochlorite (Solorzano 1969) and perchloric acid (Cawse 1967) methods, respectively (Figure A.4). The solution was completely replaced every 14 days to ensure that there was no substantial decrease

(>10%) in concentration of nitrate or ammonium over time that could increase the solution $\delta^{15}N$.

5.3.3. Sampling and natural abundance isotope analysis

After 45 days of growth, plants were randomly sampled for either nitrogen isotope mass balance or ^{15}N compartmental analysis (N=3). For natural abundance isotope analysis, samples were separated into leaves, stems and roots. Samples were flash frozen in liquid nitrogen and stored at -80 °C until freeze drying. Roots, leaves and stems were weighed for dry mass and then ground to a fine powder using a mortar and pestle and then ball milled (Fritsch Laborgeratebau, Terochem Scientific). Sub-samples of 3±0.1 mg were weighed into tin capsules (Elemental Microanalysis Ltd., 8x5 mm, D1008) and analyzed for $\delta^{15}N$ and nitrogen on a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) (University of California Stable Isotope Facility, Davis, CA).

5.3.4. ^{15}N efflux apparatus

Plants sampled for ^{15}N efflux analysis were transferred to a holding tank with nutrient composition equal to the growing solution. The holding tank was placed under fluorescent lighting (200 µmol m^{-2} s^{-1} PPFD) until plants were treated with the ^{15}N loading solution. Each plant was individually placed in 1000 mL of aerated, loading solution of 98 atom% ^{15}N for 60 minutes, after which the plant was removed and roots were allowed to gravitationally drain for 30 seconds. Roots were then rinsed over a glass funnel with the eluate flowing into 15 pre-weighed glass beakers at intervals of 3 x 15 s, 3 x 30 s, 2 x 60 s and 7 x 120 s for a total elapsed time of 18 minutes 15 seconds. Rinsing solution (identical to the original hydroponics solution) was supplied using a peristaltic pump and polyvinyl chloride tubing (Kuritec, Brantford, Ontario, Canada) at a rate of 4 mL s^{-1}. Eluate volumes were determined by mass difference. Acidified (pH 2.0) 15 mL aliquots were stored in disposable scintillation vials and stored at 2 °C until processing for isotope analysis. Roots were weighed for fresh mass then oven-dried for 2 days at 60 °C to obtain dry mass.

5.3.5. ^{15}N efflux isotope analysis

Tin capsules (~1.5 mL) were prepared from foil squares (30 mm x 30 mm, Elemental Microanalysis UK). Eluate was pipetted in 7 x 1 mL increments (~49 µg of N total) into the capsules, with drying at ~50 °C between each increment. Root samples were prepared and loaded into tin capsules as above. Nitrogen isotope analysis was performed at the University of Saskatchewan Soil Science Stable Isotope Laboratory using a Costech ECS4010 elemental analyzer coupled to a Delta V mass spectrometer with Conflo IV interface.

5.3.6. Compartmental analysis

Compartmental analysis was performed based on methods in Lee and Clarkson (1986), except release of excess ^{15}N from the root (µmol g fw^{-1} min^{-1}) was substituted for rate of ^{13}N release, as follows:

Equation 5.1

$$^{15}N\ efflux\ (nmol\ N) = \frac{(^{15}N\ Atom\%_{Eluate} - ^{15}N\ Atom\%_{Rinse})}{S_0} \times N_{eluate}$$

where, $^{15}N\ Atom\%_{Rinse}$ was equal to 0.36654% and the specific activity of the loading solution, S_0, was equal to 98%. The rate of ^{15}N efflux, expressed on a per mass basis, was then calculated as:

Equation 5.2 $$^{15}N\ efflux\ rate\ (nmol\ N\ g\ fw^{-1}\ min^{-1}) = \frac{(^{15}N\ Efflux)}{m \times t}$$

where, m = fresh mass of root, and t = time of eluate sampling and represents the elapsed sampling time for each individual solution sample.

The log of the ^{15}N efflux rate was plotted against elution time for segmental linear regression using Graphpad Prism 6 (La Jolla, CA, USA) to determine breakpoints and segment slopes (Figure 5.1). Segments represent nitrogen release from three compartments: the root surface, the apoplastic (intercellular) space and the cytoplasmic (intracellular) space (Lee and Clarkson, 1986). Half-life for each compartment was calculated as:

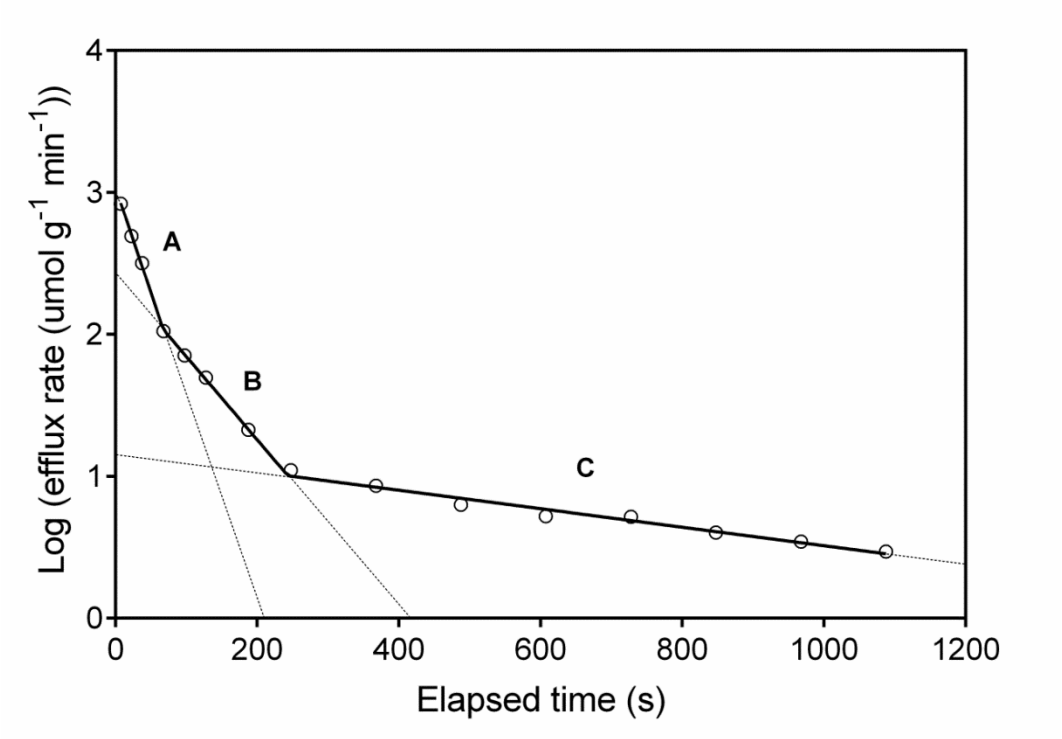

Figure 5.1. Representative segmental linear regression for determining half-lives of exchange and flux rates for the surface (A), apoplastic (B) and cytoplasmic (C) compartments. Each data point represents the log (efflux rate) at *x*-elapsed time (s).

Equation 5.3 $\boldsymbol{t_{1/2}\ (half-time\ of\ exchange) = \frac{0.693}{k}\ (min^{-1})}$

where, k is the rate constant and is equal to:

Equation 5.4 $\boldsymbol{k = \frac{\beta}{2.303}}$

where, β is the slope of the regression line. Compartmental efflux can be calculated as:

Equation 5.5 $\boldsymbol{Efflux\ (nmol\ g\ fw^{-1}\ h^{-1}) = \frac{R_0}{S_0}}$

where, R_0 is the intercept of the regression line with the ordinate for each compartment (i.e., the ^{15}N efflux rate at time zero).

Net uptake was calculated as the excess ^{15}N remaining in the roots after rinsing and expressed as µmol N g fw^{-1} h^{-1}. Efflux/influx was then:

Equation 5.6 $\boldsymbol{Efflux/Influx = \frac{Efflux}{(Efflux+Net\ Uptake)}}$

Flux from the root to the shoot could not be calculated because of the possibility of direct contamination of leaves or stems by the labelling solution during the loading process. Slight enrichment of stems and leaves was, however, observed (not shown).

Compartmental nitrate or ammonium content was calculated as the area under the exponential decay curve of a plot of time versus ^{15}N efflux rate (µmol h^{-1}), and was approximated as the total ^{15}N effluxed (µmol) during five half-lives:

Equation 5.7 $\boldsymbol{\sum_{i=1}^{5} N_i = (t_i - t_{i-1}) \times \frac{(r_i - r_{i-1})}{2}}$

where, i = i^{th} half-life, t = time and r = rate of ^{15}N efflux at i^{th} half-life. r at the i^{th} half-life was calculated by multiplying the initial rate of ^{15}N efflux by 0.5^i. Then, assuming that 5% and 10% of root volume was occupied by cytoplasmic and apoplastic space, respectively (Lee and Ratcliffe, 1983), nitrate and ammonium concentrations of these compartments were calculated.

5.3.7. Isotope mass balance flux calculations

E/*I* was calculated based on the methods in chapter 2 after correcting for carry-over of tissue nitrogen in stem cuttings (see chapter 4). *E*/*I* is a function of overall plant discrimination against ^{15}N relative to the substrate, the discrimination factor of the enzyme (nitrate reductase or glutamine synthetase for nitrate or ammonium, respectively) and the localization of assimilation in roots versus leaves. The proportion of total plant nitrogen in the leaf pool ($N_{leaf\ pool}/N_{total}$) is given by equation 2.8. Partitioning of assimilation between roots (P_{root}) and leaves ($1-P_{root}$) is a function of $N_{leaf\ pool}/N_{total}$, the difference in $\delta^{15}N$ between leaves and roots and the discrimination factor of the enzyme (Δ_{enz}) and is given by Equations 2.12 and 2.13. The discrimination factor for glutamine synthetase is thought to be near 16.8‰ (Yoneyama *et al.* 1993). Data in Ledgard *et al.* (1985) suggest a Δ_{enz} of 15.4‰ for nitrate reductase. From P_{root}, plant $\Delta^{15}N$ and Δ_{enz} of either nitrate reductase or glutamine synthetase, the estimate of *E*/*I* was obtained by Equation 2.15. The assimilation-averaged net influx of inorganic nitrogen into the root is equal to the total plant nitrogen, after correcting for contaminating nitrogen from the original cutting, divided by the root biomass (Equation 2.4).

5.3.8. Statistical analysis

To compare net uptake and *E*/*I* across the two methods, a three-way ANOVA in SAS 9.3 (SAS Institute, Cary, NC) was performed using the following model:

Equation 5.8 $$\boldsymbol{Y_{ijk} = \mu + \alpha_i + \tau_j + \rho_k + \beta_{ij} + \gamma_{ik} + \lambda_{jk} + \varepsilon_{ijk}}$$

where, μ is the overall mean response, α_i is the effect due to the genotype, τ_j is the effect due to the nitrogen source, ρ_k is the effect due to method, β_{ij} is the effect due to any interaction between the genotype and nitrogen source, γ_{ik} is the effect due to any interaction between genotype and method, λ_{jk} is the effect due to any interaction between nitrogen source and method and ε_{ijk} is the effect due to any three-way interaction between genotype, nitrogen source and method. Geometric mean

regression (Ricker 1984) was used to express the relationship between the two independent methods.

Nitrogen source and genotype fixed effects on biomass and on physiological variables unique to a particular method (i.e., root and leaf nitrogen concentrations, compartmental fluxes, half-lives of exchange and compartmental nitrogen concentrations) were tested using two-way ANOVA. The statistical model was as follows:

Equation 5.9 $$\boldsymbol{Y_{ij} = \mu + \alpha_i + \tau_j + \beta_{ij} + \varepsilon_{ij}}$$

where μ is the overall mean response, α_i is the effect due to the genotype, τ_j is the effect due to the nitrogen source and β_{ij} is the effect due to any interaction between the genotype and nitrogen source. ANOVA was carried out using Graphpad Prism 6 (La Jolla, CA, USA) followed by Tukey's multiple comparison tests to separate means. Where necessary, data were log transformed to meet assumptions of homogeneity of variance and normality. Differences between treatments described as significant are those where $P<0.05$.

5.4. Results

5.4.1. Plant growth

Plants were bigger when grown with nitrate ($P<0.05$) where mean biomass was 4.68 and 1.18 g under NO_3^- and NH_4^+, respectively (Figure 5.2). However, there was a significant interaction between genotype and nitrogen source. Biomass was greater on NO_3^- than NH_4^+ for four out of five genotypes. There were significant differences in growth among genotypes grown with NO_3^- but not NH_4^+. GIL-9 had the greatest difference in biomass between sources (6.9 and 1.19 g for NO_3^- and NH_4^+ grown plants, respectively). Biomass was not different between sources for GIL-7. No genotype was larger under NH_4^+ than NO_3^-. Root:shoot ratios were two times greater under NH_4^+ (0.26) than under NO_3^- (0.13). In contrast to biomass, there were no genotypic differences in root:shoot ratios for NO_3^- grown plants but there were for NH_4^+. Under

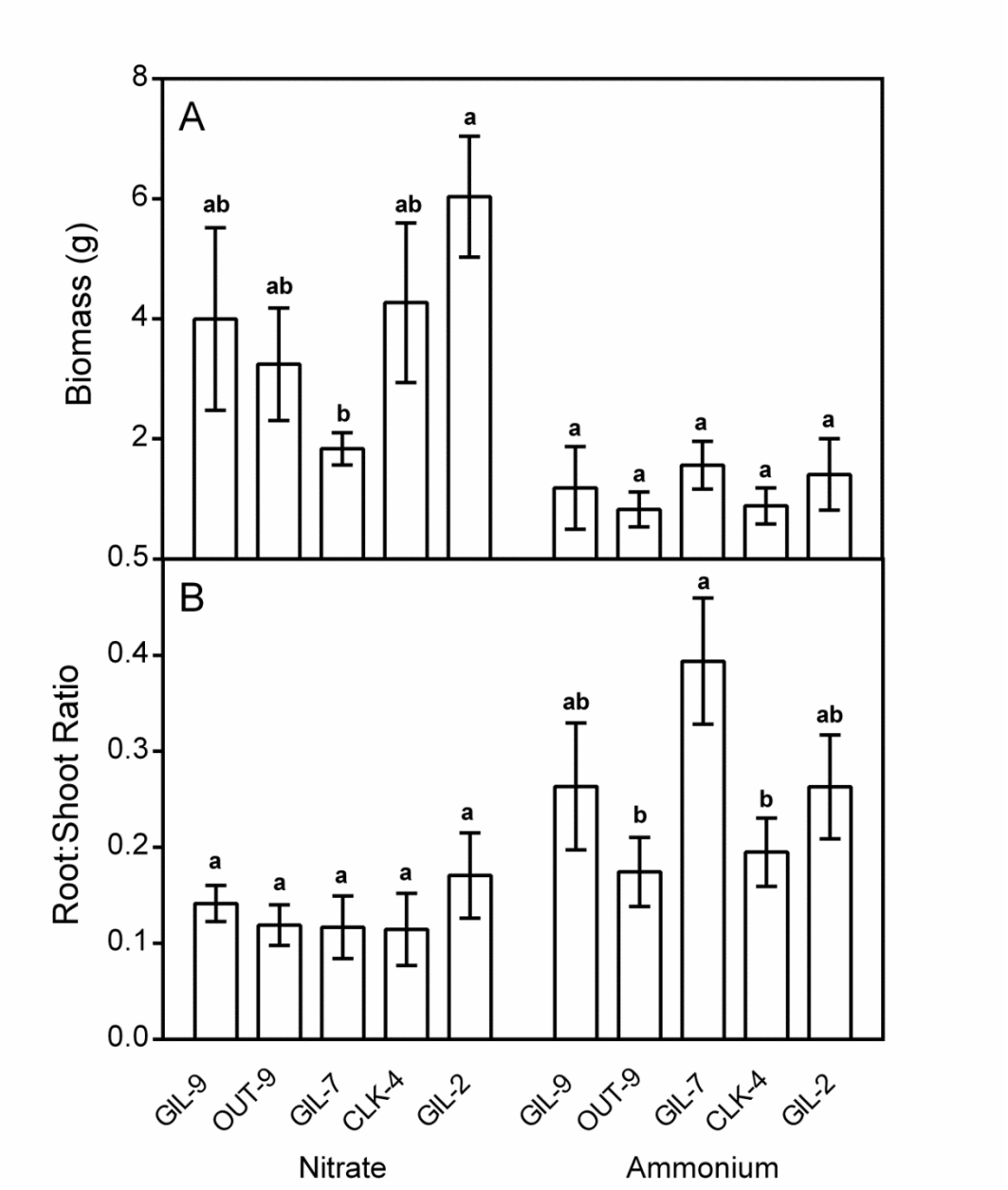

Figure 5.2. A) Total biomass and B) root: shoot ratio for five *Populus balsamifera* L. genotypes grown with either 500 µM NO_3^- of NH_4^+ for 45 days. Means ±SE (N = 5-6).

Table 5.1. Root and leaf nitrogen concentration (mmol N g dw^{-1}) for five *Populus balsamifera* L. genotypes grown with either 500 µM NO_3^- or NH_4^+ for 45 days. Means ±SE (N = 2-3).

			NO_3^-		
	GIL-9	**OUT-9**	**GIL-7**	**CLK-4**	**GIL-2**
Root (mmol N g dw^{-1})	2.68±0.05	2.79±0.08	2.27±0.13	2.34±0.02	2.44±0.08
Leaf (mmol N g dw^{-1})	2.31±0.25	2.14±0.14	1.79±0.23	1.62±0.29	2.43±0.15
			NH_4^+		
	GIL-9	**OUT-9**	**GIL-7**	**CLK-4**	**GIL-2**
Root (mmol N g dw^{-1})	2.34±0.19	2.85±0.08	2.44±0.14	2.04±0.37	2.40±0.26
Leaf (mmol N g dw^{-1})	1.68±0.41	2.36±0.48	1.82±0.36	1.31±0.15	1.67±0.36

NH_4^+, root:shoot ratios ranged from 0.18 for OUT-9 to 0.4 for GIL-7. GIL-7 and GIL-9 had significantly higher root:shoot ratios when grown with NH_4^+ ($P<0.05$).

Leaf and root nitrogen concentrations were not significantly different among genotypes or between nitrogen sources (Table 5.1). Root nitrogen concentration was approximately 0.5 mmol g dw^{-1} greater than, but significantly correlated with, leaf nitrogen concentration ($r=0.634$; $P=0.002$). Mean root and leaf nitrogen concentrations were 2.46 and 1.94 mmol N g dw^{-1}, respectively. However, there was a significant interaction between genotype and source where root nitrogen concentration for OUT-9 was significantly greater than CLK-4 when grown with NH_4^+ but not with NO_3^-.

5.4.2. Compartmental analysis

Compartmental analysis indicated that surface flux was roughly two orders of magnitude greater than flux from the apoplast, which in turn was about one order of magnitude higher than the cytoplasmic efflux (Table 5.2). The surface flux represents exchange between the boundary layer and substrate, and was not significantly different between treatments or genotypes (Table 5.2). In contrast, the flux from the apoplast averaged 0.96 and 3.37 mmol g fw^{-1} h^{-1} for NO_3^- and NH_4^+, respectively ($P=0.007$). Although the outward flux of NO_3^- from the apoplast was not significantly different between genotypes, the flux of NH_4^+ was higher in GIL-7 than GIL-9, OUT-9 and CLK-4. Similarly, cytoplasmic efflux was approximately twice as high ($P=0.0078$) for NH_4^+ (0.27 mmol g fw^{-1} h^{-1}) compared to NO_3^- (0.13 mmol g fw^{-1} h^{-1}). There were no genotypic differences in cytoplasmic efflux ($P=0.498$)

The half-lives of exchange ($t_{1/2}$) indicate the time taken for 50% of the inorganic nitrogen to be lost from a NO_3^- or NH_4^+ pool or compartment (Table 5.3). Although loss rates from the root surface were not different, the $t_{1/2}$ was significantly longer for NH_4^+ (13.8 s) than for NO_3^- (7.9 s). In contrast, the $t_{1/2}$ of the apoplastic space did not differ significantly among treatments. The overall apoplastic $t_{1/2}$ was 69.3 s, approximately six times that of the surface compartment. Half-lives of exchange for the cytoplasmic space

Table 5.2. Outward fluxes (µmol N g fw^{-1} h^{-1}) of nitrate (NO_3^-) and ammonium (NH_4^+) as estimated from compartmental analysis for five *Populus balsamifera* L. genotypes grown with either 500 µM NO_3^- or NH_4^+ for 45 days. Means ±SE (N = 2-3).

			NO_3^-		
Compartment	**GIL-9**	**OUT-9**	**GIL-7**	**CLK-4**	**GIL-2**
Surface	156.6±64.03	38.2±1.26	104.6±43.44	27.9±17.82	12.88±0.78
Apoplast	1.95±0.671	0.41±0.048	1.46±0.948	0.34±0.109	0.62±0.189
Cytoplasm	0.23±0.099	0.07±0.022	0.22±0.148	0.033±0.004	0.056±0.014
			NH_4^+		
	GIL-9	**OUT-9**	**GIL-7**	**CLK-4**	**GIL-2**
Surface	33.2±0.73	53.8±20.67	28.5±6.09	36.3±11.28	33.8±2.86
Apoplast	0.97±0.729	2.54±0.848	7.59±1.039	2.27±0.560	3.50±1.591
Cytoplasm	0.19±0.116	0.33±0.013	0.33±0.083	0.25±0.009	0.28±0.021

Table 5.3. Half-lives of exchange ($t_{1/2}$) for NO_3^- and NH_4^+ of root surface film, apoplastic space and cytoplasm estimated from compartmental analysis for five *Populus balsamifera* L. genotypes grown with either 500 μM NO_3^- or NH_4^+ for 45 days. Means ±SE (N = 2-3).

			NO_3^-		
Compartment	GIL-9	OUT-9	GIL-7	CLK-4	GIL-2
Surface (s)	7.1±3.82	6.1±0.11	4.7±0.69	9.9±5.06	12.2±1.05
Apoplast (s)	56.1±2.89	88.3±3.13	60.4±4.98	74.1±13.76	53.8±3.33
Cytoplasm (min)	56.6±16.33	47.2±29.12	19.48±4.40	12.5±5.39	2.1±0.22
			NH_4^+		
	GIL-9	OUT-9	GIL-7	CLK-4	GIL-2
Surface (s)	14.8±5.26	7.6±2.29	16.6±2.49	12.9±0.03	15.0±2.25
Apoplast (s)	126.7±12.98	61.4±22.84	37.34±7.02	71.6±13.88	82.1±25.84
Cytoplasm (min)	26.1±2.09	13.1±0.08	6.8±0.44	8.5±1.28	9.75±3.42

Table 5.4. Apoplast and cytoplasm nitrate and ammonium concentrations as estimated by compartmental analysis for five *Populus balsamifera* L. genotypes grown with either 500 µM NO_3^- or NH_4^+ for 45 days. Means ±SE (N = 2-3).

			NO_3^-		
Compartment	GIL-9	OUT-9	GIL-7	CLK-4	GIL-2
Apoplast (mM)	0.46±0.16	0.14±0.03	0.32±0.19	0.10±0.01	0.13±0.01
Cytoplasm (mM)	13.48±2.01	4.26±0.68	4.03±0.91	4.18±1.32	2.10±0.33
			NH_4^+		
	GIL-9	OUT-9	GIL-7	CLK-4	GIL-2
Apoplast (mM)	0.78±0.43	0.61±0.08	1.00±0.45	0.63±0.04	0.97±0.12
Cytoplasm (mM)	3.90±0.15	2.54±0.16	1.61±0.14	1.78±0.27	1.64±0.34

were approximately 32 and 12 min for NO_3^- and NH_4^+, respectively (P=0.0488). There were no genotypic differences in cytoplasmic half-lives of exchange (P=0.1460).

Estimated apoplastic NH_4^+ concentrations were approximately three times higher than NO_3^- concentrations (P<0.0012), but did not vary between genotypes (Table 5.4). Mean apoplastic concentrations, were 0.28 mM and 0.83 mM for NO_3^- and NH_4^+, respectively. In contrast, cytoplasmic NO_3^- concentrations were significantly greater than NH_4^+ concentrations and GIL-9 had significantly higher concentrations for both ions than the other four genotypes.

5.4.3. Independent estimates of net uptake and *E*/*I* are correlated

Estimates of net uptake were almost two times greater using the isotope mass balance model compared to compartmental analysis (Table 5.5). However, there was a positive relationship between the two methods (r=0.623; P=0.051) (Figure 5.3). Since net uptake in the isotope mass balance approach is a function of total plant nitrogen and root biomass, it is, in part, proportional to the inverse of the root:shoot ratio (r=0.87; P<0.0001). The same was not true for compartmental analysis (r=0.24; P=0.2252).

Irrespective of method, *E/I* was significantly higher for NH_4^+ than NO_3^-. However, whereas compartmental analysis indicated mean flux ratios of 0.4 and 0.22 for NH_4^+ and NO_3^-, respectively, isotope mass balance modelling gave flux ratios of 0.65 and 0.45. Despite this absolute difference in *E/I*, there was a significant positive relationship between the two measures of *E/I* across treatments and genotypes (r=0.77; P=0.0065) (Figure 5.4). Generally, genotypes that had the highest *E/I* using the isotope mass balance approach also had the highest *E/I* using the compartmental analysis method (Table 5.6), but there was significant genotypic variation in *E/I* only with NH_4^+.

5.5. Discussion

As shown here using rooted balsam poplar cuttings, the natural abundance isotope mass balance method proposed in Chapter 2 yields estimates of *E*/*I* and net nitrogen uptake that are comparable, but not identical, to compartmental analysis.

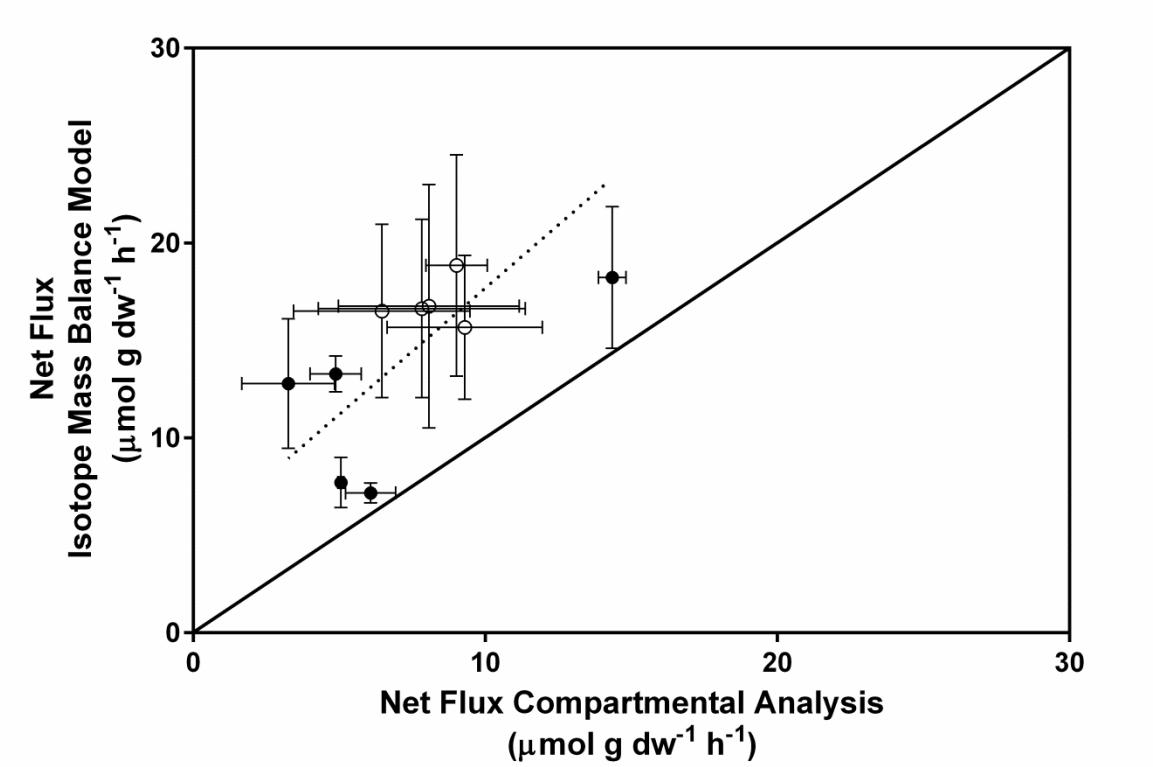

Figure 5.3. Coherence of estimates of net uptake (μmol N g dw^{-1} h^{-1}) by compartmental analysis and nitrogen isotope discrimination for five *Populus balsamifera* L. genotypes grown with either 500 μM NO_3^- (open circles) of NH_4^+ (closed circles) for 45 days. Means ±SE (N = 2-3). The dotted line indicates significant geometric mean regression (P=0.0131). The solid line shows the position of a 1:1 relationship.

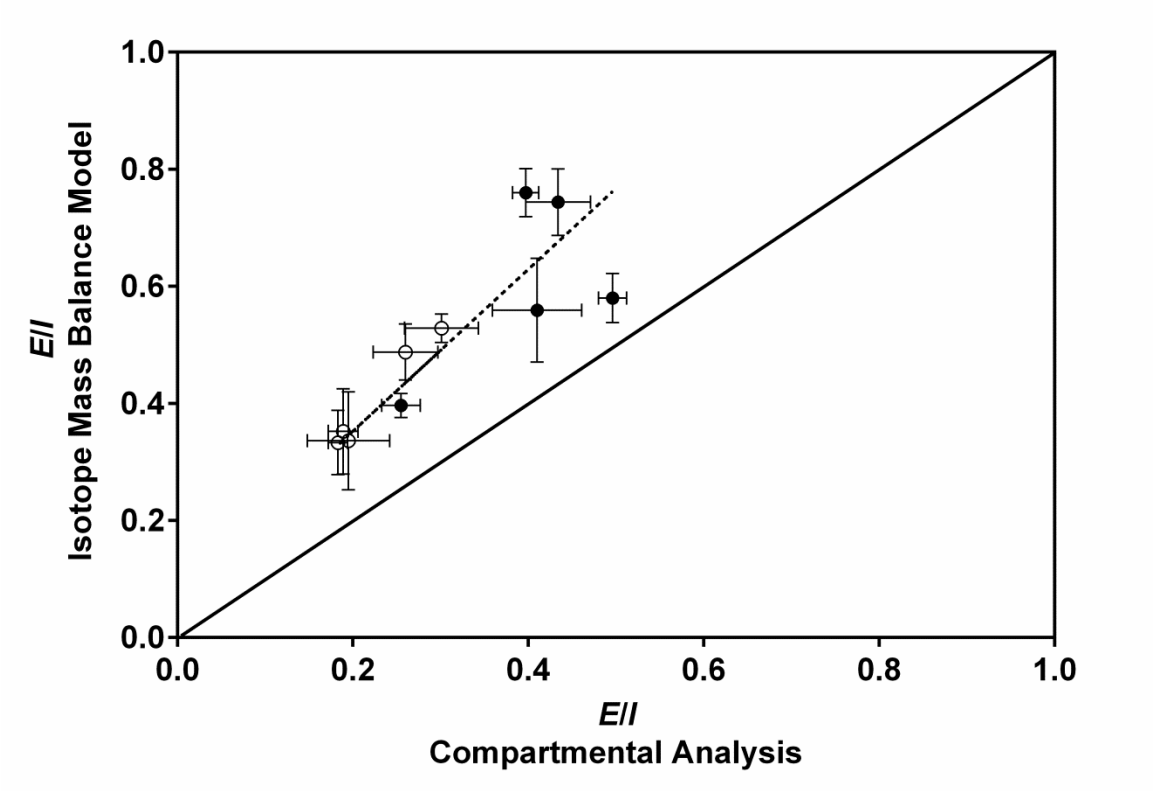

Figure 5.4. Coherence of estimates of efflux/influx by compartmental analysis and nitrogen isotope discrimination for five *Populus balsamifera* L. genotypes grown with either 500 µM NO_3^- (open circles) of NH_4^+ (closed circles) for 45 days. Means ±SE (N = 2-3). The dotted line indicates significant geometric mean regression (P=0.0065). The solid line shows the position of a 1:1 relationship.

Table 5.5. Net nitrate and ammonium uptake (μmol N g dw^{-1} h^{-1}) estimated from the isotope mass balance approach (IMB) or compartmental analysis (CA) for five *Populus balsamifera* L. genotypes grown with either 500 μM NO_3^- or NH_4^+ for 45 days. Means ±SE (N = 2-3).

			NO_3^-		
Method	**GIL-9**	**OUT-9**	**GIL-7**	**CLK-4**	**GIL-2**
IMB	15.67±3.69	18.85±5.67	16.51±4.44	16.76±8.24	16.64±4.57
CA	9.20±2.65	9.01±1.04	6.45±3.02	6.07±5.10	7.83±3.54
			NH_4^+		
	GIL-9	**OUT-9**	**GIL-7**	**CLK-4**	**GIL-2**
IMB	12.79±3.33	18.23±3.64	7.17±0.51	13.38±0.92	7.71±1.29
CA	3.25±1.58	14.34±0.47	6.07±0.86	4.88±0.88	5.05±0.11

Table 5.6. Efflux/influx estimated by isotope mass balance modelling (IMB) or by compartmental analysis (CA) for five *Populus balsamifera* L. genotypes grown with either 500 µM NO_3^- or NH_4^+ for 45 days. Means ±SE (N = 2-3).

			NO_3^-		
Method	**GIL-9**	**OUT-9**	**GIL-7**	**CLK-4**	**GIL-2**
IMB	0.35±0.073	0.33±0.055	0.49±0.048	0.46±0.024	0.34±0.083
CA	0.19±0.016	0.18±0.011	0.26±0.037	0.30±0.041	0.19±0.047
			NH_4^+		
	GIL-9	**OUT-9**	**GIL-7**	**CLK-4**	**GIL-2**
IMB	0.48±0.042	0.57±0.020	0.76±0.04	0.74±0.056	0.46±0.088
CA	0.50±0.016	0.25±0.021	0.40±0.015	0.43±0.037	0.41±0.051

Compartmental analysis by isotope labelling has for some time been the only method used for calculating *E*/*I* in addition to net uptake, but provides a more complete picture of ion flux in and out of roots.

5.5.1. Compartmental analysis using ^{15}N

Based on methods modified from Clarkson and Lee (1986) for ^{13}N, balsam poplar roots were loaded with ^{15}N-labelled nitrate and ammonium to measure net flux characteristics under steady-state conditions. Although the ^{13}N radioisotope has more frequently been used in such studies (e.g., Siddiqi *et al.* 1991; Kronzucker *et al.* 1995*a*, 1995*b*; Min *et al.* 2002), similar precision was achieved using ^{15}N. Stable isotope labelling with ^{15}N has also been successfully applied to compartmental analysis in *Triticum aestivum* L. (Devienne *et al.* 1994) and *Allium cepa* L. (Macklon *et al.* 1990). To the best of our knowledge, the present study is the first to report ^{15}N compartmental analysis of a woody plant species.

Half-lives of exchange for nitrate as compared to ammonium were longer for the cytoplasm and shorter at the root surface and the apoplast (Table 5.3). Rates of efflux from the apoplast and cytoplasm were greater for ammonium. Although differences in substrate concentrations limit direct comparison to our results, the fluxes and half-lives of exchange in balsam poplar were within the same range as reported in other tree species, including white spruce (*Picea glauca* (Moench) Voss), lodgepole pine (*Pinus contorta* Douglas), Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) and trembling aspen (*Populus tremuloides* Michx.) (Kronzucker *et al.* 1995a, b; and Min *et al.* 2002). Also, whether comparing genotypes (our study) or species (Min *et al.* 2002), variation in apoplastic and cytoplasmic fluxes were lower with ammonium than with nitrate.

Nitrate concentrations were more than two times higher than ammonium concentrations in the cytoplasm, but were lower in the apoplast (Table 5.4). This may indicate that balsam poplar has a general preference for nitrate. The better growth observed on nitrate is also consistent with a nitrate preference. For white spruce, which has an ammonium preference (Kronzucker 1995*a*, *b*), nitrate concentrations in the

cytoplasm were lower than ammonium, but the same was also true of trembling aspen (Min *et al.* 2002). In the apoplast, mean nitrate concentration was approximately half the concentration of the substrate (Table 5.4). In contrast, in spruce and aspen the apoplastic nitrate concentration was approximately equal to the substrate (Kronzucker *et al.* 1995*a*, *b*; Min *et al.* 2002). For balsam poplar, the ammonium concentration in the apoplast was greater than the substrate, although not to the degree reported for spruce and aspen. Kronzucker *et al.* (1995*a*, *b*) attributed differences between apoplastic nitrate and ammonium concentrations to the Donnan equilibrium, where the negative charge of the cell wall increases the binding capacity for cations (NH_4^+) but not anions (NO_3^-).

The compartmental concentrations reported here are lower than those observed by Kronzucker *et al.* (1995a; 1995b) and Min *et al.* (2002). These differences may in part be due to uncertainties in estimating compartmental volumes. Based on Laties (1959), Clarkson and Lee (1986) suggested that 10% of the total root volume is apoplastic free space and 5% is cytoplasm, estimates commonly used in compartmental analysis. However, volume fractions may vary with species or genotype and are known to be affected by root development and environment (McGarry, 1995).

Few, if any, studies have used compartmental analysis to examine intraspecific variation in nitrogen efflux, half-life of exchange and compartmental nitrogen concentration. Intraspecific variability exists, and in some cases, is independent of nitrogen source for traits measured using compartmental analysis. Intraspecific variability in exchange half-life, particularly in the cytoplasm, was high. GIL-9, in particular, had longer exchange half-lives on both sources compared to other genotypes. Since nitrogen concentration is a function of compartmental half-life of exchange and efflux, cytoplasmic nitrogen concentration of GIL-9 was considerably higher than the other genotypes on both sources. The reasons underlying these variations are not entirely clear. However, they could be related to physiological or morphological variations at the root level.

5.5.2. Compartmental analysis and isotope mass balance estimates of *E/I*

Efflux expressed relative to influx not only describes bidirectional movement of inorganic nitrogen between the root and rhizosphere but also indicates the efficiency of uptake. The measure has utility because it reflects species preferences (Kronzucker *et al.* 1997), possible toxicity (Kronzucker *et al.* 2001; Britto and Kronzucker, 2006), and how well external supplies meet internal demands (Pritchard & Guy 2005). Although there was a significant relationship between *E/I* calculated from compartmental analysis and the isotope mass balance approach, *E/I* was lower by approximately 33-50% using compartmental analysis (Figure 5.4). There are several possible explanations for the difference. First, the two methods operate over different scales in time and, to some degree, space. Second, the $\delta^{15}N$ of the root organic fraction may deviate from $\delta^{15}N$ of the whole tissue. Third, there are uncertainties in discrimination factors for the assimilatory enzymes. Fourth, there may be a small amount of discrimination associated with membrane transport. None of these are mutually exclusive.

In the first instance, compartmental analysis monitors nitrogen flux over a period of several minutes, whereas the isotope mass balance approach integrates over growing time (i.e., several weeks). With the latter, developmental and diurnal variation in *E*/*I* would be assimilation-averaged according to when the nitrogen was acquired. Increased efflux during night was observed in pearl millet (*Pennisetum glaucum* (L.) R. Br.) with nitrate nutrition (Pearson *et al.* 1981) and in Italian ryegrass (*Lolium perenne* L.) with ammonium (MacDuff and Jackson 1992). Using ^{15}N labelling, net uptake rates decrease during the night for both ammonium and nitrate but are species dependent (Delhon *et al.* 1995; Gessler *et al.* 2002). Kumar *et al.* (2003) showed changes in the transcript abundance of ammonium transporters and a subsequent decrease in ammonium uptake at night in rice (*Oryza sativa* L.) plants. Decreased uptake rates combined with increased efflux rates at night may increase *E*/*I* at night (Scheurwater *et al.* 1999). Since compartmental analysis did not account for *E*/*I* during the night period, this could, in part, explain lower estimates of *E*/*I* compared to the isotope mass balance approach.

Spatially, the two techniques differ because the isotope mass balance method accounts for the translocation of inorganic nitrogen from the root to the shoot, whereas this flux was not measurable for compartmental analysis. However, the inclusion of xylem flux in compartmental analysis would only lead to an increase in net uptake and a further decrease in *E*/*I*.

As per assumption #3 in chapter 2, it was assumed that the $\Delta^{15}N$ of the organic nitrogen in the root was equal to the bulk tissue $\Delta^{15}N$ in the root. However, there will likely be a small portion of inorganic nitrogen in the root that leads to an underestimation of root $\Delta^{15}N$. If significant, this omission would cause *E*/*I* to be underestimated, not overestimated, and therefore cannot account for the discrepancy between the two methods.

Underestimation of enzymatic discrimination factors for the assimilatory enzymes could result in an increase in estimated *E/I* using the nitrogen isotope mass balance approach. Data in Figures 5.3 & 5.4 and Tables 5.5 & 5.6 assume discrimination factors of 15.4‰ for nitrate reductase (Ledgard *et al.* 1985) and 16.8‰ for glutamine synthetase (Yoneyama *et al.* 1993). However, more recent work has suggested that the discrimination factor of nitrate reductase is closer to 22-26‰ (Needoba *et al.* 2002; Tcherkez and Farquhar, 2006; Karsh *et al.* 2012). If *E/I* is recalculated using 22‰ for both enzymes, the agreement between methods is considerably improved (Figure 5.5A). Although discrimination by glutamine synthetase has not been similarly reassessed, the improved coherence of *E/I* on both nitrate and ammonium indicates that the discrimination factors of both assimilatory enzymes may indeed be underestimated.

There is no evidence that nitrogen isotope discrimination occurs during membrane transport (Handley and Raven 1992; Evans 2001), but the possibility remains that there may be some slight fractionation associated with this step. I considered this possibility by adjusting the observed plant $\Delta^{15}N$ as follows:

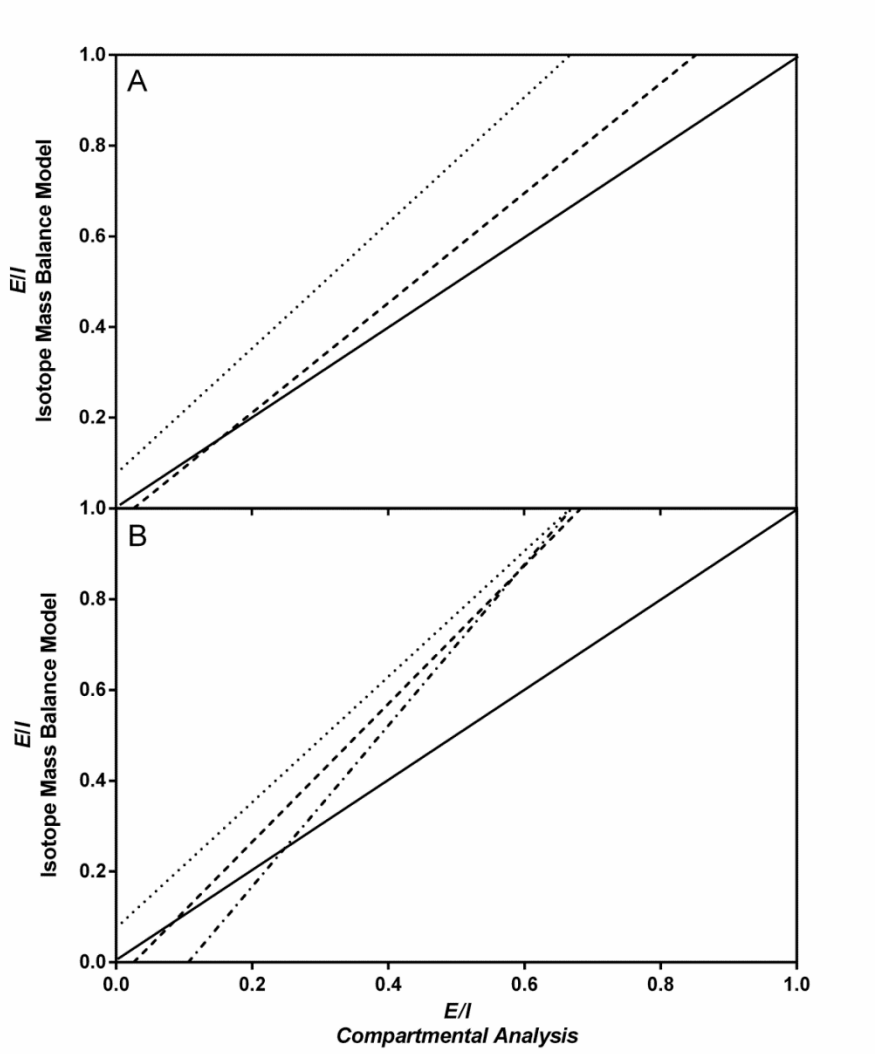

Figure 5.5. Effects of changing the discrimination factor of the assimilatory enzyme A) or adjusting for discrimination in membrane transport B) on the coherence between estimates of efflux/influx by isotope mass balance modelling and compartmental analysis. In panel A, the dotted line shows the relationship when enzyme discrimination factors of 15.4 and 16.8‰ (dotted line) are used for nitrate reductase and glutamine synthetase, respectively, while the dashed line shows the relationship when 22.6‰ is used for both. In panel B, the dotted line is as above, with adjustments for ^{15}N discrimination during membrane transport of 1 and 2‰ shown by dashed and dashed-dotted lines, respectively. The solid line in both panels shows the position of a 1:1 relationship.

Equation 5.10 $$\textbf{Adjusted Plant } \boldsymbol{\Delta^{15}}\mathbf{N} = \left(\frac{\boldsymbol{\Delta_{transport} - Plant\ \Delta^{15}N}}{\boldsymbol{\Delta_{transport} - (\Delta_{enz} \times P_{root})}}\right) \times \mathbf{P}_{\boldsymbol{root}} \times \boldsymbol{\Delta_{enz}}$$

where, Δ_{enz} is equal to the discrimination factor of the enzyme and $\Delta_{transport}$ is the discrimination factor for transport. This calculation assumes that discrimination in transport applies equally to influx and efflux, thus as plant Δ^{15}N approaches 0, the adjustment is maximized and as plant Δ^{15}N becomes more negative and closer to Δ_{enz}, the adjustment approaches 0‰. When *E/I* is recalculated assuming $\Delta_{transport}$ of either 1 or 2‰ (Figure 5.5B) there is improved convergence between the isotope mass balance approach and compartmental analysis at low *E*/*I*, but the effect is otherwise marginal.

5.5.3. Advantages of the isotope mass balance approach

Considering all the above, it is not surprising that there are absolute differences in flux estimates and *E*/*I* as determined by either compartmental analysis or by isotope mass balance. The concurrence observed, however, at the treatment level and at the genotype level, helps validate the isotope mass balance approach, which has some significant advantages over compartmental analysis.

Although cell or tissue specific at the root level, compartmental analysis requires the use of either radioactive ^{13}N or stable ^{15}N. Specialized equipment and training is required to use ^{13}N for compartmental analysis and access to ^{13}N ($t_{1/2}$ = 9.97 minutes) is limited to just a few labs worldwide. Using stable ^{15}N in the loading solution is equally effective. However, as with ^{13}N, at least 15 samples must be taken per assay to have enough data for segmental linear regression, and this is labour intensive and costly. On the other hand, establishing proper growth conditions for the isotope mass balance approach requires considerable care and attention and is also quite labour intensive. Advantages include reduced costs for isotope analysis (2-4 samples per assay; i.e., starting material, leaves, stems and roots) and opportunities for scaling up to the simultaneous assay of large numbers of plants under a variety of environmental conditions. Additionally, it provides time-integrated estimates of several other whole plant and organ level nitrogen use traits.

5.6. Conclusion

Coherence is significant between the established, time-point specific compartmental analysis approach and a new, time-integrated isotope mass balance approach for measuring net nitrogen uptake and *E/I*. Regardless of the method used to study the flux of inorganic nitrogen across the root plasma membrane, the information obtained is crucial for understanding whole-plant nitrogen dynamics. Increasing the confidence in measurements of nitrogen fluxes and increasing the understanding of the relationship of the fluxes to other nitrogen-use processes contributes to a further understanding of nitrogen-use efficiency. Through reduced isotopic sampling and more universal access, and because experiments can be scaled up to better evaluate genetic variation in flux and nitrogen-use traits, the isotope mass balance approach may facilitate efforts to improve the utilization of nitrogen.

6. Nitrogen isotope discrimination indicates genotypic variation in nitrogen source preference, uptake and assimilation in *Populus balsamifera* L.

6.1. Summary

As interest in using poplar as a biofuel feedstock increases, it is important that increases in yields do not require proportionate increases in nitrogen fertilization. Intraspecific variability in nitrogen use has not been assessed in a natural poplar species. The nitrogen isotope mass balance approach was used to assess intraspecific variability in uptake, assimilation and allocation traits in five climatically dispersed provenances of *Populus balsamifera* L. grown with either nitrate or ammonium. Balsam poplar was able to grow well with either ammonium or nitrate. Genotypic variation within provenances exceeded significant provenance level variation. Genotypes with rapid growth on nitrate achieved similar growth with ammonium, and root:shoot ratio was greater under ammonium. However, there were genotypes where root:shoot ratio and biomass were affected by nitrogen source. Tissue nitrogen concentration was greater for ammonium in the leaves and stems but not the roots. There was extensive genotypic variation in organ-level nitrogen isotope composition. Root nitrogen isotope composition was more depleted under nitrate than ammonium, but leaf nitrogen isotope discrimination was not different between plants on different sources. This is indicative of variation in partitioning of nitrogen assimilation, efflux/influx (E/I) and root or leaf assimilation rates. The proportion of nitrogen assimilated in roots was lower under nitrate than ammonium. In contrast, E/I was higher for nitrate than ammonium. With the exception of E/I, nitrogen use traits for nitrate were correlated with the same traits when grown with ammonium. Using the nitrogen isotope mass balance model, a high degree of genotypic variation in nitrogen use traits was identified at both the provenance and more importantly, the clonal level. To improve on nitrogen-use in poplar, time-integrated information based on nitrogen isotope composition can be incorporated into traditional breeding and phenotypic screening efforts to explore the relationship between calculated nitrogen use traits under controlled conditions and field level variation in performance.

6.2. Introduction

There is increasing interest in using *Populus* species or their hybrids as feedstock for the growing biofuel industry (Yamshanov *et al.* 2008; Sannigrahi *et al.* 2010). Rapid growth, ease of propagation and the ability to grow on marginal soils make poplar suitable for sustainable production of biofuel feedstock. However, as efforts to improve yields increase, the nitrogen demand of new high-yielding cultivars will increase. Nitrogen is considered the limiting nutrient in most natural ecosystems and the largest input in agricultural systems. Nitrogen-use efficiency must be maintained or even improved upon to establish poplar as a bioenergy feedstock than can be grown with minimal inputs and, at the same time, produce rapid biomass growth. The first step to improving nitrogen-use efficiency is to adequately determine the degree of intraspecific variation in nitrogen-use traits. Cost, time and complexity are all limitations to current assays available for measuring many of these traits. Although intraspecific variation in nitrogen use has been reported for a number of cereal species (Hirel *et al.* 2007) and Arabidopsis (Masclaux-Daubresse *et al.* 2010), the lack of an integrated approach to evaluate nitrogen-use traits limits interpretation of this genotypic variation (Hirel *et al.* 2007). Nitrogen isotope discrimination at natural abundance has the potential to be used as an integrated measure of nitrogen use in plants (Robinson 2001; Evans 2001).

For woody plants grown under steady-state conditions, species level variation in nitrogen isotope composition has only been described in white spruce (*Picea glauca* (Moench) Voss) (Pritchard and Guy 2005). In the field, intraspecific variation in nitrogen isotope composition has been reported in European beech (*Fagus sylvatica* L.) (Peuke et al 2006) and Norway spruce (*Picea abies* (L.) Karst) (Gebauer and Schulze 1991). However, interpretation of nitrogen isotope composition under field conditions is typically affected by chemical, spatial and temporal heterogeneity in soil nitrogen. Interpretation of intraspecific variation in nitrogen isotope composition at the whole plant and organ level requires careful control over the nitrogen substrate and measurement of organ level isotope composition. In herbaceous plants under steady-state conditions, intraspecific variation in nitrogen isotope composition has been

reported in barley (*Hordeum vulgare* L.) (Handley *et al.* 1997; Robinson *et al.* 2000; Kolb and Evans, 2003), wheat (*Triticale aestivum* L.) (Yousfi *et al.* 2009; Yousfi *et al.* 2012; Yousfi *et al.* 2013), and rice (*Oryza sativa* L.) (Yoneyama *et al.* 2001). This variation has been attributed to possible differences in nitrogen uptake or assimilation patterns that were a function of environment and genotype. The nitrogen isotope mass balance model introduced in Chapter 2 can be used to interpret results obtained from such experiments, providing integrated information on nitrogen use at the whole plant and organ level that is difficult to measure using traditional assays.

The nitrogen isotope mass balance model developed in this thesis uses measurements of organ level nitrogen isotope fractionation under steady-state nitrogen conditions in combination with *a priori* knowledge of source $\delta^{15}N$. In addition to organ-level biomass and unprocessed nitrogen isotope composition, traits related to both, nitrogen uptake and assimilation, were calculated. Assimilation partitioning is a function of the difference in isotope composition between the two primary sites of assimilation (roots and leaves) and the proportion of overall nitrogen in the leaves. Partitioning of assimilation, efflux/influx (*E*/*I*) and translocation of inorganic nitrogen to the shoot can be calculated to reflect the ease of movement and supply relative to demand under steady-state nitrogen conditions. *E*/*I* is obtained as a function of whole plant nitrogen isotope composition, partitioning of assimilation and the discrimination associated with the assimilatory enzyme. More detail of the nitrogen isotope mass balance approach can be found in Chapter 2.

In general, poplars have been widely described as model systems for woody plant biology (Bradshaw *et al.* 2000; Jansson and Douglas, 2007). Balsam poplar (*Populus balsamifera* L.) has a geographic range extending through much of the boreal forest region across North America, extending from the Atlantic coast of Canada and New England to Alaska and above the Arctic Circle (Petersen and Petersen, 1992). This large geographic range contains latitudinal gradients in adaptive traits (Soolanayakanahally *et al.* 2009). I used five climatically dispersed provenances of balsam poplar that extend from the prairie transition in the dry range of the species to the boreal forest-tundra

transition zone to determine whether nitrogen source preference varies along a climatic gradient, and whether intraspecific variation exists for nitrogen-use traits when grown with nitrate or ammonium.

6.3. Materials and methods

6.3.1. Plant material and experimental design

First year branches of 25 genotypes of balsam poplar ranging from 51 °N to 56 °N were obtained from the AAFC-AESB Agroforestry Development Centre at Indian Head, Saskatchewan, Canada and stored at 4 °C for approximately three months to fulfill chilling requirements. The five provenances reflected a climatic gradient that extends from a prairie ecosystem to the Canadian Shield boreal forest. The five provenances starting from prairie were: Outlook (OUT), Saskatchewan (51.1 °N, 106.2 °W), Saskatoon (SKN) (2.2 °N, 106.4 °W), Saskatchewan, Turtleford (TUR), Saskatchewan (53.2 °N, 108.3 °W), Cold Lake (CLK), Alberta (54.2 °N, 110.1 °W) and Gillam (GIL), Manitoba (56.4 °N, 94.7 °W). Two-node cuttings, approximately 6-8 cm long were weighed for fresh weight and arranged in a randomized complete block design with three blocks of two nitrogen treatments supplied as either 500 μM nitrate or 500 μM ammonium. Plants were grown for 45 days in a hydroponics solution until harvest. Complementary samples of each genotype were collected as reference samples (N = 3) and analyzed for starting nitrogen isotope composition and concentration.

6.3.2. Hydroponics system

The hydroponics system was comprised of six 1000L containers lined with 45 mil rubber pond liner (Firestone, USA) constructed in a greenhouse under ambient light conditions supplemented by sodium halide lighting (600 μmol m^{-2} s^{-1}) and 18/6 h day/night photoperiod. Temperatures in the greenhouse were maintained between 20 and 24 °C. For the first experiment, solution temperatures averaged approximately 20°C. For the second experiment to confirm genotypic differences, solution temperatures averaged approximately 23 to 24°C (Figure A.2). Each container had a floating raft that had a capacity for up to 32 plants. Unused plugs in the raft and the rest

of the container were covered with black plastic to prevent algal growth from light infiltration into the hydroponics solution. The hydroponics solution was a modified 1/10th strength Johnson's solution (Johnson *et al.* 1957) supplemented with either 250 μM $Ca(NO_3)_2$ or 250 μM $(NH_4)_2SO_4$. Final nutrient composition, excluding the nitrogen salts, was: 200 μM KH_2PO_4, 200 μM K_2S0_4, 100 μM $MgSO_4$, 100 μM $CaSO_4$, and micronutrients: 5 μM Cl, 2.5 μM B, 0.2 μM Mn, 0.2 μM Zn, 0.1 μM Mo, 0.05 μM Cu, and 50 μM Fe^{2+}. A centrifugal pump, with a pumping capacity of approximately 20 L per minute, provided circulation and aeration of the solution for each container. The solution was monitored periodically for oxygen levels, pH (Figure A.2) and temperature (Figure A.3). Powdered calcium carbonate ($CaCO_3$) was added to buffer pH in the range of 6-7.5. Media NH_4^+ and NO_3^- concentrations were assayed using the phenol:hypochlorite (Solorzano 1969) and perchloric acid (Cawse 1967) methods, respectively (Figure A.4). The solution was completely replaced every 14 days to ensure that there was no substantial decrease (no more than 10%) in concentration of nitrate or ammonium over time that could increase the solution $\delta^{15}N$.

6.3.3. Sampling and natural abundance isotope analysis

After 45 days of growth, plants were separated into leaves, stems, roots and the original cutting. Samples were flash frozen in liquid nitrogen and stored at -80 °C until samples could be freeze-dried at -50 °C for two days. Once dried, roots, leaves, stems, cuttings and the reference cuttings were weighed. Samples were ground to a fine powder using a mortar and pestle and then ball milled (Fritsch Laborgeratebau, Terochem Scientific). Sub-samples of 3±0.1 mg were weighed into tin capsules (Elemental Microanalysis Ltd., 8x5 mm, D1008) and analyzed for $\delta^{15}N$ and nitrogen concentration on a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) (University of California Stable Isotope Facility, Davis, CA). Isotopic composition is expressed as $\delta^{15}N$:

Equation 6.1 $$\boldsymbol{\delta^{15}N = \left(\frac{R_{sample}}{R_{standard}} - 1\right) * 1000}$$

where, R_{sample} is the $^{15}N/^{14}N$ isotope ratio of the sample and $R_{standard}$ is the isotope ratio of a known standard (air). Tissue nitrogen isotope composition was corrected for contaminating nitrogen from cuttings using the protocol described in Chapter 4. Isotope discrimination is usually denoted by the equation (Farquhar *et al*. 1989):

Equation 6.2 $$\boldsymbol{\Delta^{15}N_{sample} = \left[\frac{(\delta^{15}N_{source} - \delta^{15}N_{sample})}{\left(1 + \frac{\delta^{15}N_{sample}}{1000}\right)}\right]}$$

The ammonium and nitrate salts used for the growth media had a $\delta^{15}N$ of -0.96 and +58.5‰, respectively. Leaf, root, stem and cutting $\delta^{15}N$ were converted to discrimination values prior to model calculations.

6.3.4. Mass balance calculations

After correcting for pre-existing nitrogen remobilized to growing tissues from vegetative cuttings following the approach described in Chapter 4, the isotope mass balance model described in Chapter 2 was used to obtain several nitrogen-use traits from measurements of nitrogen isotope composition, nitrogen concentration and biomass of roots and leaves.

6.3.5. Statistical analysis

Biomass, root:shoot ratio, $\delta^{15}N$, nitrogen concentration, total nitrogen of root, stem and leaf tissues, and model calculations were compared using a nested ANOVA with nitrogen source, genotype and provenance as fixed effects with genotype nested within provenance. The statistical model is as follows:

Equation 6.3 $$\boldsymbol{Y_{ij} = \mu + \alpha_i + \tau_j + \tau(\gamma)_{jk} + \beta_{ij} + \varepsilon_{ijk}}$$

where, μ is the overall mean response, α_i is the effect due to the genotype, τ_j is the effect due to the nitrogen source, $\tau(\gamma)_{jk}$ is the effect due to genotype nested within provenance and β_{ij} is the effect due to any interaction between the genotype and

nitrogen source. Analysis of variance procedure was carried out using Graphpad Prism 6 (La Jolla, CA, USA) to obtain estimates of the means, followed by Tukey's multiple comparison tests to separate means. Where necessary, data were log transformed to meet assumptions of homogeneity of variance and normality. Differences between treatments described as significant are those where P <0.05. For correlations, significance is indicated using ˙, *, **, and *** to denote significance when P<0.10, P<0.05, P<0.01 and P<0.001.

6.3.6. Replication of genotypic differences in calculated nitrogen-use traits predicted from the nitrogen isotope mass balance model.

To determine whether genotypic ranking within each source for nitrogen-use traits was repeatable, a second experiment with only seven genotypes was compared to the results from the first experiment in raw measured traits and overall calculated traits. To compare the two experiments, a three-way ANOVA using SAS 9.3 (SAS Institute, Cary, NC) was used with genotype, nitrogen source and experiment as fixed effects. The statistical model is as follows:

Equation 6.4 $$\boldsymbol{Y_{ijk} = \mu + \alpha_i + \tau_j + \rho_k + \beta_{ij} + \gamma_{ik} + \lambda_{jk} + \varepsilon_{ijk}}$$

where, μ is the overall mean response, α_i is the effect due to the genotype, τ_j is the effect due to the nitrogen source, ρ_k is the effect due to experiment, β_{ij} is the effect due to any interaction between the genotype and nitrogen source, γ_{ik} is the effect due to any interaction between genotype and experiment, λ_{jk} is the effect due to any interaction between nitrogen source and experiment and ε_{ijk} is the effect due to any three-way interaction between genotype, nitrogen source and experiment. Geometric mean regression was used to determine the relationship between the two experiments using Microsoft Excel and plotted using Graphpad Prism 6 (La Jolla, CA, USA).

6.1. Results

6.1.1. Root:shoot ratio and leaf nitrogen concentration was affected by nitrogen source but biomass was not

Growth averaged approximately 15% more when grown with NO_3^- as compared to NH_4^+, but this difference was not significant (P=0.0772) (Figure 6.1). Genotypes with high biomass under NO_3^- also had high biomass under NH_4^+ (r=0.543; P=0.0059) (Table 6.1). Because of a significant increase in leaf and stem biomass but not an increase in root biomass under NO_3^-, root:shoot ratio was higher in all provenances when grown with NH_4^+ than with NO_3^- (Figure 6.1). The difference between root:shoot ratio for NH_4^+ and NO_3^- grown plants decreased for provenances from the boreal forest versus provenances from the prairies. Similar to biomass, although there were greater root:shoot ratios when plants were grown on NH_4^+, differences between genotypes were correlated across sources (r=0.581; P=0.0023).

Nitrogen concentration was significantly higher for plants grown with NH_4^+ than NO_3^- for leaves and stems but not roots (Table 6.2). Mean nitrogen concentration was 2.51, 0.42 and 2.03 µmol mg dw^{-1} under NO_3^- and 2.55, 0.76, and 2.18 µmol mg dw^{-1} under ammonium for roots, stems and leaves, respectively. Nitrogen concentrations in roots and stems were less variable than leaves (Table 6.1). The range in mean genotype nitrogen concentration was greater under NO_3^- for roots, under NH_4^+ for stems and equal for leaves (Table 6.1). There were no clinal trends in nitrogen concentration for the provenances. The fraction of total plant nitrogen found in the leaves ($N_{leaf\ pool}/N_{total}$) was not higher for NO_3^- compared to NH_4^+. Since net root uptake is partly proportional to the inverse of the root:shoot ratio, net root uptake was lower under NH_4^+ than NO_3^-. Although there were genotypic differences in net root uptake of nitrogen, there were no differences between provenances (Table 6.2).

6.1.2. Source did not affect whole plant $\Delta^{15}N$ but organ level $\Delta^{15}N$ was affected

All genotypes were depleted in ^{15}N relative to their respective hydroponic solutions at the individual organ and whole plant level, showing discrimination against

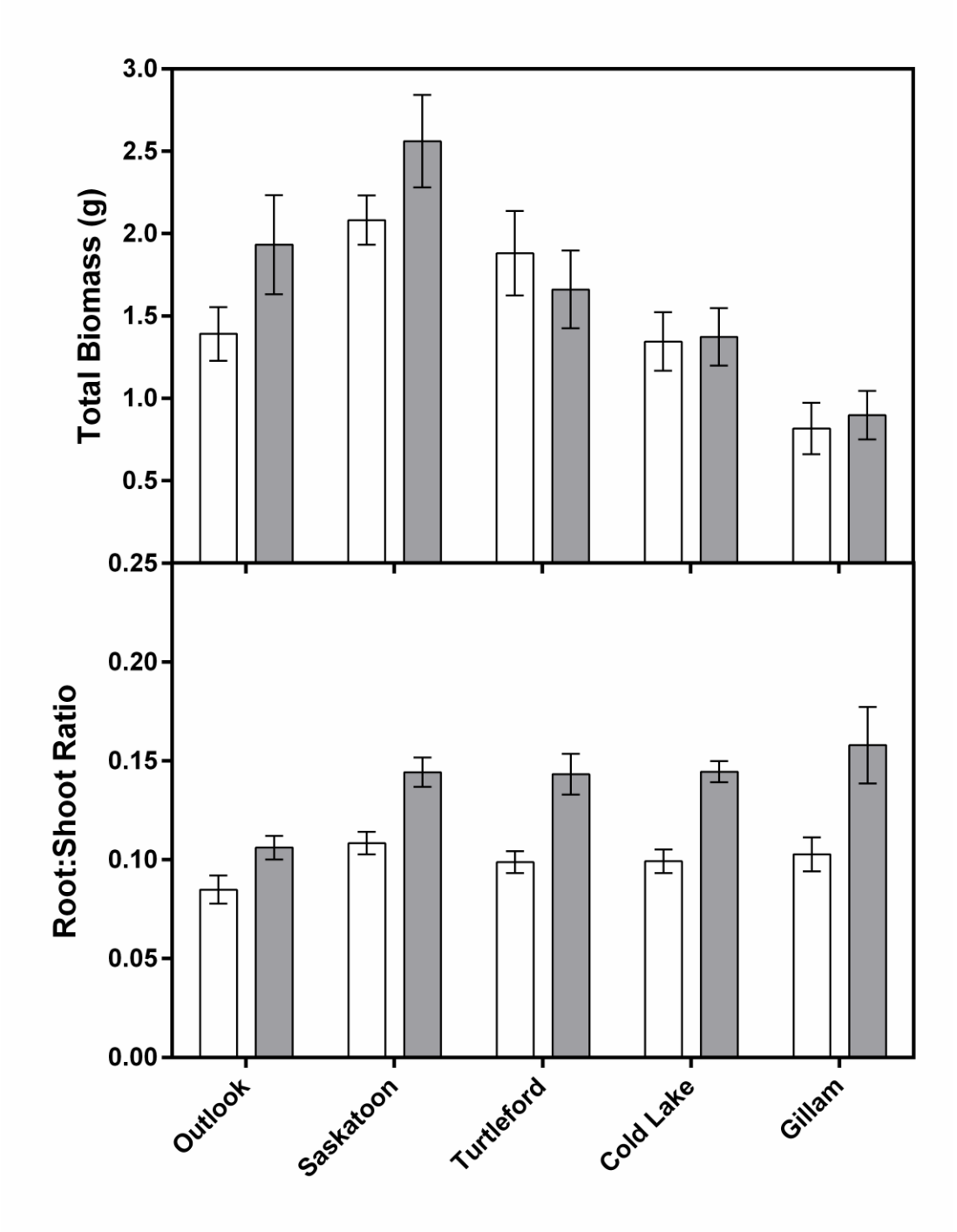

Figure 6.1. Total plant biomass ±SE (N=9-15) (top) and root:shoot ratio ±SE (bottom) for five provenances of *Populus balsamifera* L. grown with either 500 µM nitrate (white bars) or ammonium (grey bars).

Table 6.1. Mean (±SE) (N=9-15) provenance tissue nitrogen concentration (root [N], stem [N] and leaf [N]) (µmol mg dw^{-1}), the proportion of plant nitrogen allocated to the leaf ($N_{leaf\ pool}/N_{total}$), and net root uptake (µmol mg dw^{-1} h^{-1}) for five climatically dispersed provenances of *Populus balsamifera* L. grown with either 500 µM nitrate or ammonium.

	Provenance				
	Outlook	**Saskatoon**	**Turtleford**	**Cold Lake**	**Gillam**
Nitrate					
Root [N]	2.54±0.11	2.45±0.06	2.54±0.08	2.48±0.10	2.52±0.08
Stem [N]	0.42±0.02	0.43±0.01	0.39±0.02	0.40±0.02	0.46±0.03
Leaf [N]	2.13±0.08	2.03±0.06	2.06±0.06	1.96±0.08	2.06±0.11
$N_{leaf\ pool}/N_{total}$	0.81±0.01	0.77±0.01	0.79±0.01	0.78±0.01	0.82±0.01
Net Root Uptake	0.56±0.04	0.43±0.02	0.46±0.02	0.43±0.04	0.46±0.03
Ammonium					
Root [N]	2.67±0.07	2.51±0.05	2.49±0.06	2.57±0.12	2.53±0.05
Stem [N]	0.87±0.09	0.80±0.07	0.65±0.05	0.71±0.07	0.81±0.10
Leaf [N]	2.28±0.08	2.17±0.07	2.15±0.07	2.13±0.11	2.24±0.13
$N_{leaf\ pool}/N_{total}$	0.83±0.01	0.78±0.01	0.77±0.01	0.77±0.01	0.76±0.02
Net Root Uptake	0.49±0.02	0.37±0.02	0.37±0.02	0.38±0.02	0.37±0.03

Table 6.2 Mean and genotype minimum and maximum total biomass (g), root:shoot ratio, tissue nitrogen concentration (root [N], stem [N] and leaf [N]) (µmol mg dw^{-1}), the proportion of plant nitrogen allocated to the leaf pool ($N_{leaf\ pool}/N_{total}$), net root uptake (µmol mg dw^{-1} h^{-1}), tissue $\Delta^{15}N$, the proportion of leaf organic nitrogen assimilated in the leaf (*Ti*/*Tt*), the proportion of root assimilated nitrogen exported to the leaves as organic nitrogen (*To*/*Tr*), the proportion of plant nitrogen assimilation in the roots (P_{root}), efflux/influx (*E*/*I*), and tissue assimilation (µmol mg dw^{-1}).

		Nitrate			Ammonium				
	Measurement	Mean±SE	Genotype Minimum	Genotype Maximum	Mean±SE	Genotype Minimum	Genotype Maximum	Source Effect[1]	Source Relationship[2]
Growth	Total Biomass	1.86±0.14	0.51	4.46	1.64±0.10	0.33	3.48	0.077	0.543**
	Root:shoot ratio	0.10±0.00	0.06	0.15	0.14±0.00	0.09	0.20	<0.0001	0.581**
Nitrogen Partitioning	Root [N]	2.50±0.04	2.08	2.93	2.55±0.03	2.28	2.96	0.3082	0.205
	Stem [N]	0.42±0.01	0.33	0.52	0.76±0.03	0.47	1.25	<0.0001	0.329·
	Leaf [N]	2.04±0.03	1.58	2.42	2.18±0.04	1.63	2.65	0.0005	0.831***
	$N_{leaf\ pool}/N_{total}$	0.79±0.01	0.72	0.84	0.78±0.01	0.72	0.85	0.0613	0.157
	Net Root Uptake	0.46±0.01	0.29	0.64	0.39±0.01	0.29	0.55	<0.0001	0.616***
Nitrogen Isotopic Discrmination	Root $\Delta^{15}N$	10.1±0.25	6.88	13.44	8.75±0.19	5.64	11.80	<0.0001	0.342·
	Stem $\Delta^{15}N$	7.37±0.25	4.21	11.54	6.56±0.20	3.27	9.35	0.0101	0.162
	Leaf $\Delta^{15}N$	4.42±0.23	2.41	6.79	4.83±0.19	1.62	6.84	0.1562	0.076
	Plant $\Delta^{15}N$	5.59±0.21	3.32	7.94	5.70±0.18	2.52	8.11	0.66947	0.180
Model Calculations	*Ti*/*Tt*	0.26±0.01	0.14	0.45	0.23±0.01	0.14	0.41	0.163	0.501*
	To/*Tr*	0.64±0.01	0.58	0.73	0.63±0.01	0.41	0.69	0.1285	0.462*
	P_{root}	0.80±0.01	0.64	0.88	0.82±0.01	0.71	0.89	0.018	0.591*
	E/*I*	0.31±0.01	0.17	0.44	0.42±0.01	0.20	0.58	<0.0001	0.280
	Leaf Assimilation	0.63±0.03	0.44	1.01	0.59±0.02	0.37	0.95	0.8561	0.573*
	Root Assimilation	18.36±0.60	11.16	26.84	16.12±0.43	11.62	21.97	<0.0001	0.496*

[1] P-value reported from a nested three-way ANOVA. [2] Source relationship is the correlation for a genotypic mean for trait for NO_3^- versus NH_4^+. Pearson correlation coefficient (r-value) reported from linear regression analysis, significance denoted as *** P<0.001, ** P<0.01, * P<0.05, · P<0.10.

^{15}N on both nitrogen sources (Figure 6.2). Average $\Delta^{15}N$ was 10.1, 7.37 and 4.42‰ under NO_3^- and 8.75, 6.56 and 4.83‰ under NH_4^+ for roots, stems and leaves, respectively. Whole plant $\Delta^{15}N$ was weighted more towards leaf $\Delta^{15}N$ since leaf nitrogen accounted for approximately 80% of total plant nitrogen. There were no clinal trends in nitrogen isotope composition among provenances (Figure 6.3). Root and stem $\Delta^{15}N$ grown with NO_3^- was greater than plants grown with NH_4^+ and differences between provenances were consistent across sources (Figure 6.3). There were no significant relationships for leaf and stem $\Delta^{15}N$ when grown with NO_3^- compared to NH_4^+ but there was for root $\Delta^{15}N$ (Table 6.2) ($P<0.10$).

6.1.3. Proportioning of nitrogen assimilation to the leaves was higher for NO_3^- than NH_4^+, but genotypic ranking was maintained on either source

Ti/*Tt* was greater for plants grown with NO_3^- than NH_4^+ (Table 6.1). Between 14 and 45% of leaf nitrogen was assimilated in leaves under NO_3^- compared to 14 to 41% under NH_4^+ (Figure 6.4). There was no clinal trend between provenances. From the product of *Ti*/*Tt* and $N_{leaf\ pool}/N_{total}$, an estimate for the partitioning of nitrogen (P_{root}) assimilation was calculated. Since $N_{leaf\ pool}/N_{total}$ was not different between sources, P_{root} was largely a reflection of *Ti*/*Tt* where P_{root} was greater for plants grown with NO_3^- compared to NH_4^+ (Table 6.1). Relative to NO_3^-, P_{root} under NH_4^+ was fairly constant and was not different between provenances (Figure 6.5). P_{root} under nitrate ranged from 0.64 to 0.88, indicating that for some genotypes, up to one-third of assimilation occurred in the leaves. Genotypic differences in both *Ti*/*Tt* and P_{root} on the different sources were correlated with each other (Table 6.2) ($P<0.05$), indicating that ranks are maintained and clonal variation in these traits is independent of source.

Although root assimilation accounted for between 60 and 80% of total plant nitrogen assimilation, since root biomass was much smaller than leaf biomass, assimilation rates per unit dry mass were approximately 15 times higher in roots than in leaves. Root assimilation was similar among provenances and was not significantly different between nitrogen sources (Figure 6.7A) ($P<0.05$). However, leaf assimilation

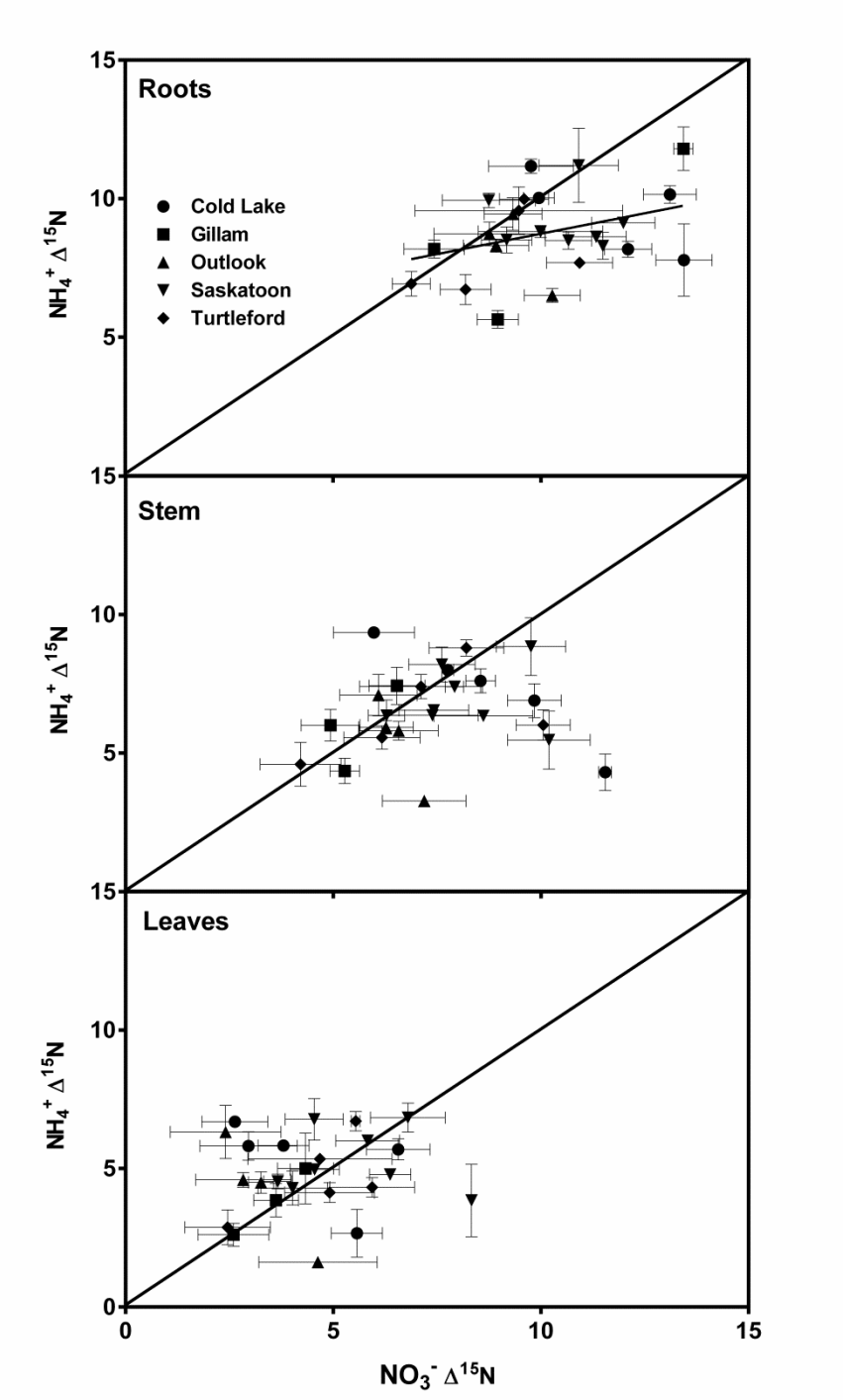

Figure 6.2. Root, stem and leaf $\Delta^{15}N$ (N = 3) ±SE for 25 genotypes of *Populus balsamifera* L. from five provenances grown with either 500 µM nitrate (x axis) or ammonium (y axis).

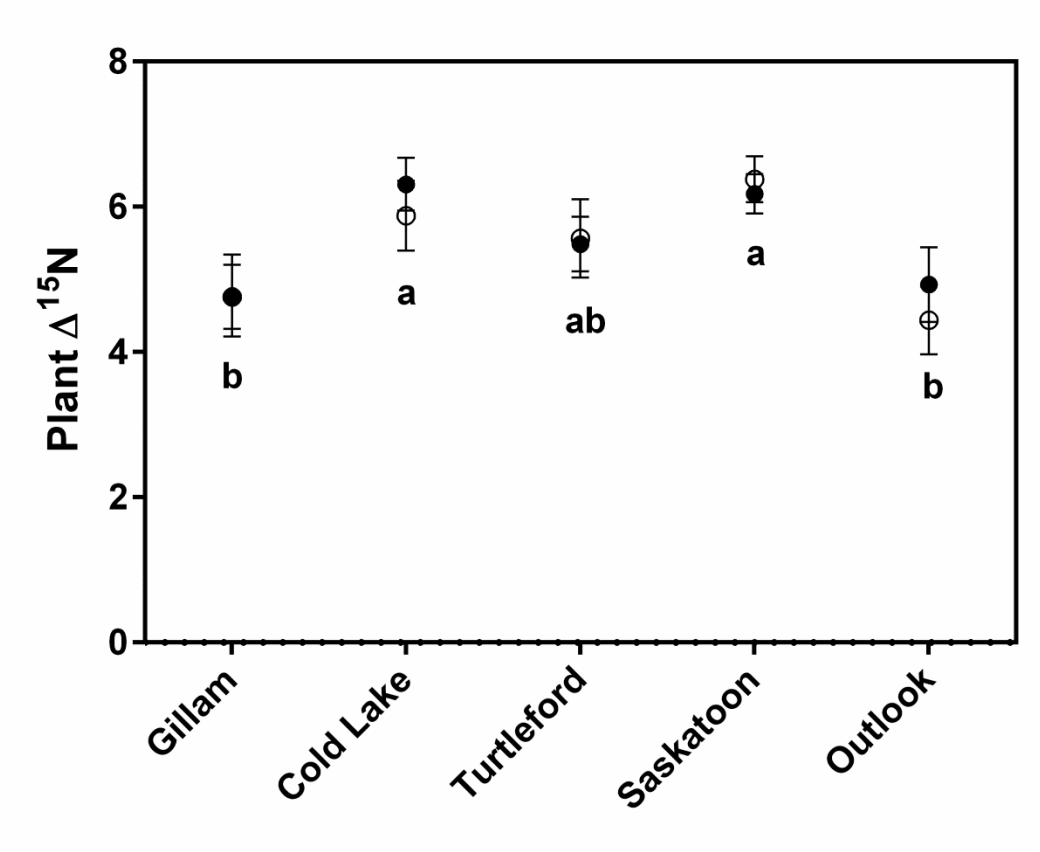

Figure 6.3. Plant $\Delta^{15}N$ ±SE (N=9-15) of five provenances of *Populus balsamifera* L. grown with either 500 µM nitrate (open circles) or ammonium (closed circles).

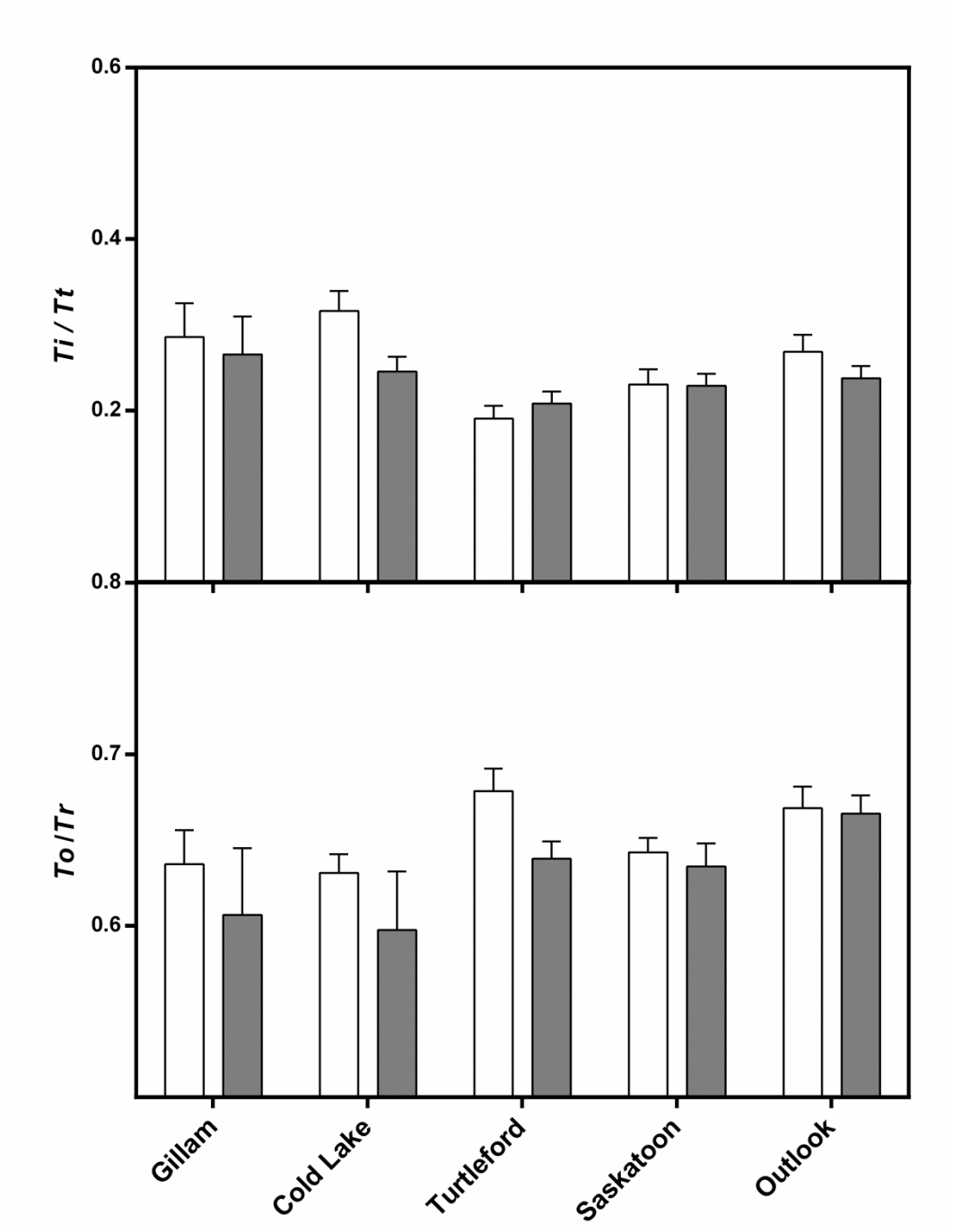

Figure 6.4. The proportion of leaf organic nitrogen assimilated in the leaf (*Ti*/*Tt*) (upper) and the proportion of root assimilated nitrogen exported to the leaves (*To*/*Tr*) (lower) ±SE (N=9-15) of five provenances of *Populus balsamifera* L. grown with either 500 µM nitrate (white bars) or ammonium (grey bars).

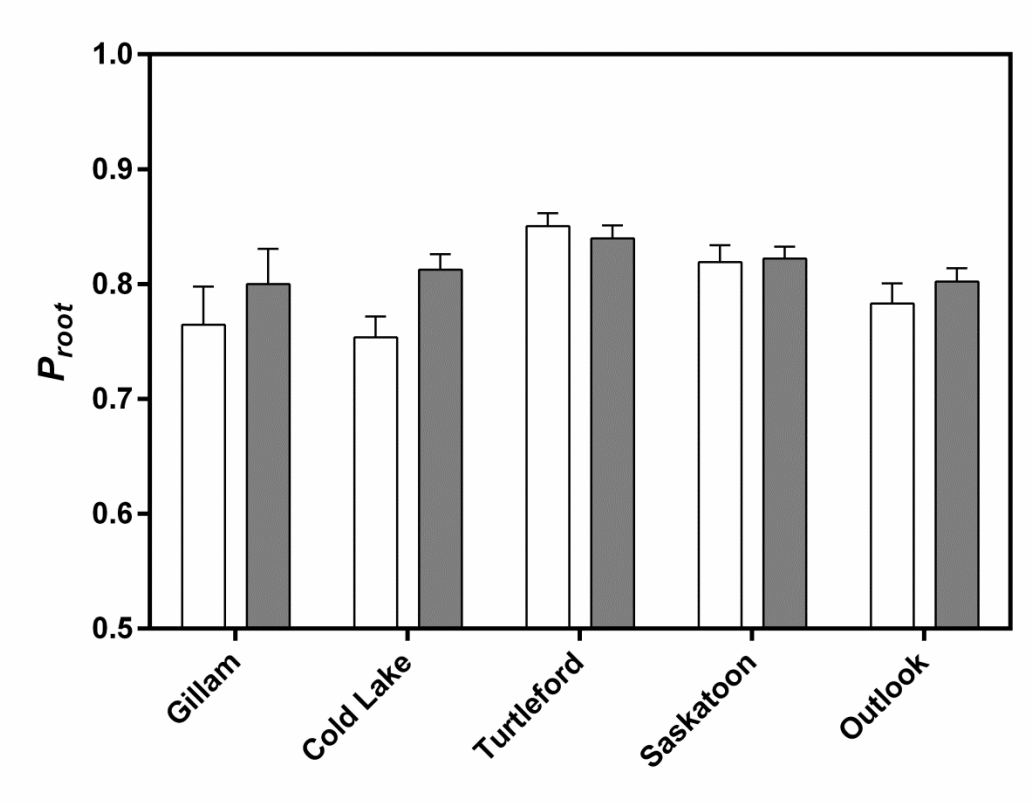

Figure 6.5. The proportion of nitrogen assimilated in the root (P_{root}) ±SE (N=9-15) of five provenances of *Populus balsamifera* L. grown with either 500 µM nitrate (white bars) or ammonium (grey bars).

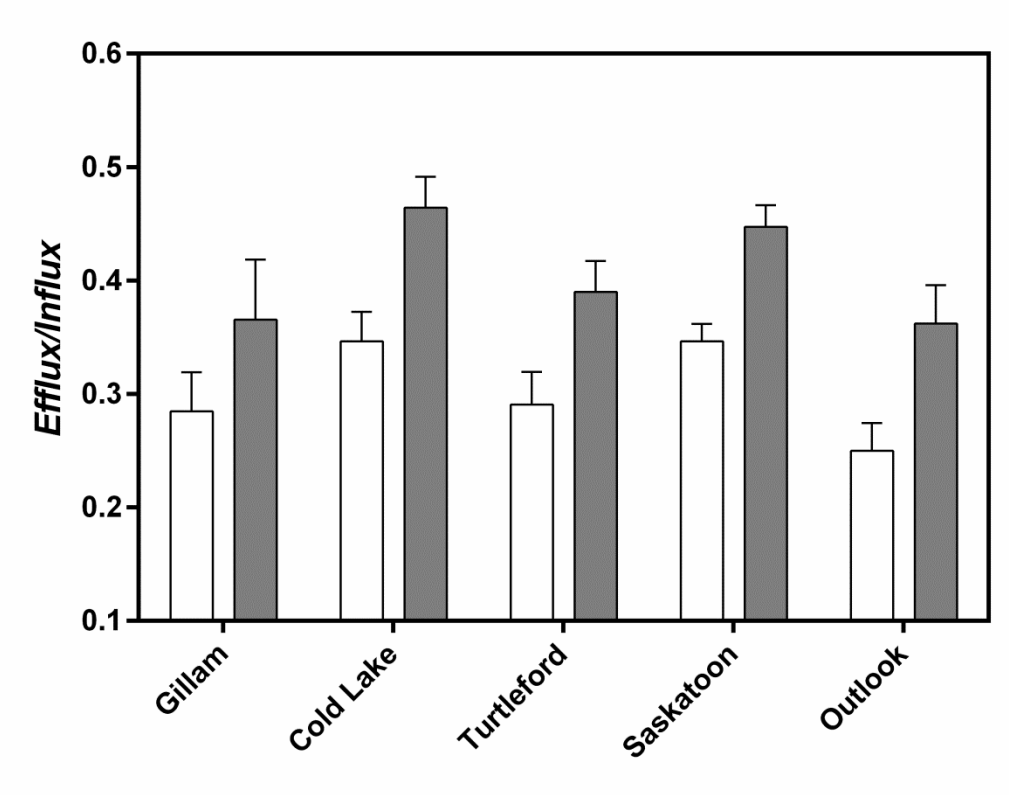

Figure 6.6. Efflux/influx (*E*/*I*) ±SE (N=9-15) of five provenances of *Populus balsamifera* L. grown with either 500 µM nitrate (white bars) or ammonium (grey bars).

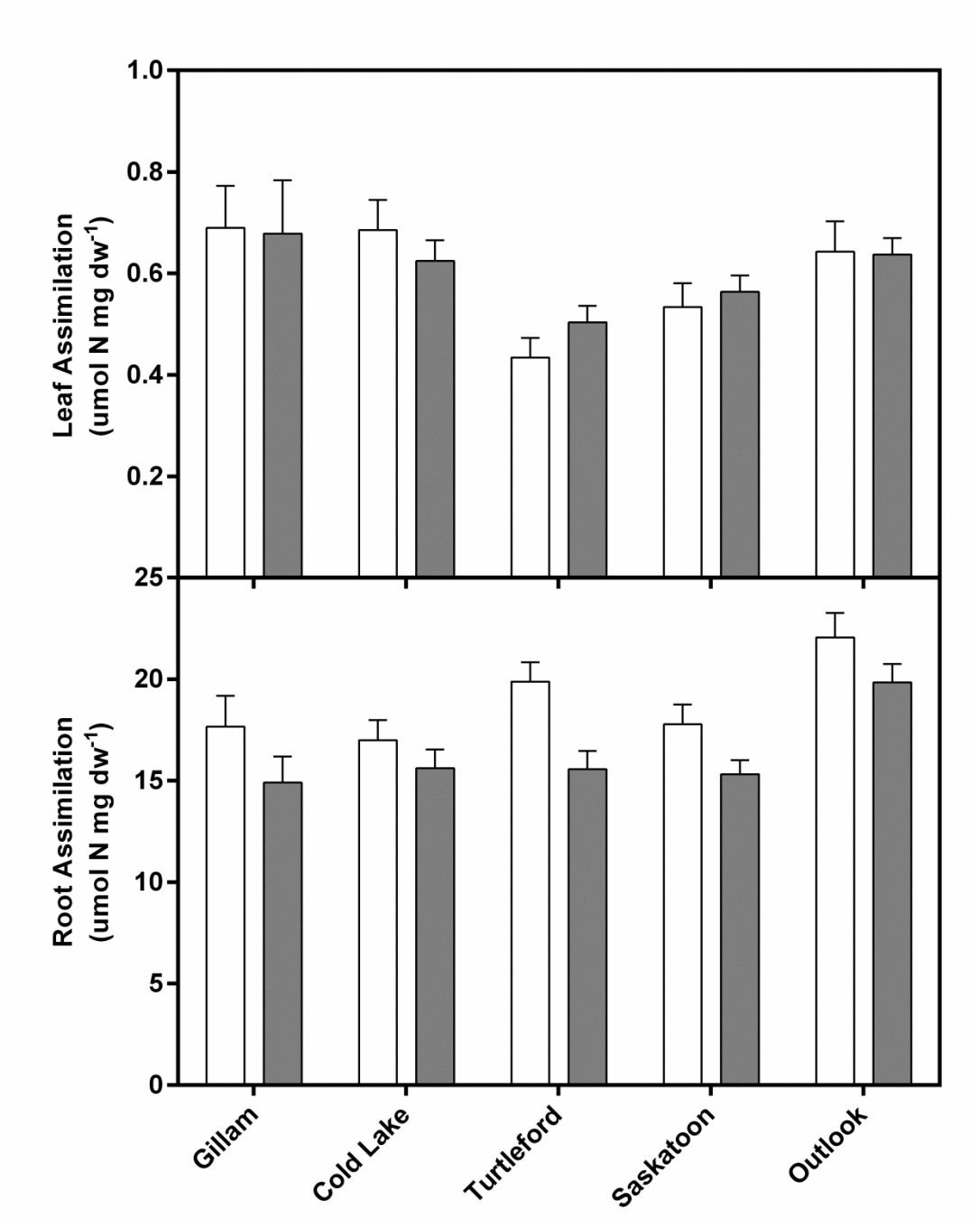

Figure 6.7 Root and leaf assimilation rates (µmol N mg dw^{-1}) (lower) ±SE (N=9-15) of five provenances of *Populus balsamifera* L. grown with either 500 µM nitrate (white bars) or ammonium (grey bars).

rates were approximately 60% higher under NO_3^- than NH_4^+ (Figure 6.7B). Both root and leaf assimilation rates were similar between sources (Table 6.2).

6.1.1. Efflux/influx was lower for NO_3^- than NH_4^+ and genotypic differences were not similar between sources.

Efflux/Influx (*E*/*I*) was lower under NO_3^- than NH_4^+ (Figure 6.6). So, since net nitrogen uptake was higher with NO_3^- there was greater *E*/*I*. For NH_4^+, *E*/*I* ranged from0.24 to 0.58 and for NO_3^-, it ranged from 0.19 to 0.48. However, there was a significant source by genotype interaction where some genotypes had higher *E*/*I* under NO_3^- than NH_4^+. However, at the population level, provenances with high *E*/*I* under NO_3^- also had high *E*/*I* under NH_4^+ (Table 6.2). There were no clinal patterns in *E*/*I* between provenances.

6.1.2. Genotypic and source effects were reproducible in independent experiments

When the experiment was repeated using seven genotypes, there was a significant relationship for genotypes and source combinations between the two experiments (Figure 6.8 and 6.9). However, there were also significant effects of experiment on *E*/*I*, but not on partitioning of nitrogen assimilation. There was a significant interaction for *E*/*I* between source and experiment, whereby the *E*/*I* for NH_4^+ was much higher in the second experiment than the first, whereas the *E*/*I* for NO_3^- was more-or-less unaffected. Genotypic ranks were largely unchanged between experiments ($P<0.05$).

6.2. Discussion

The objective of this study was to identify intraspecific variation in nitrogen isotope composition and consequently, nitrogen fluxes, assimilation and allocation for balsam poplar grown with either steady-state nitrate or ammonium nutrition. Strong genotypic, primarily clonal, variation was uncovered in growth, nitrogen isotope discrimination and nitrogen-use traits calculated from the isotope mass balance model

presented in Chapter 2. Intraspecific differences in nitrogen-use traits have not been described in many species. Masclaux-Daubresse *et al.* (2010) described intraspecific

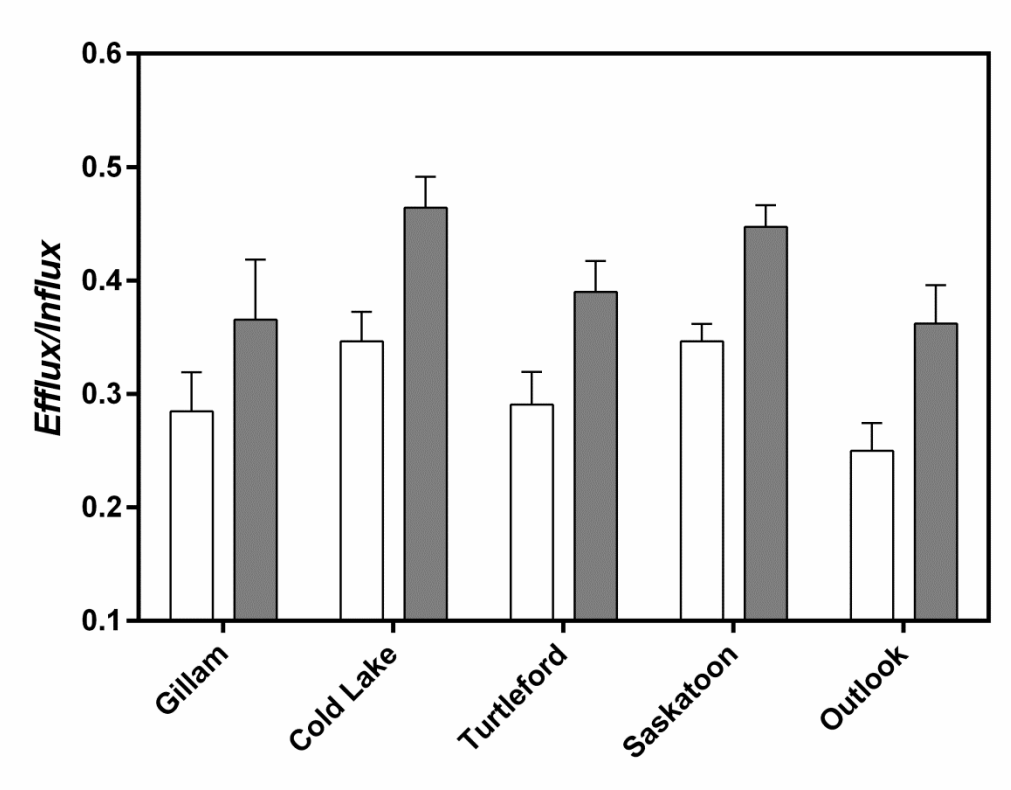

Figure 6.8. Efflux/influx (*E*/*I*) ±SE (N=9-15) of five provenances of *Populus balsamifera* L. grown with either 500 µM nitrate (white bars) or ammonium (grey bars).

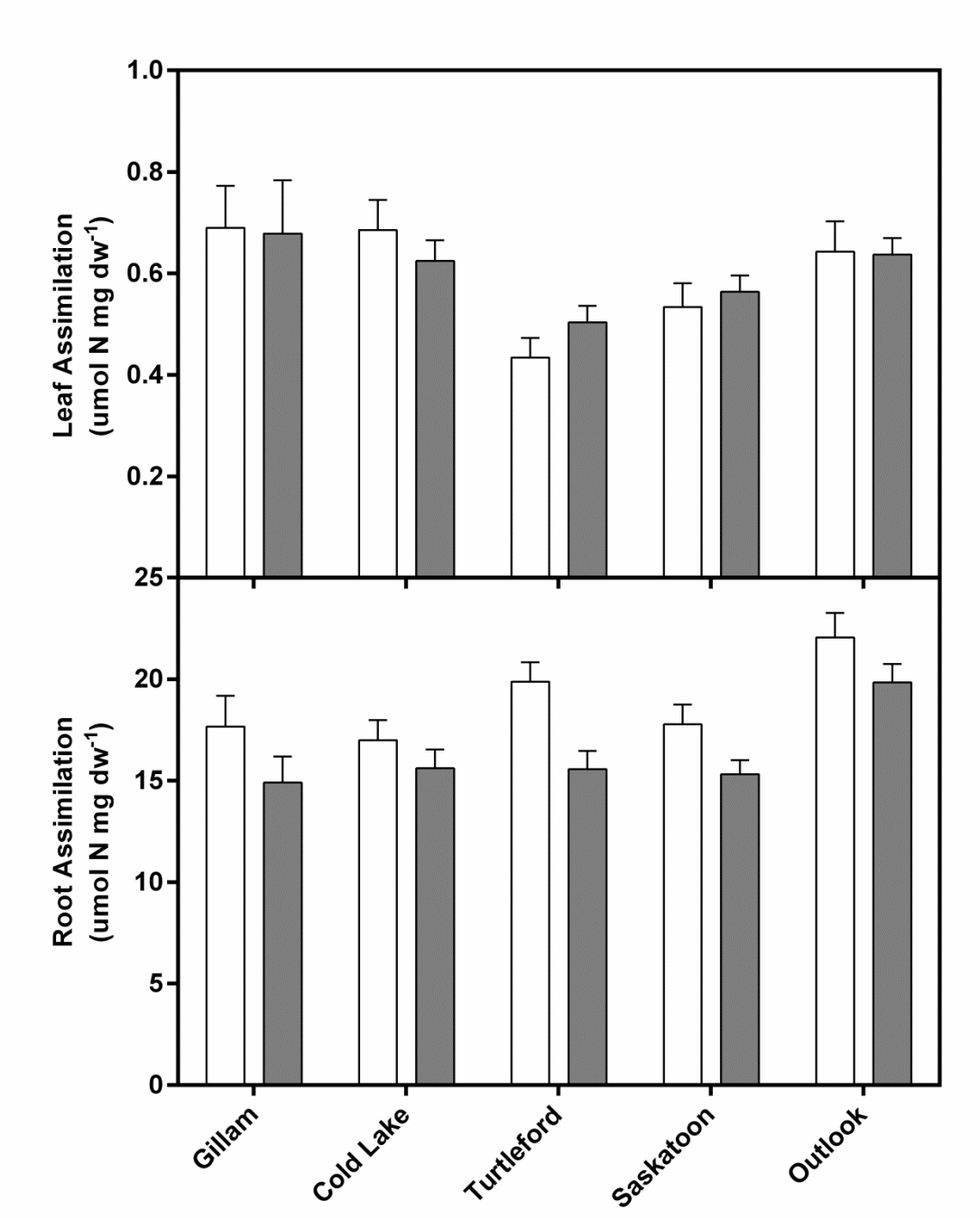

Figure 6.9 Root and leaf assimilation rates (µmol N mg dw^{-1}) (lower) ±SE (N=9-15) of five provenances of *Populus balsamifera* L. grown with either 500 µM nitrate (white bars) or ammonium (grey bars).

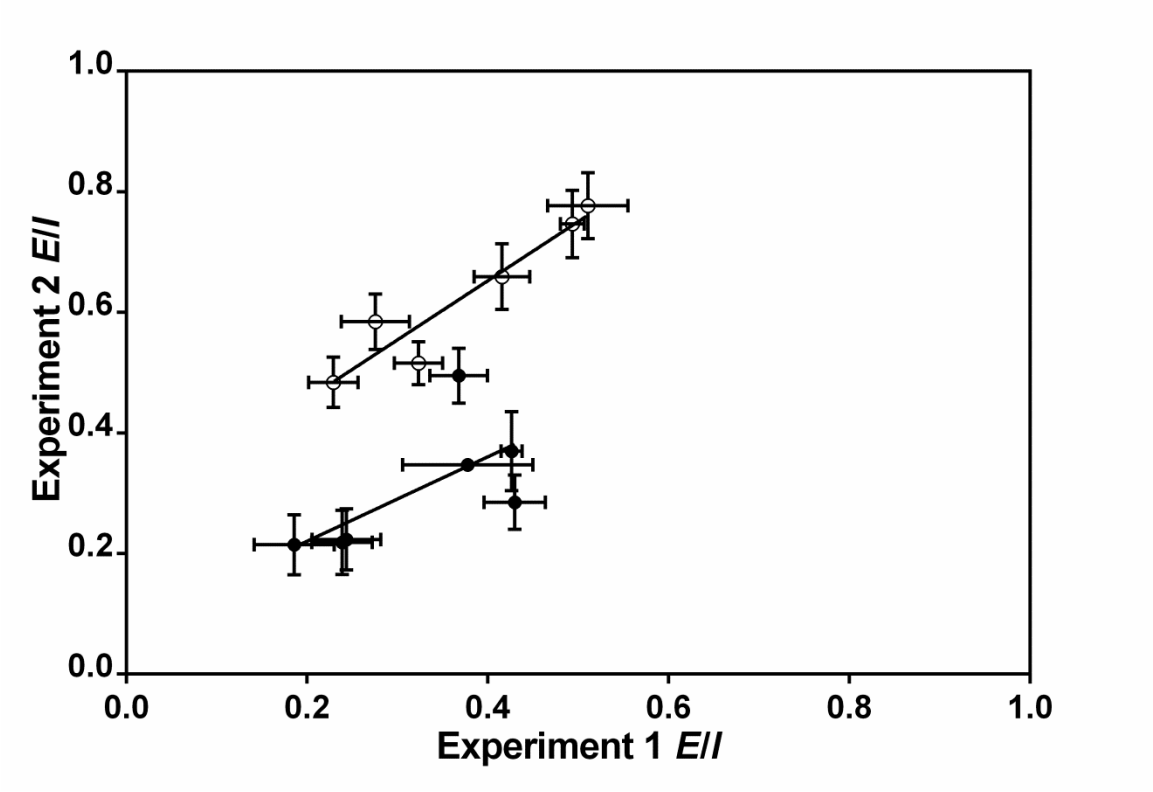

Figure 6.10 Estimates of efflux/influx ±SE (N=3) for two experiments for seven genotypes of *Populus balsamifera* L. grown with either 500 µM nitrate (solid circles) or ammonium (empty circles).

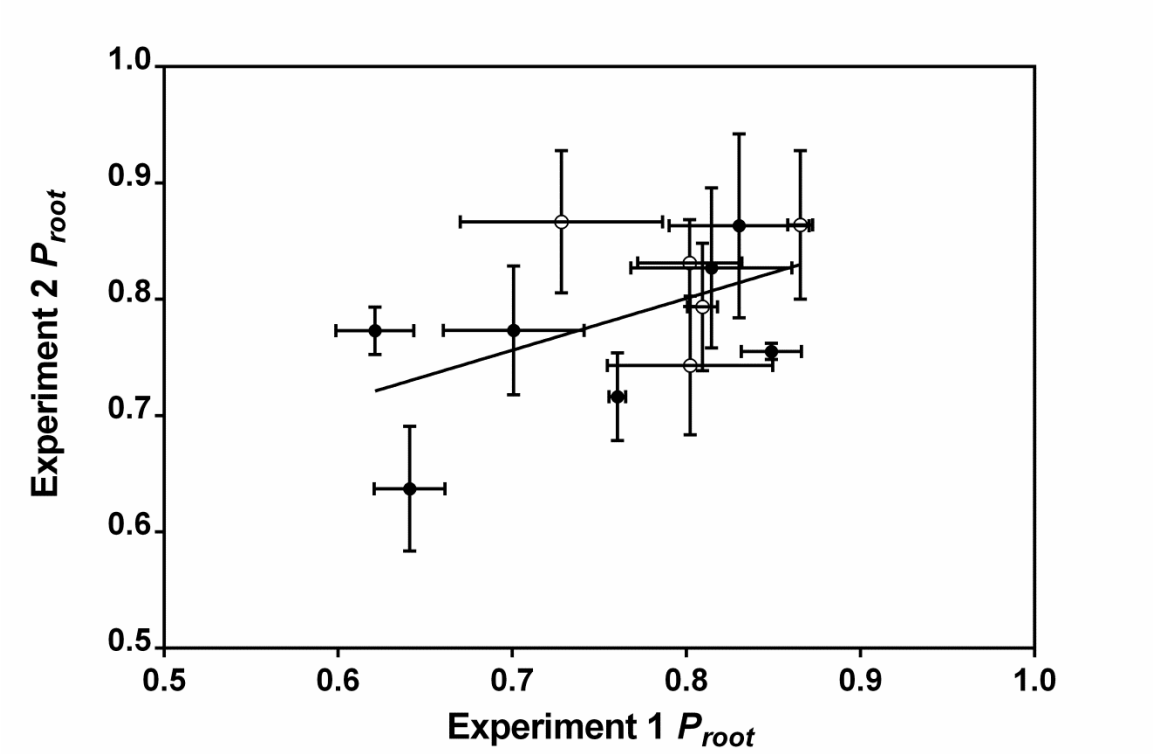

Figure 6.11 Estimates of P_{root} ±SE (N=3) for two experiments for seven genotypes of *Populus balsamifera* L. grown with either 500 µM nitrate (solid circles) or ammonium (empty circles).

variation in nitrogen-use traits in Arabidopsis and reported high variability among populations under nitrate. Since poplar is a potential agricultural crop to supply growing bioenergy demand, the ability to grow on marginal soils low in nitrogen availability, and/or to use applied nitrogen efficiently is important to being an effective carbon uptake system. The high degree of natural variation in balsam poplar suggests that selection for improved nitrogen use efficiency is possible as part of poplar breeding efforts.

6.2.1. Balsam poplar grew well on both sources of nitrogen

Here, irrespective of provenance or genotype within provenance, ramets grown on ammonium were approximately 15% smaller than on nitrate, indicating a general preference for nitrate. However, healthy growth on both sources is consistent with balsam poplar being a generalist species (Peterson and Peterson 1992) that has the ability to acquire multiple forms of nitrogen. In contrast, Desrochers *et al.* (2007) reported greater growth with ammonium than nitrate for pot-grown poplar genotypes, but this response was genotype-dependent. Under ammonium, plant biomass corresponded well with previous measurements of hydroponically grown *Populus trichocarpa* for a similar period of time (Chapter 2). Between species, Falkengren-Gerup (1995) reported large differences in biomass between nitrate and ammonium-grown plants where in many cases, greater growth occurred with nitrate nutrition, particularly in species that are found in less acidic soils. Furthermore, Cox and Reisenauer (1973) reported that *Triticum aestivum* was smaller when grown with ammonium compared to nitrate. In contrast, Pritchard and Guy (2005) reported higher growth with ammonium than nitrate for white spruce, a species known to prefer ammonium. Ariz *et al.* (2011) reported increased nitrogen isotope discrimination when grown with ammonium and a decreased nitrate:ammonium biomass ratio for species known to be sensitive to ammonium. Here, I observed provenance level differences in growth, nitrogen isotope composition and calculated nitrogen use traits. However, genotypic variation within provenances often exceeded variation between provenances (Table 6.1). Previously, within and between-provenance variation was reported for phenological traits in balsam

poplar and followed a clinal trend indicating selection for adaptive traits (Farmer 1993; Soolanayakanahally *et al.* 2009). The limitation on the number of genotypes and provenances used in this experiment prevents any firm conclusions regarding local adaptation to different nitrogen sources, but the wide geoclimatic range of balsam poplar used here produced no evidence of such. However, there appears to be a high degree of clonal variation and differential genotypic responses producing a range in growth and root:shoot ratios under nitrate and ammonium that may be indicative of variability in source preference.

6.2.2. Time-averaged nitrogen use traits are independent of growth and nitrogen source

Genotype means for isotope discrimination and root-to-shoot differences in isotope discrimination were similar between nitrogen sources. This is one of very few reports of genotypic differences in nitrogen use traits that extend across more than a single inorganic nitrogen source. Partitioning of assimilation between roots and leaves, leaf and root assimilation rates, translocation of inorganic and organic nitrogen to the shoot all show a significant relationship between genotypic means under ammonium and nitrate. Interestingly, these variables are not related to growth. Therefore, there must be some unknown commonality that explains the observed genotypic consistency in nitrogen-use traits that is independent of nitrogen source. Genetic variation in root architecture may be one explanation. Variation in nitrogen uptake efficiency has been attributed to variation in root architecture (Hirel *et al.* 2007; Garnett *et al.* 2009), a trait that may contribute to nitrogen isotope composition under steady-state conditions. In the present study, root thickness and branching frequency appeared to vary between genotypes (not shown) and genetic differences in root thickness, density and/or surface area may produce similar responses between the two nitrogen sources. Another possible explanation would be genotypic variation in nitrogen demand. Regardless of source, genotypic differences in biomass accretion were similar therefore assimilatory demand would be similar. Assimilatory demand is thought to impact rates of efflux

relative to influx, and therefore net nitrogen isotope discrimination, regardless of source.

6.2.3. Differences in efflux/influx and net root uptake between experiments may be explained by temperature-source interactions

Given the significant correlations between experiments seen in the proportions of nitrogen assimilation in the root, net nitrogen uptake and *E*/*I*, the genotypic differences reported here are repeatable and robust. However, the decrease in net uptake and increase in *E*/*I* from the first experiment to the second, particularly for ammonium, may indicate that other environmental variables influence nitrogen use traits in balsam poplar. In particular, media temperatures were warmer in the second experiment relative to the third, and this may have differentially affected NH_4^+ versus NO_3^- uptake mechanisms. In both experiments, the temperature of the hydroponics media were warmer than what would be experienced in native soil environments; therefore, this interaction may not occur under natural conditions but warrants consideration nonetheless. Although little work has been done on the effects of temperature on ion uptake, a handful of studies have found decreased uptake at lower temperatures (Clarkson and Warner, 1979; Garnett and Smethurst 1999). For example, Garnett and Smethurst (1999) reported a reduced uptake of both ammonium and nitrate at lower temperatures in shining gum (*Eucalyptus nitens* H. Deane & Maiden). However, the temperature conditions in the present study were warmer than used in previous experiments. Membrane permeability may increase and cytoplasmic ammonium control may decrease with higher temperatures resulting in increased *E*/*I*. Britto and Kronzucker (2006) suggested higher efflux leads to futile cycling which has high respiratory costs. Greater futile cycling of ammonium, and not nitrate, at higher root temperatures would result in relatively greater energetic costs for this treatment than at lower temperatures. This interpretation is consistent with reduced growth observed with ammonium at higher temperatures. In accordance with observed increased growth of poplar under ammonium at lower temperatures, better relative growth on ammonium has also been observed in conifers at low (10-15 °C) versus warm

temperatures (Barbara Hawkins, personal communication). Since net nitrogen uptake decreased and *E*/*I* increased, efflux on a root basis increased with increased temperatures for ammonium but not nitrate in the present study.

6.2.4. Using genotypic differences in calculated nitrogen use traits to improve nitrogen use in poplar

Though very fundamental, our understanding of nitrogen uptake and assimilation in plants, particularly trees, is incomplete. Assessment of time-integrated genotypic and environmental variation in nitrogen uptake, assimilation and allocation under steady-state conditions is a first step towards improving nitrogen-use in poplar. Here, I report on the inherent variation in these traits using an approach that provides an opportunity for screening current cultivars and species used for breeding of hybrid poplar for multiple uses, including biomass feedstock. As a time-integrated approach, the nitrogen isotope mass balance method is promising given that it can measure multiple traits in a single experiment. However, the tightly controlled conditions needed for these analyses, as presently implemented, do not reflect the physiochemical and biotic variability that is normal to a natural environment. Further testing of the relationship between measured genotypic variation under controlled, common environment conditions to field-level growth and nitrogen-use will be required

6.3. .Conclusion

Poplar has the capacity to grow well on both nitrate and ammonium when provided at realistic rates of supply. However, there is considerable intraspecific variation in growth performance and in isotope discrimination at both whole plant and organ levels. Intraspecific variation was repeatable and indicates that observed variation was genetically controlled. Some of this variation is the same across inorganic nitrogen sources, suggesting that nitrogen demand rather than source-specific supply contributes to uptake, assimilation and allocation of nitrogen within balsam poplar. The exception to this was cycling of nitrogen across the root membrane where isotope mass balance

modelling showed that genotypes responded differently to each source, indicating possible intraspecific variation in source preference.

7. Conclusion

Over the past 75 years, plant production has doubled, largely corresponding to a seven-fold increase in nitrogen fertilizer use (Hirel *et al.* 2007). A large portion of applied fertilizer is lost through leaching, volatilization or denitrification (Vitousek *et al.* 1997). Improving nitrogen-use efficiency will be critical for future food security and reducing emissions and environmental impacts from agricultural activities. Over the last three decades, advances in molecular physiology have contributed to our understanding of nitrate uptake and assimilation mechanisms at the functional and cellular level. However, to date, gains in nitrogen-use efficiency have been limited by the complexity of nitrogen uptake and assimilation. The importance of developing an integrated measure of nitrogen-use in plants is underscored by the need to reduce fertilizer requirements to produce optimum yields and reduce emissions from global fertilizer production. Poplar has the potential to be a primary feedstock for the growing biofuel sector. To optimize carbon sequestration efficiencies, fertilizer inputs must be minimized while, at the same time, achieving rapid growth. First, the genotypic variability in nitrogen-use traits within relevant poplar species used for breeding needs to be considered.

Nitrogen isotope discrimination can provide time-integrated information on nitrogen fluxes, assimilation and allocation in roots similar to how carbon isotope composition can provide information on time-integrated carbon assimilation in leaves. Here, I improved, tested and applied a nitrogen isotope mass balance model used to calculate time-integrated nitrogen-use traits in balsam poplar and other plants. Nitrogen isotope discrimination is a function of external and internal supply and physiological demand from multiple assimilatory sinks. Tests designed to alter nitrogen isotope discrimination through modification of nitrogen supply and demand, either environmentally or genetically, showed results that were interpretable within the context of the nitrogen isotope mass balance model. Furthermore, estimates of cycling across the root membrane using the isotope mass balance model are comparable to results obtained using an independent, previously established technique. This thesis

addressed the absence of a detailed, integrated measure of nitrogen-use in plants and used this approach to evaluate the variability in nitrogen-use traits among five climatically dispersed provenances of *Populus balsamifera*.

7.1. Deriving integrated nitrogen-use traits from organ level and whole plant nitrogen isotope composition

Initial observations of nitrogen isotope discrimination in pearl millet during nitrate uptake and assimilation (Mariotti *et al.* 1982) led to research working towards understanding the contributions of nitrogen use physiology to nitrogen isotope composition in plants. Contributions of Yoneyama (1993), Evans *et al.* (1996), Robinson *et al.* (1998), Yoneyama (2001), Evans (2001), Comstock (2001), Robinson (2001), Yoneyama (2003), Tcherkez and Farquhar (2006), Buschhaus (2007), Cernusak *et al.* (2009) and Tcherkez (2011) were all central to the development of the theory described in this thesis. These contributions provided the theoretical framework on which the proposed model is based. Comstock (2001) and Evans (2001) provided the most complete models to date explaining nitrogen isotope discrimination in plants. This thesis has built on that theory and combined it with mass balance nitrogen measurements to produce a tractable model explaining the relationship between nitrogen-use physiology and nitrogen isotope discrimination under steady-state conditions.

The heterogeneous nitrogen environment in natural ecosystems can limit interpretation of whole plant and organ level nitrogen isotope composition. Under closed source conditions, such as those that exist in natural environments, plant nitrogen isotope composition largely reflects that of the source nitrogen. However, not all environments are completely closed systems and some degree of discrimination may occur that may contribute to error in the interpretation of nitrogen isotope composition of plants in natural environments. However, without knowing the integrated nitrogen isotope composition of multiple nitrogen sources, interpretation is not possible. Under open source conditions where the source $\delta^{15}N$ is homogeneous, stable and known, plants discriminate against ^{15}N and the plant becomes depleted relative to the

substrate. This change in nitrogen isotope composition is a function of internal nitrogen-use physiology. From the model, multiple nitrogen-use traits can be derived from a single experiment.

Measured traits such as growth, nitrogen concentration and isotope composition indicated that although there may be no visible growth phenotype, there may be isotopic phenotypes that indicate variability in nitrogen-use. Growth was not closely related to nitrogen isotope composition in poplar or Arabidopsis. Previously, efforts to use nitrogen isotope discrimination as an indicator of nitrogen uptake and assimilation in plants were confounded by non-steady-state conditions and unknown source isotope composition, or through complicating assumptions that limited the scope of interpretation. Here, by making physiologically reasonable assumptions, the isotope mass balance model describing nitrogen isotope composition was simplified. This approach provides information on nitrogen-use physiology that would normally require careful and extensive sampling using more traditional assays.

7.2. Modifying nitrogen isotope composition of plants through changes in inorganic nitrogen supply or demand

Nitrogen or carbon supply can modify nitrogen isotope composition in plants grown under steady-state nitrogen conditions. These changes are a result of impacts on net nitrogen uptake and export of inorganic nitrogen from the root to shoot. More simply, changes to internal supply or demand of inorganic nitrogen from the root or shoot produce changes in nitrogen isotope composition. These changes could be induced environmentally as was observed in Chapters 2 (*Populus trichocarpa*), 3 (*Arabidopsis thaliana*), 4 (*Populus balsamifera*) and 6 (*Populus balsamifera*), or genetically as in Chapters 3 (*Arabidopsis thaliana*) and 6 (*Populus balsamifera*). Changes were interpretable within the context of the nitrogen isotope mass balance model where changes in efflux/influx or partitioning of assimilation were functions of changes in nitrogen source, concentration, carbon supply and possibly temperature. Arabidopsis lines showed differences in nitrogen isotope composition that were repeatable across

nitrate concentrations and experiments. Similarly, poplar genotypes showed repeatable differences in nitrogen isotope discrimination under different nitrogen sources that indicate variability in nitrogen source preference and nitrogen demand. By validating the model through a series of tests, a greater degree of confidence in the model was acquired for purposes of identifying intraspecific variation in nitrogen-use traits.

7.3. Comparisons of efflux/influx (*E*/*I*) acquired by independent experiments

One of the limitations of the nitrogen isotope discrimination model was that it was not grounded to other measures of nitrogen-use. In Chapter 5, I compared estimates of *E*/*I* using the isotope mass balance model to estimates using compartmental analysis tracer efflux. There was a positive relationship between *E*/*I* measured using the two approaches. However, *E*/*I* was greater using the isotope mass balance method indicating that there may be errors in the discrimination factor of the enzyme, diurnal variation in fluxes or a kinetic isotope effect associated with transport across the membrane. When a greater enzyme discrimination factor was used for nitrate reductase or glutamine synthetase, measures of *E*/*I* were more alike. *E*/*I* was greater when grown with ammonium, consistent with a preference of poplar for nitrate. The coherence in estimates of *E*/*I* between independent methods suggests that leakage or efflux of inorganic nitrogen along the root occurs regardless of source, and is indicative of source preference and overall nitrogen assimilatory demand.

7.4. Genotypic variation in nitrogen fluxes, assimilation and allocation in balsam poplar

After testing the model through environmental and genetic modification of nitrogen isotope discrimination and validating the estimation of *E*/*I* using an independent method, intraspecific variation in time-integrated nitrogen-use traits were assessed using balsam poplar grown under steady-state conditions with either nitrate or ammonium. There was considerable variation in growth, uptake and assimilation traits. These traits were related between sources but not related to growth, indicating that

these traits are driven by internal nitrogen demand and/or external nitrogen supply unrelated to source. There was a high degree of genotypic variation that was repeatable across experiments.

7.5. Limitations of research

One of the primary limitations of this approach to measuring integrated nitrogen-use traits is the need for carefully controlled, hydroponic conditions. Plants are not likely to express these isotopic phenotypes if grown in any substrate that restricts mixing around the root because of the heterogeneity of nitrogen isotope composition and concentration in these environments, and because localized isotopic enrichment would occur in the rhizosphere if unassimilated nitrogen cannot freely diffuse away. Substrates with multiple sources make interpretation of individual flux, assimilation and allocation traits rather difficult. Like any phenotyping experiment, controlled conditions allow for genotypic comparisons that minimize environmental effects. There is a gap in knowledge between measurements of nitrogen-use under controlled conditions in the lab or greenhouse that limits the extension of results to field conditions. Hydroponically-grown plants can only be grown under single-source nitrogen that does not address interactions between sources that can occur, particularly between nitrate and ammonium.

Second, this method is not a high-throughput approach and requires extensive sampling of all plant parts to provide the input data necessary for calculation of model outputs. Although sampling intensity is lower than other isotope-based experiments, it is still intensive. However, there is the potential to phenotype lines or genotypes of plants in relatively large numbers. The system for hydroponics needs to be of sufficient volume to enable close control of substrate concentration and to stabilize its isotopic composition. Larger plants require larger volumes. For the experiments in Chapters 4-6, this necessitated the construction of 1000 L tanks to grow only 32 plants/tank and required multiple solution replacements over the course of each experiment. In turn, experiments had to be limited to a greenhouse with sufficient growing space but poor

control of air temperature, humidity, etc. Plants were given additional light, essential in the winter, but on sunny days during the summer where experiments were conducted for Chapter 5 and the second experiment for Chapter 6, air temperatures would reach up to 26 or 27°C and the temperature of the hydroponics solution would subsequently rise, sometimes reaching over 24°C (See Figure A.4). This appeared to have an adverse effect on plant growth under ammonium but not nitrate.

The model is able to derive time-integrated variables that are reflections of nitrogen fluxes, assimilation and allocation but these values are still relative comparisons and should be interpreted with a certain degree of caution. The assumptions required to derive these time-integrated variables involved simplifying the isotope end-members of the model. Specifically, measured root bulk tissue δ^{15}N that is a combination of inorganic and organic N δ^{15}N signatures, was considered to be only organic N. The fraction of inorganic N may be as high as 10% in some cases, which would cause the δ^{15}N taken for root organic nitrogen to be slightly more positive than it actually is. This does not negate our basic conclusions from the model or relative interpretation of the results, but does inject some error into the estimate of Ti/Tt, P_{root} and E/I.

7.6. Applications and Future Research

Although the work presented here advanced the use of nitrogen isotope discrimination as a time-integrated proxy of various nitrogen-use traits, refinement of the model for discrimination is still not complete. There were a number of assumptions presented here that allowed derivation of nitrogen traits from isotope composition at the whole plant and organ level which were justified based on previous physiological estimates in the literature. Ideally, these assumptions could be theoretically and empirically tested to determine the influence of variation in these parameters on nitrogen isotope discrimination measurements. Specifically, assumptions that be worthwhile to test include: 1) Quantifying better constrained isotope discrimination factors for glutamine synthetase and nitrate reductase, 2) Determining a time-

integrated estimate of inorganic and organ nitrogen concentration and isotope composition in xylem exudate 3) Determining the isotopic composition of remobilized nitrogen from cuttings of vegetatively propagated plants 4) Determining whether there is some discrimination that occurs during transport of inorganic nitrogen across the plasma membrane 5) Finding the contribution of inorganic nitrogen to total root nitrogen 6) Testing the influence that volatilization of ammonia or exudation of organic nitrogen from the root may have on leaf and root isotopic composition and 7) Determining whether there is organic nitrogen translocated from shoots to roots of actively growing plants.

The findings in this thesis prompt many questions regarding the morphological, physiological and environmental influences on nitrogen isotope composition in plants. These include:

1) Do root morphology and anatomy influence nitrogen uptake and assimilation in such a way that nitrogen isotope discrimination and model outputs are universally expressed independent of source?

2) Is there an interaction between temperature and source for nitrogen uptake?

3) Do measurements of nitrogen-use using the nitrogen isotope mass balance approach correspond to measures of nitrogen-use efficiency and uptake efficiency in the field?

4) Can this approach be combined with traditional measures of nitrogen-use to produce a comprehensive model of nitrogen-use extending from the cellular to the whole-plant level?

In Chapter 6, a close relationship was observed between nitrogen-use traits on nitrate compared to those on ammonium with the exception of efflux/influx. I hypothesized that assimilatory demand, regardless of nitrogen source or growth rate, appeared to be driving this relationship. To address this question, assimilatory demand expressed on a per mass basis would need to be quantified. This could be approached from a cellular/molecular biology or an anatomical/whole plant perspective. Root-based traits have been suggested as contributors of whole-plant nitrogen-use (Garnett *et al.*

2009). To address this, measurements of root-based traits such as thickness, surface area to volume ratio, cortex thickness, and cell size may provide some indication of the relationship between root anatomy and nitrogen fluxes and assimilation partitioning. A contributing role for root architecture in determining nitrogen movement through the root and into the xylem could be empirically tested by taking advantage of intra- or inter-specific variation in root form. One could explore this in more detail by incorporating non-destructive *in vivo* measures of nitrogen movement through the root in both inorganic and organic forms. NMR microimaging has been shown to detect stable isotope signatures in biochemical components within the cell at a resolution as low as 50 µM. Using non-destructive methods to measure nitrogen movement through the root and comparing that to complementary anatomical measurements may provide an indication of how and where inorganic nitrogen is moved through the root during uptake and assimilation under different conditions.

Also in Chapter 6, there were indications of a source by temperature interaction whereby efflux/influx was greater with ammonium at warmer solution temperatures but not with nitrate. This observation suggests that ammonium toxicity becomes more problematic at higher temperatures. A similar effect has been observed in conifer species (B. Hawkins; Personal Comm.). Further work could be done to address this hypothesis.

Thirdly, it is not known whether the variation in nitrogen-use traits observed under tightly controlled, hydroponic conditions is expressed in the field. Ideally, this would be best addressed using crop plants that rely on relatively high concentrations of soil inorganic nitrogen for optimum growth and development. By exploring variation in nitrogen-use traits using the isotopic approach, and then comparing these data to performance in the field, a relationship between the two may be apparent. Although root architecture is likely to change and the heterogeneous environment may result in different uptake patterns compared to those in hydroponics, any correlation between the two would encourage application of the isotope mass balance approach as a

phenotyping tool for screening in breeding programs. Furthermore, genotypic variation under controlled conditions can be used in combination with genomic variation to produce associations between phenotypes and genes responsible for nitrogen fluxes, assimilation and allocation within the plant. The advantage this approach as compared to other phenotyping approaches is that multiple nitrogen use traits can be acquired in a single experiment that can then be associated with genomic data to produce candidate genes responsible for genotypic variation in those traits.

Although isotope discrimination/mass balance modelling can provide time-integrated measures of nitrogen-use, it does not provide measures of nitrogen use that reflect diurnal or developmental changes in fluxes, assimilation or allocation. By using careful sampling protocols described in detail in Chapter 4, changes in nitrogen-use traits could be calculated for different developmental stages including seedling growth, vegetative growth, anthesis and fruit development for herbaceous species. Furthermore, examining spatial variation in nitrogen fluxes, assimilation and allocation at the cellular and tissue level will be critical for obtaining an accurate model of nitrogen use at the whole plant level. By developing a complete model of nitrogen-use at the cellular, tissue and whole plant levels that predicts whole plant nitrogen use efficiency, improvements in nitrogen use efficiency in poplar and other agricultural crops can be achieved.

References

Ahlgren S, Baky A, Bernesson S, Nordberg Å, Norén O, & Hansson PA (2008) Ammonium nitrate fertiliser production based on biomass–environmental effects from a life cycle perspective. *Bioresource Technology* 99: 8034-8041

Alber A, Ehlting B, Ehlting J, Hawkins BJ & Rennenberg H (2012) Net NH_4^+ and NO_3^- flux, and expression of $NH_4^+$and NO_3^- transporters in roots of *Picea glauca*. *Trees-Structure and Function* 26: 1403-1411

Andrews M (1986) The partitioning of nitrate assimilation between root and shoot of higher plants. *Plant, Cell & Environment* 9: 511-519

Andrews M, Morton JD, Lieffering M & Bisset L (1992) The partitioning of nitrate assimilation between root and shoot of a range of temperate cereals and pasture grasses. *Annals of Botany* 70: 271-276

Arabidopsis GI (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* 408: 796-815

Ariz I, Cruz C, Moran JF, González-Moro MB, García-Olaverri C, González-Murua C & Aparicio-Tejo PM (2011) Depletion of the heaviest stable N isotope is associated with NH_4^+/NH_3 toxicity in NH_4^+-fed plants. *BMC Plant Biology* 11: 83

Bassirirad H, Constable JVH, Lussenhop J, Kimball BR, Norby RJ, Oechel WC, Reich PB, Schlesinger WH, Zitzer S, Sehtiya HL & Silim S (2003) Widespread foliage $\delta^{15}N$ depletion under elevated CO_2: Inferences for the nitrogen cycle. *Global Change Biology* 9: 1582-1590

Black BL, Fuchigami LH & Coleman GD (2002) Partitioning of nitrate assimilation among leaves, stems and roots of poplar. *Tree Physiology* 22: 717-724

Bloom AJ, Chapin FS & Mooney HA (1985) Resource limitation in plants: an economic analogy. *Annual Review of Ecology and Systematics* 16: 363-392

Bloom AJ, Randall L, Taylor AR & Silk WK (2012) Deposition of ammonium and nitrate in the roots of maize seedlings supplied with different nitrogen salts. *Journal of Experimental Botany* 63: 1997-2006

Bollmark L, Sennerby-Forsse L & Ericsson T (1999) Seasonal dynamics and effects of nitrogen supply rate on nitrogen and carbohydrate reserves in cutting-derived *Salix viminalis* plants. *Canadian Journal of Forest Research* 29: 85-94

Bradshaw HD, Ceulemans R, Davis J & Stettler R (2000) Emerging model systems in plant biology: poplar (*Populus*) as a model forest tree. *Journal of Plant Growth Regulation* 19: 306-313

Britto DT & Kronzucker HJ (2002) NH_4^+ toxicity in higher plants: A critical review. *Journal of Plant Physiology* 159: 567-584

Britto DT, Siddiqi MY, Glass ADM & Kronzucker HJ (2001) Futile transmembrane NH_4^+ cycling: a cellular hypothesis to explain ammonium toxicity in plants. *Proceedings of the National Academy of Sciences* 98: 4255-4258

Britto DT & Kronzucker HJ (2006) Futile cycling at the plasma membrane: a hallmark of low-affinity nutrient transport. *Trends in Plant Science* 11: 529-534

Brooks PD, JM Stark, BB McInteer & T Preston (1989) Diffusion method to prepare soil extracts for automated nitrogen-15 analysis. *Soil Science Society of America Journal* 53: 1707–1711

Brunner AM, Busov VB & Strauss SH (2004) Poplar genome sequence: functional genomics in an ecologically dominant plant species. *Trends in Plant Science* 9: 49-56

Buschhaus HA (2007) ^{15}N discrimination as an indicator of nitrogen dynamics in *Populus trichocarpa*. M.Sc. Thesis. University of British Columbia. Vancouver, BC, Canada. 70 pages.

Callesen I, Raulund-Rasmussen K, Westman CJ & Tau-Strand L (2007) Nitrogen pools and C:N ratios in well-drained soil related to climate and soil texture. *Boreal Environment Research* 12: 681-692

Campbell WH (1988) Nitrate reductase and its role in nitrate assimilation in plants. *Physiologia Plantarum* 74: 214-219

Campbell WH (1999) Nitrate reductase structure, function and regulation: Bridging the gap between biochemistry and physiology. *Annual Review of Plant Physiology* 50: 277-303

Carvalhais LC, Dennis PG, Fedoseyenko D, Hajirezaei MR, Borriss R & von Wirén N (2011) Root exudation of sugars, amino acids, and organic acids by maize as affected by nitrogen, phosphorus, potassium, and iron deficiency. *Journal of Plant Nutrition and Soil Science* 174: 3-11

Cawse PA (1967) The determination of nitrate in soil solution by ultraviolet spectrometry. *Analyst* 92: 311-315

Cerezo M, Tillard P, Filleur S, Muños, Daniel-Vedele F & Gojon A (2001) Major alterations of the regulation of root NO_3^- uptake are associated with the mutation of NRT2.1 and NRT2.2 genes in Arabidopsis. *Plant Physiology* 127: 262-271

Cernusak LA, Winter K & Turner BL (2009) Plant $\delta^{15}N$ correlates with the transpiration efficiency of nitrogen acquisition in tropical trees. *Plant Physiology* 151: 1667-1676

Chardon F, Barthélémy J, Daniel-Vedele F & Masclaux-Daubresse C (2010) Natural variation of nitrate uptake and nitrogen use efficiency in *Arabidopsis thaliana* cultivated with limiting and ample nitrogen supply. *Journal of Experimental Botany* 61: 2293-2302

Clarkson DT & Warner AJ (1979) Relationships between root temperature and the transport of ammonium and nitrate ions by Italian and perennial ryegrass (*Lolium multiflorum* and *Lolium perenne*). *Plant Physiology* 64: 557-561

Coleman GD (2004) Physiology and regulation of seasonal nitrogen cycling in woody plants. *Journal of Crop Improvement* 10: 237-259

Coleman JS, McConnaughay KDM & Bazzaz FA (1993) Elevated CO_2 and plant nitrogen-use: is reduced tissue nitrogen concentration size-dependent? *Oecologia* 93: 195-200

Comstock JP (2001) Steady-state isotopic fractionation in branched pathways using plant uptake of NO_3^- as an example. *Planta* 214: 220-234

Cooke JE & Weih M (2005) Nitrogen storage and seasonal nitrogen cycling in *Populus*: bridging molecular physiology and ecophysiology. *New Phytologist* 167: 19-30

Couturier J, Montanini B, Martin F, Brun A, Blaudez D & Chalot M (2007) The expanded family of ammonium transporters in the perennial poplar plant. *New Phytologist* 174: 137-150

Cox WJ & Reisenauer HM (1973) Growth and ion uptake by wheat supplied nitrogen as nitrate, or ammonium, or both. *Plant and Soil* 38: 363-380.

Crawford NM, Smith M, Bellissimo D & Davis RW (1988) Sequence and nitrate regulation of the *Arabidopsis thaliana* mRNA encoding nitrate reductase, a metalloflavoprotein with three functional domains. *Proceedings of the National Academy of Sciences* 85: 5006-5010

Dawson TE, Mambelli S, Plamboeck AH, Templer PH & Tu KP (2002) Stable isotopes in plant ecology. *Annual Review of Ecology and Systematics* 33: 507-559

Delhon P, Gojon A, Tillard P & Passama L (1995) Diurnal regulation of NO_3^- uptake in soybean plants I. Changes in NO_3^- influx, efflux, and N utilization in the plant during the day/night cycle. *Journal of Experimental Botany* 46: 1585-1594

DeLuca, TH, Zackrisson O, Nilsson MC & Sellstedt A (2002) Quantifying nitrogen-fixation in feather moss carpets of boreal forests. *Nature* 419: 917-920

DesRochers A, van den Driessche R & Thomas B (2006) NPK fertilization at planting of three hybrid poplar clones in the boreal region of Alberta. *Forest Ecology and Management* 232: 216-225

DesRochers ADA, van den Driessche R & Thomas BR (2007) The interaction between nitrogen source, soil pH, and drought in the growth and physiology of three poplar clones. *Canadian Journal of Botany* 85: 1046-1057

Devienne F, Mary B & Lamaze T (1994) Nitrate transport in intact wheat roots: I. Estimation of cellular fluxes and NO_3^- distribution using compartmental analysis from data of $^{15}NO_3^-$ efflux. *Journal of Experimental Botany* 45: 667-676

Dong S, Cheng L, Scagel CF & Fuchigami LH (2004) Nitrogen mobilization, nitrogen uptake and growth of cuttings obtained from poplar stock plants grown in different N regimes and sprayed with urea in autumn. *Tree Physiology* 24: 355-359

Evans RD (2001) Physiological mechanisms influencing nitrogen isotope composition. *Trends in Plant Science* 6: 121-126.

Evans RD, Bloom AJ, Sukrapanna SS & Ehleringer JR (1996) Nitrogen isotope composition of tomato (*Lycopersicon esculentum* Mill. Cv. T-5) grown under ammonium or nitrate nutrition. *Plant, Cell & Environment* 19: 1317-1323

Falkengren-Grerup U (1995) Interspecies differences in the preference of ammonium and nitrate in vascular plants. *Oecologia* 102: 305-311

Fan X, Gordon-Weeks R, Shen Q & Miller AJ (2006) Glutamine transport and feedback regulation of nitrate reductase activity in barley roots leads to changes in cytosolic nitrate pools. *Journal of Experimental Botany* 57: 1333-1340

Farmer RE (1993) Latitudinal variation in height and phenology of balsam poplar. *Silvae Genetica* 42: 148-148

Farquhar GD, O'Leary MH & Berry JA (1982) On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Australian Journal of Plant Physiology* 9: 121-137

Farquhar GD, Ehleringer JR & Hubick KT (1989) Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology* 40: 503-537

Feng H, Yan M, Fan X, Li B, Shen Q, Miller AJ & Xu G (2011) Spatial expression and regulation of rice high-affinity nitrate transporters by nitrogen and carbon status. *Journal of Experimental Botany* 62: 2319-2332

Filleur S, Dorbe MF, Cerezo M, Orsel M, Granier F, Gojon A & Daniel-Vedele F (2001) An Arabidopsis T-DNA mutant affected in NRT2 genes is impaired in nitrate uptake. *FEBS Letters* 489: 220-224

Garnett TP & Smethurst PJ (1999) Ammonium and nitrate uptake by *Eucalyptus nitens*: effects of pH and temperature. *Plant and Soil* 214: 133-140

Garnett T, Conn V & Kaiser BN (2009) Root based approaches to improving nitrogen use efficiency in plants. *Plant, Cell & Environment* 32: 1272-1283

Gauthier PPG, Lamothe M, Mahé A, Molero G, Nogués S, Hodges M & Tcherkez G (2012) Metabolic origin of $\delta^{15}N$ values in nitrogenous compounds from *Brassica napus* L. leaves. *Plant, Cell & Environment*, In Press

Gebauer G & Schulze ED (1991) Carbon and nitrogen isotope ratios in different compartments of a healthy and a declining *Picea abies* forest in the Fichtelgebirge, NE Bavaria. *Oecologia* 87: 198-207

Gedroc JJ, McConnaughay KDM & Coleman JS (1996) Plasticity in root/shoot partitioning: optimal, ontogenetic, or both? *Functional Ecology* 10: 44-50

Gessler A, Kreuzwieser J, Dopatka T & Rennenberg H (2002) Diurnal courses of ammonium net uptake by the roots of adult beech (*Fagus sylvatica*) and spruce (*Picea abies*) trees. *Plant and Soil* 240: 23-32

Giesler R, Högberg M & Högberg P (1998) Soil chemistry and plants in Fennoscandian boreal forest as exemplified by a local gradient. *Ecology* 79: 119-137

Giesler R, Petersson T & Högberg P (2002) Phosphorus limitation in boreal forests: effects of aluminum and iron accumulation in the humus layer. *Ecosystems* 5: 300-314

Glass ADM, Britto DT, Kaiser BN, Kinghorn JR, Kronzucker HJ, Kumar A, Okamoto M, Rawat S, Siddiqi MY, Unkles SE & Vidmar JJ (2002) The regulation of nitrate and ammonium transport systems in plants. *Journal of Experimental Botany* 53: 855-864

Gomez L & Faurobert M (2002) Contribution of vegetative storage proteins to seasonal nitrogen variations in the young shoots of peach trees (*Prunus persica* L. Batsch). *Journal of Experimental Botany* 53: 2431-2439

Greenwood JS, Stinissen HM, Peumans WJ & Chrispeels MJ (1986) *Sambucus nigra* agglutinin is located in protein bodies in the phloem parenchyma of the bark. *Planta* 167: 275-278

Guo FQ, Wang R & Crawford NM (2002) The Arabidopsis dual-affinity nitrate transporter gene AtNRT1.1 (CHL1) is regulated by auxin in both shoots and roots. *Journal of Experimental Botany* 53: 835-844

Hale MG, Foy CL & Shay FJ (1975) Factors affecting root exudation. *Advances in Agronomy* 23: 89-109

Handley LL & Raven JA (1992) The use of natural abundance of nitrogen isotopes in plant physiology and ecology. *Plant, Cell & Environment* 15: 965-985

Handley LL, Austin AG, Robinson D, Scrimgeour CM, Raven JA, Heaton THE, Schmidt S & Stewart GR (1999) The ^{15}N natural abundance ($\delta^{15}N$) of ecosystem samples reflects measures of water availability. *Australian Journal of Plant Physiology* 26: 185-199

Handley LL, Robinson D, Forster BP, Ellis RP, Scrimgeour CM, Gordon DC, Nevo E, Raven JA (1997) Shoot $\delta^{15}N$ correlates with genotype and salt stress in barley. *Planta* 201: 100-102

Harrison J, Hirel B & Limami AM (2004) Variation in nitrate uptake and assimilation between two ecotypes of *Lotus japonica* and the recombinant inbred lines. *Physiologia Plantarum* 120: 124-131

Harrison KA, Bol R & Bardgett RD (2007) Preferences for different nitrogen forms by coexisting plant species and soil microbes. Ecology 88: 989-999

Hart SC (2006) Potential impacts of climate change on nitrogen transformations and greenhouse gas fluxes in forests: a soil transfer study. *Global Change Biology* 12: 1032-1046

Hawkins BJ & Robbins S (2010) pH affects ammonium, nitrate and proton fluxes in the apical region of conifer and soybean roots. *Physiologia Plantarum* 138: 238-247

Hayes JM (2001) Fractionation of carbon and hydrogen isotopes in biosynthetic processes. In: Stable Isotope Geochemistry. JW Valley and D Cole, eds. *Reviews in Mineralogy and Geochemistry* 43: 225-277

Henriksen GH, Bloom AJ & Spanswick RM (1990) Measurement of net fluxes of ammonium and nitrate at the surface of barley roots using ion-selective microelectrodes. *Plant Physiology* 93: 271-280

Henriksen GH, Raman DR, Walker LP & Spanswick RM (1992) Measurement of net fluxes of ammonium and nitrate at the surface of barley roots using ion-selective microelectrodes II. Patterns of uptake along the root axis and evaluation of the microelectrode flux estimation technique. *Plant Physiology* 99: 734-747

Henry BK, Atkin OK, Day DA, Millar AH, Menz RI & Farquhar GD (1999) Calculation of the oxygen isotope discrimination factor for studying plant respiration. *Australian Journal of Plant Physiology* 26:773–780

Hietz P, Wanek W, Wania R, Nadkarni NM (2002) Nitrogen-15 natural abundance in a montane cloud forest as an indicator of nitrogen cycling and epiphyte nutrition. *Oecologia* 131: 350-355

Hirel B, Goius JL, Ney B & Gallais A (2007) The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches. *Journal of Experimental Botany* 58: 2369-2387

Hirel B, Bertin P, Quilleré I, Bourdoncle W, Attagnant C, Dellay C, Guoy A, Cadiou S, Retailliau C, Falque M & Gallais A (2001) Towards a better understanding of the genetic and physiological basis for nitrogen-use efficiency in maize. *Plant Physiology* 125: 1258-1270

Hobbie EA, Sanchez FS, Rygiewicz PT (2004) Carbon use, nitrogen use and isotopic fractionation of ectomycorrhizal and saprotrophic fungi in natural abundance and ^{13}C-labelled cultures. *Mycological Research* 108: 725-736

Högberg P (1997) ^{15}N natural abundance in soil-plant systems. *New Phytologist* 137: 179-203

Holmes RM, McClelland JW, Sigman DM, Fry B & Peterson BJ (1998) Measuring $^{15}N–NH_4^+$ in marine, estuarine and fresh waters: an adaptation of the ammonia diffusion method for samples with low ammonium concentrations. *Marine Chemistry* 60: 235–243

Houlton BZ, Sigman DM, Schuur EAG & LO Hedin (2007) A climate driven switch in plant nitrogen acquisition within tropical forest communities. *Proceedings of the National Academy of Sciences* 104: 8902-8906

Huang Y, Drengstig T, Ruoff P (2012) Integrating fluctuating nitrate uptake and assimilation to robust homeostasis. *Plant, Cell & Environment*, In press.

Hunter WJ (1985) Soyabean in vivo stem nitrate reductase activity. *Annals of Botany* 55: 759-761

Hymus GJ, Baker NR & Long SP (2001) Growth in elevated CO_2 can both increase and decrease photochemistry and photoinhibition of photosynthesis in a predictable manner. *Dactylis glomerata* grown in two levels of nitrogen nutrition. *Plant Physiology* 127: 1204-1211

Jansson S & Douglas CJ (2007) *Populus*: a model system for plant biology. *Annual Review of Plant Biology* 58: 435-458

Johnson CM, Stout PR, Broyer TC & Carlton AB (1957) Comparative chlorine requirements of different plant species. *Plant and Soil* 8: 337–353

Kahmen A, Wanek W & Buchmann N (2008) Foliar $\delta^{15}N$ values characterize soil N cycling and reflect nitrate or ammonium preference of plants along a temperate grassland gradient. *Oecologia* 156: 861-870

Kaiser WM & Huber SC (2001) Post-translational regulation of nitrate reductase: mechanism, physiological relevance and environmental triggers. *Journal of Experimental Botany* 52: 1981-1989

Karsh KL, Granger J, Kritee K, Sigman DM (2012) Eukaryotic assimilatory nitrate reductase fractionates N and O isotopes with a ratio near unity. *Environmental Science and Technology* 46: 5727-5735

Khan SN, Mulvaney RL, Mulvaney CS (1997) Accelerated diffusion methods for inorganic nitrogen analysis of soil extracts and water. *Soil Science Society of America Journal* 61: 936–947

Kolb KJ & Evans RD (2003) Influence of nitrogen source and concentration on nitrogen isotope discrimination in two barley genotypes (*Hordeum vulgare* L.). *Plant, Cell & Environment* 26: 1431-1440

Kolb KJ & Evans RD (2003) Implications of leaf nitrogen recycling on the nitrogen isotope composition of deciduous plant tissues. *New Phytologist* 156: 57-64

Kronzucker HJ, Britto DT, Davenport R & Tester M (2001) Ammonium toxicity and the real cost of transport. *Trends in Plant Science* 6: 335-337

Kronzucker HJ, Siddiqi MY & Glass ADM (1995) Compartmentation and flux characteristics of ammonium in spruce. *Planta* 196: 691-698

Kronzucker HJ, Siddiqi MY & Glass ADM (1997) Conifer root discrimination against soil nitrate and the ecology of forest succession. *Nature* 385: 59-61

Laugier E, Bouguyon E, Mauriès A, Tillard P, Gojon A & Lejay L (2012) Regulation of high-affinity nitrate uptake in roots of Arabidopsis depends predominantly on posttranscriptional control of the NRT2. 1/NAR2. 1 transport system. *Plant Physiology* 158: 1067-1078

Lea PJ & Azevedo RA (2006) Nitrogen use efficiency: 1. Uptake of nitrogen from the soil. *Annals of Applied Botany* 149: 243-247

LeBauer DS & Treseder KK (2008) Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. *Ecology* 89: 371-379

Ledgard SF, Woo KC & Bergersen FJ (1985) Isotopic fractionation during reduction of nitrate and nitrite by extracts of spinach leaves. *Australian Journal of Plant Physiology* 12: 631–640

Lee RB & Clarkson DT (1986) Nitrogen-13 studies of nitrate fluxes in barley roots I. Compartmental analysis from measurements of ^{13}N efflux. *Journal of Experimental Botany* 37: 1753-1767

Li W, Wang Y, Okamoto M, Crawford NM, Siddiqi MY & Glass ADM (2007) Dissection of the AtNRT2.1:AtNRT2.2 inducible high-affinity nitrate transporter gene cluster. *Plant Physiology* 143: 425-433

Lin S, Kuo H, Canivenc G, Lin C, Lepetit M, Hsu P, Tillard P, Lin H, Wang Y, Tsai C, Gojon A & Tsay Y (2008) Mutation of the Arabidopsis NRT1.5 nitrate transporter causes defective root-to-shoot nitrate transport. *Plant Cell* 20: 2514–2528

Loqué D & von Wiren N (2004) Regulatory levels for the transport of ammonium in plant roots. *Journal of Experimental Botany* 55: 1293-1305

Macklon, AES, Ron MM & Sim A (1990) Cortical cell fluxes of ammonium and nitrate in excised root segments of *Allium cepa* L; Studies using ^{15}N. *Journal of Experimental Botany* 41: 359-370

Macko SA, Fogel ML, Hare PE & Hoering TC (1987) Isotopic fractionation of nitrogen and carbon in the synthesis of amino acids by microorganisms. *Chemical Geology* 65: 79-92

Malaguti D, Millard P, Wendler R, Hepburn A & Tagliavini M (2001) Translocation of amino acids in the xylem of apple (*Malus domestica* Borkh.) trees in spring as a consequence of both N remobilization and root uptake. *Journal of Experimental Botany* 52: 1665-1671

Mariotti A, Germon JC, Hubert P, Kaiser P, Letolle R, Tardieux A & Tardieux P (1981) Experimental determination of nitrogen kinetic isotope fractionation: some principles; illustration for the denitrification and nitrification processes. *Plant and Soil* 62: 413-430

Mariotti A, Mariotti F, Champigny ML, Amarger N & Moyse A (1982) Nitrogen isotope fractionation associated with nitrate reductase activity and uptake of NO_3^- by pearl millet. *Plant Physiology* 69: 880-884

Mariotti A (1983) Atmospheric nitrogen is a reliable standard for natural ^{15}N abundance measurements. *Nature* 303: 685-687

Marmann P, Wendler R, Millard P, Heilmeier H (1997) Nitrogen storage and remobilization in ash (*Fraxinus excelsior*) under field and laboratory conditions. *Trees: Structure and Function* 11: 298-305

Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gaufichon L & Suzuki A (2010) Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. *Annals of Botany* 105: 1141-1157

McFarland JW, Ruess RW, Kielland K & Doyle AP (2002) Cycling dynamics of NH_4^+ and amino acid nitrogen in soils of a deciduous boreal forest ecosystem. *Ecosystems* 5: 775-788

McKee KL, Feller IC, Popp M & Wanek W (2002) Mangrove isotopic $\delta^{15}N$ and $\delta^{13}C$ fractionation across a nitrogen versus phosphorus limitation gradient. *Ecology* 83: 1065-1075

Millard P & Grelet GA (2010) Nitrogen storage and remobilization by trees: ecophysiological relevance in a changing world. *Tree Physiology* 30: 1083-1095

Millard P & Thomson CM (1989) The effect of the autumn senescence of leaves on the internal cycling of nitrogen for the spring growth of apple trees. *Journal of Experimental Botany* 40: 1285-1289

Miller AJ & Smith S (2008) Cytosolic ion homeostasis: could it have a role in sensing nitrogen status? *Annals of Botany* 101: 485-489

Miller AJ & Zhen RG (1991) Measurement of intracellular nitrate concentrations in *Chara* using nitrate-selective microelectrodes. *Planta* 184: 47-52

Miller AE & WD Bowman (2002) Variation in nitrogen-15 natural abundance and nitrogen uptake traits among co-occurring alpine species: do species partition by nitrogen form? *Oecologia* 130: 609-616

Miller AJ & MD Cramer (2005) Root nitrogen acquisition and assimilation. *Plant and Soil* 274: 1-36

Min X, Siddiqi MY, Guy RD, Glass ADM & Kronzucker HJ (2002) A comparative study of fluxes and compartmentation of nitrate and ammonium in early-successional tree species. *Plant, Cell & Environment* 22: 821-830

Moll RH, Kamprath EJ & Jackson WA (1982) Analysis and interpretation of factors which contribute to efficiency of nitrogen-utilization. *Agronomy Journal* 74: 562–564.

Morgan MA, Volk RJ & Jackson WA (1973) Simultaneous influx and efflux of nitrate during uptake by perennial ryegrass. *Plant Physiology* 51: 267-272

Näsholm T, Ekblad A, Nordin A, Giesler R, Högberg M & Högberg P (1998) Boreal forest plants take up organic nitrogen. *Nature* 392: 914-916

Nazoa P, Vidmar JJ, Tranbarger TJ, Mouline K, Damiani I, Tillard P, Zhuo D, Glass ADM & Touraine B (2003) Regulation of the nitrate transporter gene AtNRT2. 1 in *Arabidopsis thaliana*: responses to nitrate, amino acids and developmental stage. *Plant Molecular Biology* 52: 689-703

Needoba JA, Sigman DM & Harrison PJ (2004) The mechanism of isotope fractionation during algal nitrate assimilation as illuminated by the $^{15}N/^{14}N$ of intracellular nitrate. *Journal of Phycology* 40: 517-522

Nordin A, Höberg P & Näsholm T (2001) Soil nitrogen form and plant nitrogen uptake along a boreal forest productivity gradient. *Oecologia* 129: 125-132

Okamoto M, Vidmar JJ & Glass ADM (2003) Regulation of NRT1 and NRT2 gene families of *Arabidopsis thaliana*: responses to nitrate provision. *Plant and Cell Physiology* 44: 304-317

O'Leary MH (1981) Carbon isotope fractionation in plants. *Phytochemistry* 20: 553-567

Orsel M, Filleur S, Fraiser V & Daniel-Vedelle F (2002) Nitrate transport in plants: which genes and which control? *Journal of Experimental Botany* 53: 825-833

Ouborg NJ & WJ Vriezen (2006) An ecologist's guide to ecogenomics. *Journal of Ecology* 95: 8-16

Paungfoo-Lonhienne C, Lonhienne TGA, Rentsch D, Robinson N, Christie M, Webb RI, Gamage HK, Carroll BJ, Schenk PM & Schmidt S (2008) Plants can use protein as a nitrogen source without assistance from other organisms. *Proceedings of the National Academy of Sciences* 105: 4524-4529

Peterson BJ & Fry B (1987) Stable isotopes in ecosystem studies. *Annual Review of Ecology and Systematics* 18: 293-320

Peterson EB & Peterson NM (1994) Ecology, management, and use of aspen and balsam poplar in the prairie provinces, Canada. Forestry Canada, Northwest Region, Northern Forest Centre, Edmonton, Alberta. *Special Report 1*.

Peuke AD, Gessler A & Rennenberg H (2006) The effect of drought on C and N stable isotopes in different fractions of leaves, stems and roots of sensitive and tolerant beech ecotypes. *Plant, Cell & Environment* 29: 823-835

Plett D, Toubia J, Garnett T, Tester M, Kaiser BN & Baumann U (2010) Dichotomy in the NRT gene families of dicots and grass species. *PloS one* 5: e15289

Pritchard ES & Guy RD (2005) Nitrogen isotope discrimination in white spruce fed with low concentrations of ammonium and nitrate. *Trees: Structure and Function* 19: 89-98

Raun WR & Johnson GV (1999) Improving nitrogen use efficiency for cereal production. *Agronomy Journal* 91: 357-363

Raven JA & Smith FA (1976) Nitrogen assimilation and transport in vascular land plants in relation to intercellular pH regulation. *New Phytologist* 76: 415-431

Rawat SR, Silim SN, Kronzucker HJ, Siddiqi MY & Glass ADM (1999) AtAMT1 gene expression and NH_4^+ uptake in roots of *Arabidopsis thaliana*: evidence for regulation by root glutamine levels. *Plant Journal* 19: 143-152

Redinbaugh MG & Campbell WH (1991) Higher plant responses to environmental nitrate. *Physiologia Plantarum* 82: 640-650

Reich PB, Hobbie SE, Lee T, Ellsworth DS, West JB, Tilman D, Knops JMH, Naeem S & Trost J (2006) Nitrogen limitation constrains sustainability of ecosystem response to CO_2. *Nature* 440: 922-925

Ricker WE (1984) Computation and uses of central trend lines. *Canadian Journal of Zoology* 62: 1897–1905

Robinson D (2001) $\delta^{15}N$ as an integrator of the nitrogen cycle. *Trends in Ecology and Evolution* 16: 153-162

Robinson D, Handley LL & Scrimgeour CM (1998) A theory for $^{15}N/^{14}N$ fractionation in nitrate-grown vascular plants. *Planta* 205: 397-406

Robinson D, Handley LL, Scrimgeour CM, Gordon DC, Forster BP & Ellis RP (2000) Using stable isotope natural abundances ($\delta^{15}N$ and $\delta^{13}C$) to integrate the stress responses of wild barley (*Hordeum spontaneum* C. Koch.) genotypes. *Journal of Experimental Botany* 51: 41-50

Rossato L, Laine P & Ourry A (2001) Nitrogen storage and remobilization in *Brassica napus* L. during the growth cycle: nitrogen fluxes within the plant and changes in soluble protein patterns. *Journal of Experimental Botany* 52: 1655-1663

Sage RF, Sharkey TD & Seemann JR (1989) Acclimation of photosynthesis to elevated CO_2 in five C_3 species. *Plant Physiology* 89: 590-596

Sannigrahi P, Ragauskas AJ & Tuskan GA (2010) Poplar as a feedstock for biofuels: A review of compositional characteristics. *Biofuels, Bioproducts and Biorefining* 4: 209-226

Schenk MK (1996) Regulation of nitrogen uptake on the whole plant level. *Plant and Soil* 181: 131-137

Scheurwater I, Clarkson DT, Purves JV, Van Rijt G, Saker LR, Welschen R & Lambers H (1999) Relatively large nitrate efflux can account for the high specific respiratory costs for nitrate transport in slow-growing grass species. *Plant and Soil* 215: 123-134

Schjoerring JK & Mattson M (2001) Quantification of ammonia exchange between agricultural cropland and the atmosphere: Measurements over two complete growth cycles of oilseed rape, wheat, barley and pea. *Plant and Soil* 228: 105-115

Schjoerring JK, Husted S, Mack G & Mattson M (2002) The regulation of ammonium translocation in plants. *Journal of Experimental Botany* 53: 883-890

Selle A, Willmann M, Grunze N, Gessler A, Weih M & Nehls U (2005) The high-affinity poplar ammonium importer PttAMT1. 2 and its role in ectomycorrhizal symbiosis. *New Phytologist* 168: 697-706

Siddiqi MY, Glass ADM & Ruth TJ (1991) Studies of the uptake of nitrate in barley III. Compartmentation of NO_3^-. *Journal of Experimental Botany* 42: 1455-1463

Smith BN & Epstein S (1971) Two categories of $^{13}C/^{12}C$ ratios for higher plants. *Plant Physiology* 47: 380-384

Solorzano L (1969) Determination of ammonia in natural waters by the phenol-hypochlorite method. *Limnology and Oceanography* 14: 799-801

Soolanayakanahally RY, Guy RD, Silim, SN, Drewes EC & Schroeder WR (2009) Enhanced assimilation rate and water use efficiency with latitude through increased photosynthetic capacity and internal conductance in balsam poplar (*Populus balsamifera* L.). *Plant, Cell & Environment* 32: 1821-1832

Soolanayakanahally RY (2010) Latitudinal Gradients in Adaptive Traits in *Populus*. Ph.D. Dissertation. University of British Columbia. Vancouver, BC, Canada. 144 pages

Stanton BJ, Neale DB & Li S (2010) *Populus* breeding: from the classical to the genomic approach. In: Jansson S, Rishikesh P, Bhalerao RP & Groover AT, eds. Genetics and Genomics of *Populus*. Springer, New York. pp 309–348

Steingröver E, Ratering P & Siesling J (2006) Daily changes in uptake, reduction and storage of nitrate in spinach grown at low light intensity. *Physiologia Plantarum* 66: 550-556

Stitt M & Feil R (1999) Lateral root frequency decreases when nitrate accumulates in tobacco transformants with low nitrate reductase activity: consequences for the regulation of biomass partitioning between shoots and root. *Plant and Soil* 215: 143-153

Stitt M & Krapp A (1999) The interaction between elevated carbon dioxide and nitrogen nutrition: The physiological and molecular background. *Plant, Cell and Environment* 22: 583-621

Tcherkez G (2011) Natural $^{15}N/^{14}N$ isotope composition in C_3 leaves: are enzymatic isotope effects informative for predicting the ^{15}N-abundance in key metabolites? *Functional Plant Biology* 38: 1-12

Tcherkez G, Farquhar GD (2006) Isotopic fractionation by plant nitrate reductase, twenty years later. *Functional Plant Biology* 33: 531-537

Tschoep H, Gibon Y, Carillo P, Armengaud P, Szecowka M, Nunes-Neni A, Fernie AR, Koehl K & Stitt M (2009) Adjustment of growth and central metabolism to a mild but sustained nitrogen-limitation in Arabidopsis. *Plant, Cell and Environment* 32: 300-318

Tuskan GA, DiFazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U, Putnam N, Ralph S, Rombauts S, Salamov A et al (2006) The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science* 313: 1596–1604

Vitousek PM, Aber JD, Howarth RW, Likens GE, Matson PA, Schindler DW, Schlensinger WH & Tilman DG (1997) Human alteration of the global nitrogen cycle: sources and consequences. *Ecological Applications* 7: 737-750

Wallsgrove RM, Keys AJ, Lea PJ & Miflin BJ (1983) Photosynthesis, photorespiration and nitrogen metabolism. *Plant, Cell & Environment* 6: 301-309

Wang C, Wan S, Xing X, Zhang L & Han X (2006) Temperature and soil moisture interactively affected soil net N mineralization in temperate grassland in northern China. *Soil Biology and Biochemistry* 38: 1101-1110

Weigelt A, Bol R & Bardgett RD (2005) Preferential uptake of soil nitrogen forms by grassland plant species. *Oecologia* 142: 627-635

Werner RA & Schmidt HL (2002) The *in vivo* nitrogen isotope discrimination among organic plant compounds. *Phytochemistry* 61: 465-484

Wilkinson JQ & Crawford NM (1993) Identification and characterization of a chlorate-resistant mutant of *Arabidopsis thaliana* with mutations in both nitrate reductase structural genes NIA1 and NIA2. *Molecular Genomes and Genetics* 239: 289-297

Williams L & Miller AJ (2001) Transporters responsible for the uptake and partitioning of nitrogenous solutes. *Annual Review of Plant Physiology and Plant Molecular Biology* 52: 659-688

Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV & Provart NJ (2007) An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. *PLOS One* 8: 718-726

Wirth J, Chopin F, Santoni V, Viennois G, Tillard P, Krapp A, Lejay L, Daniel-Vedele F & Gojon A (2007) Regulation of root nitrate uptake at the NRT2. 1 protein level in *Arabidopsis thaliana*. *Journal of Biological Chemistry* 282: 23541-23552

Xu G, Fan X & Miller AJ (2012) Plant nitrogen assimilation and use efficiency. *Annual Review of Plant Biology* 63: 153-182

Yemshanov D & McKenney D (2008) Fast-growing poplar plantations as a bioenergy supply source for Canada. *Biomass and Bioenergy* 32: 185-197

Yoneyama T, Kamachi K, Yamaya T & Mae T (1993) Fractionation of nitrogen isotopes by glutamine synthetase isolated from spinach leaves. *Plant Cell Physiology* 34: 489-491

Yoneyama T, Matsumaru T, Usui K & Engelaar WMHG (2001) Discrimination of nitrogen isotopes during absorption of ammonium and nitrate at different nitrogen concentrations by rice (*Oryza sativa* L.) plants. *Plant, Cell & Environment* 24: 133-139

Yoneyama T, Kamachi K, Yamaya T & Mae T (1993) Fractionation of nitrogen isotopes by glutamine synthetase isolated from spinach leaves. *Plant Cell Physiology* 34: 489-491

Yousfi S, Serret MD & Araus JL (2009) Shoot $\delta^{15}N$ gives a better indication than ion concentration or $\delta^{13}C$ of genotypic differences in the response of durum wheat to salinity. *Functional Plant Biology* 36: 144-155

Yousfi S, Serret MD, Márquez AJ, Voltas J & Araus JL (2012) Combined use of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ tracks nitrogen metabolism and genotypic adaptation of durum wheat to salinity and water deficit. *New Phytologist* 194: 230-244

Yousfi S, Serret MD & Araus JL (2013) Comparative response of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ in durum wheat exposed to salinity at the vegetative and reproductive stages. *Plant, Cell & Environment*, In Press

Yuan JS, Tiller KH, Al-Ahmad H, Stewart NR & Stewart CN (2008) Plants to power: bioenergy to fuel the future. *Trends in Plant Science* 13: 421-429

Appendix A – Supplemental measurements

Table A. 1 Mean temperature and pH ±SD (N=14) in hydroponics solutions used in Chapter 3.

Experiment	Nitrate Concentration	Temperature	pH
1	100	20.7±0.30	6.51±0.116
	1000	20.7±0.22	6.50±0.127
2	100	20.6±0.19	6.50±0.097
	1000	20.7±0.26	6.52±0.090

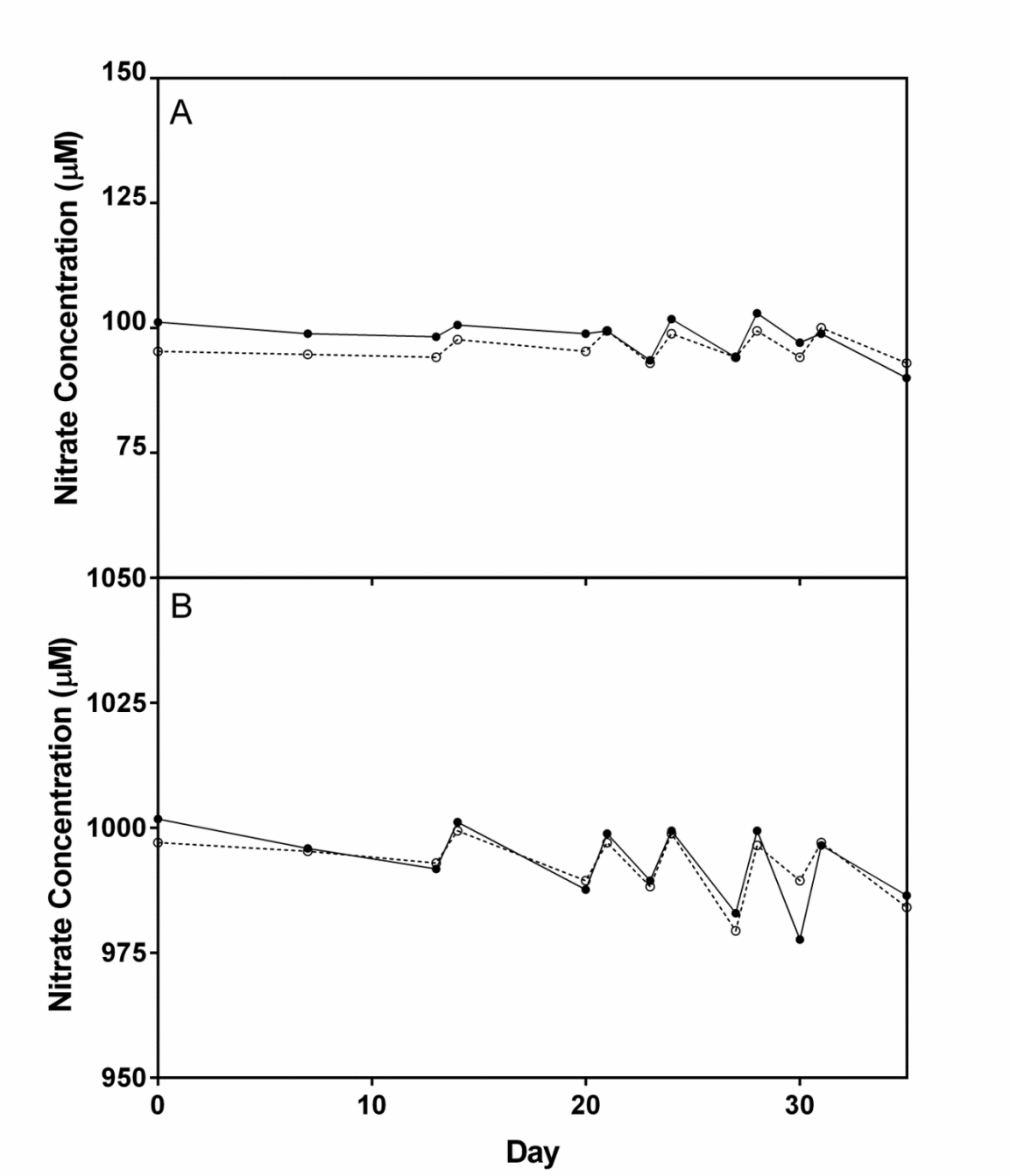

Figure A. 1 Nitrate concentrations (μM) in hydroponics solutions during growth of *Arabidopsis thaliana* for 35 days (Chapter 3) determined using the perchloric acid method. A, 100 μM NO_3^- treatment. B, 1000 μM NO_3^- treatment.

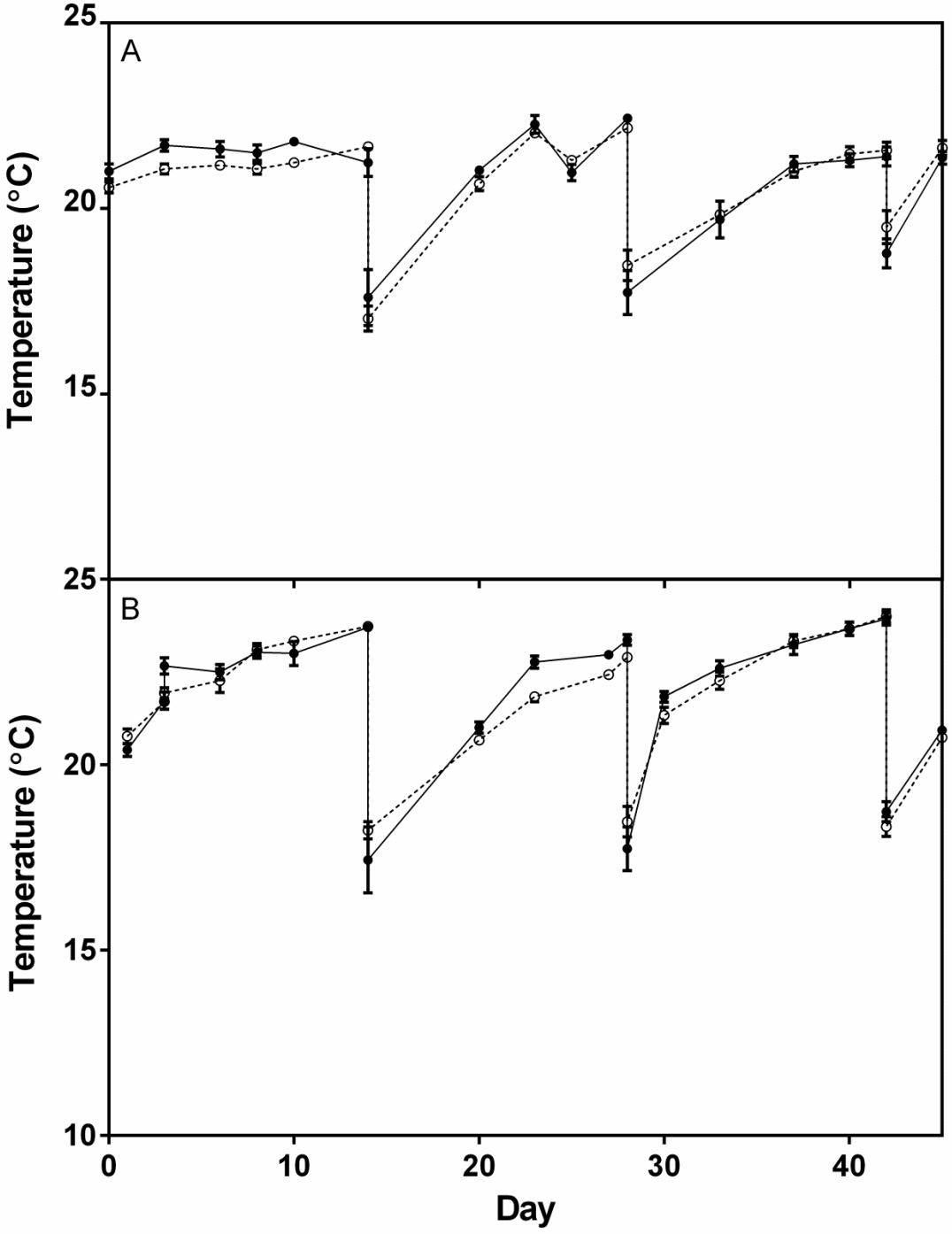

Figure A. 2 Mean solution temperatures for nitrate (solid circles) and ammonium (clear circles) substrates (N=3) over 45 days in experiment 1 (A) and experiment 2 (B) from Chapters 4, 5 and 6.

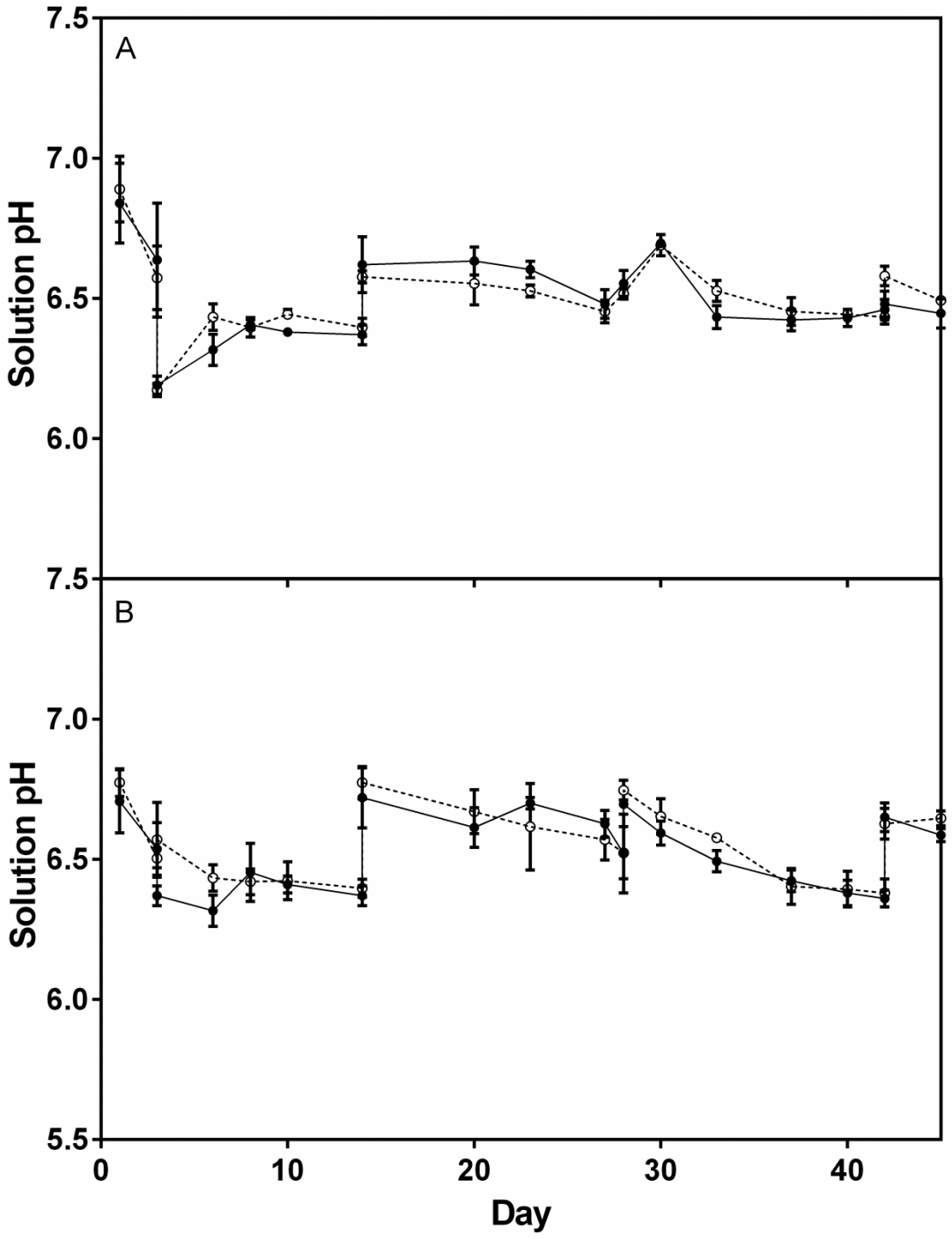

Figure A. 3 Mean solution pH for nitrate (solid circles) and ammonium (clear circles) substrates (N=3) over 45 days in experiment 1 (A) and experiment 2 (B) from Chapters 4, 5 and 6.

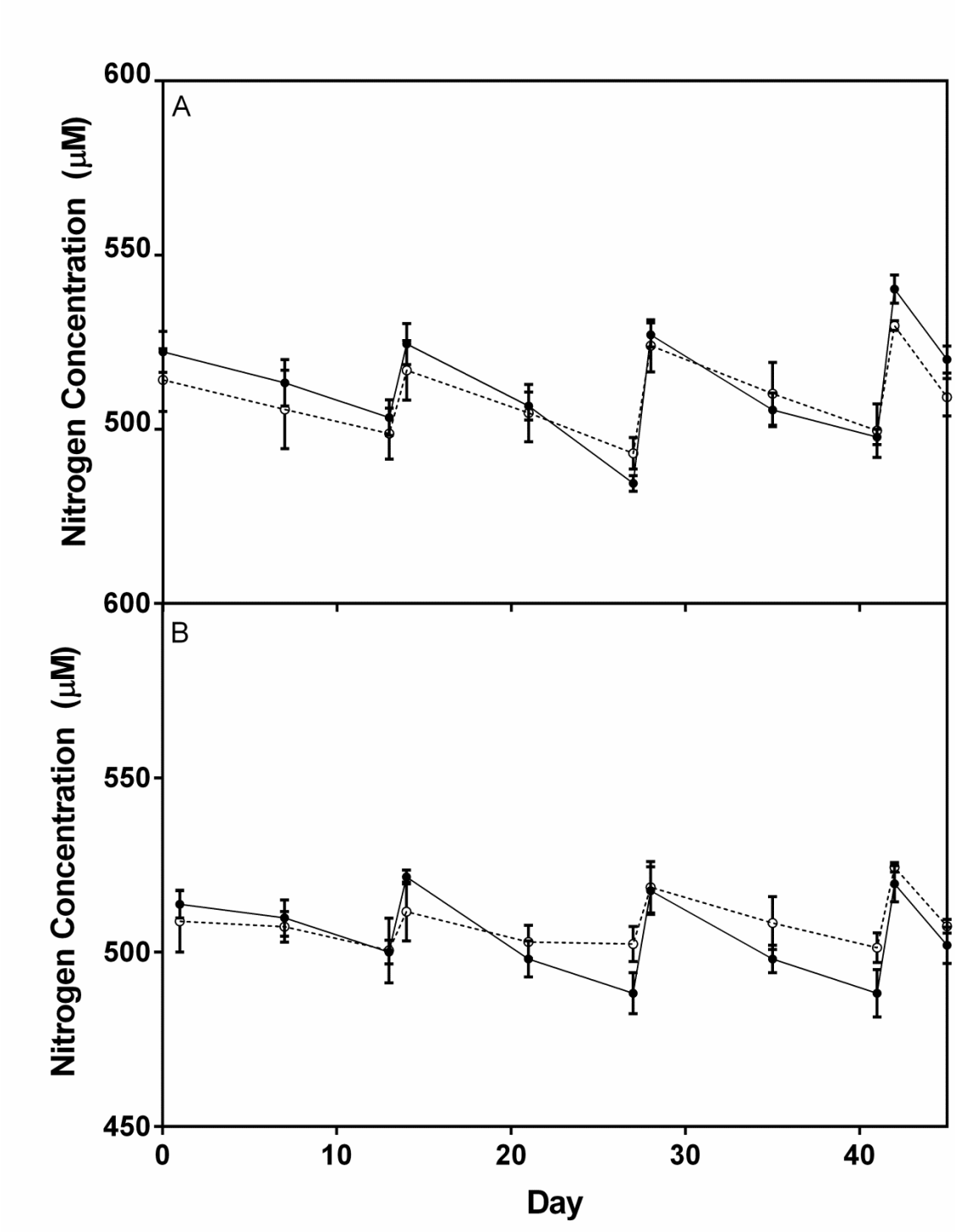

Figure A. 4 Mean nitrate (solid circles) and ammonium (clear circles) concentrations (N=3) over 45 days in experiment 1 (A) and experiment 2 (B) from Chapters 4, 5 and 6.