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IMPROVING NITROGEN MANAGEMENT IN AGRICULTURE:

OPPORTUNITIES GLEANED FROM ORGANIC SYSTEMS

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ABSTRACT

Achieving sustainable intensification goals for agriculture hinges on two essential and seemingly contradictory improvements in nitrogen (N) management: reducing N losses while increasing crop productivity. Conventional agricultural systems have attained prolific productivity, built upon tremendous inputs of inorganic N fertilizer. But, these production increases have largely stalled, and much of the N fertilizer used to achieve these yields escapes, polluting air and water. Organic agriculture takes a different approach to N management and productivity. Built on the philosophical foundation of systemic design, organic agriculture aims to use biological processes to provide N and other services necessary to support crop productivity. This organic approach results in systems with a fundamentally different structure than their conventional counterparts. In organic systems, these contrasting structural elements include: perennials, rotation diversity/complexity, legumes, and manure; which we hypothesized impart improvements in N retention and/or provisioning. Goals of this research were to: (1) determine whether annual or perennial forage systems better mitigated potential greenhouse gas (GHG) losses related to grazing and extreme rainfall events, (2) assess efficacy of alternative organic N management strategies in providing inorganic N and supporting forage yields, (3) evaluate the availability of organic N in soils of organic systems and to the crop species grown in them. I relied on a 2012-2014 organic forage systems experiment to characterize effects of N management practices employed in these systems in terms of the N they provided in the soil during the growing season (soil NH_4^+, NO_3^-, amino acids and other free primary amines), as well as the N that escaped (N_2O losses, NH_3 volatization, NO_3^- leaching).

While N management in both conventional and organic agriculture aims to provide N in

inorganic forms, emerging evidence suggests organic forms of N may also be plant-available,

however quantifying the contribution of N compounds, especially organic ones, to plant N

nutrition is methodologically challenging. I used a large greenhouse experiment to indirectly test

the ability of 3 N environments to support growth of four forage crop species, and then directly

test the capacity of plants from each of these 12 growth phase treatments to take up labeled

forms of inorganic N and amino acids using 2 alternative tracer methodologies. I found perennial

organic forage systems exhibit multiple N management benefits over annual systems: lower N_2O

fluxes, lower soil NO_3^- leaching, and greater inorganic N use efficiency, although annual systems

exhibited higher absolute yields. Among annual organic systems, those that relied on a legume

N provisioning strategy, rather than a manure N provisioning strategy, displayed superior

inorganic and organic N availability and yields. All forage species demonstrated amino acid N

uptake abilities that increased when the plants were grown in the presence of amino acids. Thus,

the greater use of perennial and legume crops may increase amino acid N availability and

facilitate direct crop plant utilization of this previously unrecognized soil N resource, reducing N

losses while maintaining or increasing crop productivity.

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Chapter 1
Introduction

N management in agriculture has radically altered global N cycling (Galloway et al. 2002, Erisman et al. 2013). The largest agricultural N changes stem from decoupling animal and crop production, which led to stockpiled manures losing two thirds of their N and exponentially increasing inorganic N use for crop production (Mosier et al. 2002). Locally, increases in reactive, inorganic N in the troposphere and subsequent ozone production cause acute adverse human and plant health effects. Regionally, inorganic N losses change ecosystem structure, degrade ecosystem functioning, and cause nitrate levels to exceed EPA human health limits in 57% of the nation's groundwater (EPA 1990). Globally, agriculture leads climate changing N_2O production, a gas with 310 times the global warming potential (GWP) of CO_2 (Robertson and Vitousek 2009). Continued agricultural productivity relies on N, but less than 50% of the N applied to agricultural systems currently supports agricultural productivity and the majority escapes (Robertson and Vitousek 2009), inflicting the multitude of costs. Galloway and Cowling (2002) estimated human N needs only equate to 10% of reactive, inorganic N produced. To reduce costs of N losses, improving N management is essential.

Organic systems, a rapidly expanding agricultural sector, display fundamentally divergent ecosystem structures from conventional agriculture systems, largely related to their different approach to N management (Figure 1-1). Rather than relying on external inputs of inorganic N, organic systems largely rely on internal cycling on N from organic sources to provision crops with N. Ecosystem structure drives ecosystem function, especially in terms of N retention and loss, and more diversified agricultural systems, such as those under organic

management, accumulate N(Drinkwater et al. 1998, Galloway et al. 2003). Therefore, I

hypothesized these structural elements characteristic of organic agroecosystems support

improvements in N functioning. To address this, we sought to identify underlying elements of N

management in organic systems and assess whether they might represent opportunities for N

management improvement across agriculture.

Elements of organic agroecosystems hypothesized to confer N management

improvements include: grazing-based livestock production, use of manure and/or legumes for N

provisioning, and facilitating direct plant uptake of organic forms of N. Positive metrics of N

management performance include soil AA-N, NO_3^--N and NH_4^+-N availability; as well as

satisfactory yields. Negative metrics of N management shortcomings include N_2O fluxes, NO_3^-

leaching, NH_3 volatization, and low yields.

There is a dearth of research documenting the function of organic systems, especially

grazing-based systems in the Northeast region. So, a primary objective of this work was to

better characterize the environmental performance of organic forage systems exposed to

grazing. As these systems expand in this region, there has also been an increase in the frequency

and intensity of rain events (Spierre and Wake 2010). Therefore, a secondary objective was to

assess system function in terms of environmental performance in the face of extreme rain

events.

Re-evaluating N availability in organic forage systems was another major objective. Yield

gaps in organic systems are often attributed to low inorganic N availability, therefore we sought

to first assess the sufficiency of inorganic N provisioning across two alternative organic N

provisioning strategies: legume-based and manure-based. However, increasing evidence, largely

from natural ecosystems, indicates plants are not only able to take up inorganic N, but can also take up organic forms of N directly (Nasholm et al. 2009). This research led us to hypothesize that crop plants in organic forage systems take up organic N directly, and that direct crop plant utilization of organic N in organic systems represents a strategic opportunity to improve N management across agriculture.

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FIGURES
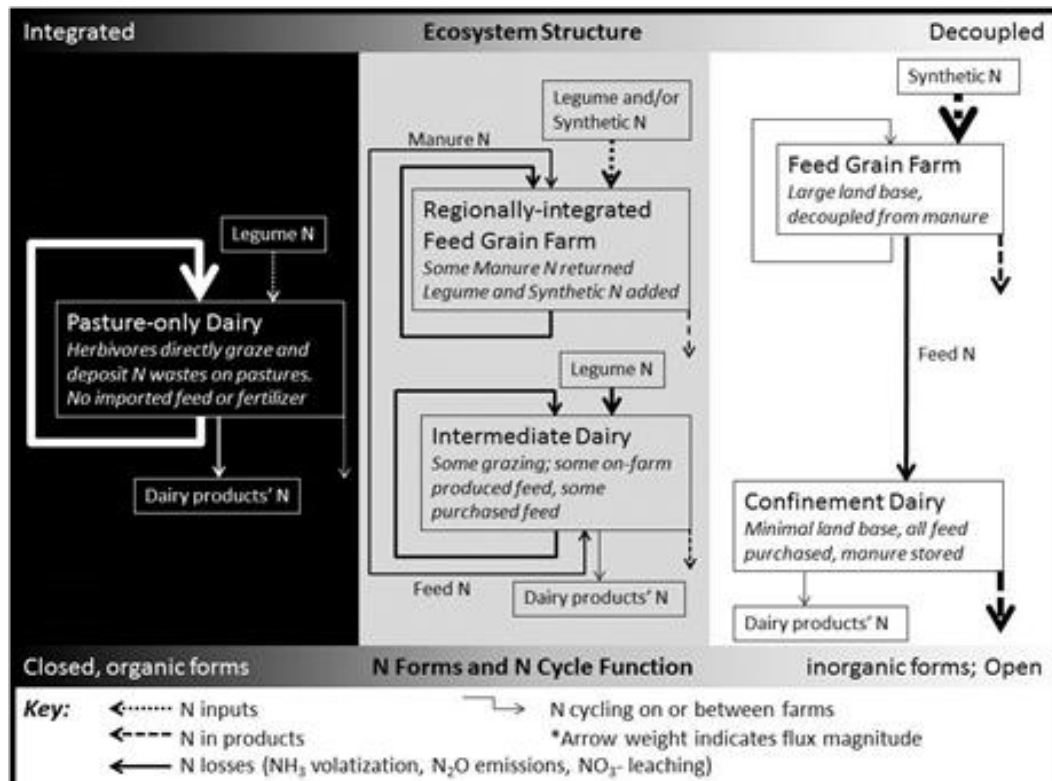
Figure 1-1. *Gradient in agroecosystem structure and hypothesized function.* Ecosystem structure creates a gradient in organic N use and cycling, impacting N form, retention and loss. The ecosystems represent a gradient from completely decoupled (right); to integrated N pools, but with spatial and temporal separation (center); to an integrated agroecosystem where N pools are adjacent and coupled with carbon pools (left)

Chapter 2
Enhanced environmental performance from perennials over annuals in organic forage systems exposed to extreme rain and grazing

ABSTRACT

Expanding grazed, organic crop-livestock systems can provide many environmental benefits, yet no research has measured their greenhouse gas (GHG) emissions in the wake of increasingly common extreme (>25.3 mm 24 hrs^{-1}) rain events in the Northeast. We aimed to fill this critical gap by quantifying environmental performance of organic annual and perennial systems exposed to field-applied grazing and extreme rain treatments three times per year in two years. In addition to monitoring soil-atmosphere nitrous oxide (N_2O), carbon dioxide (CO_2), and ammonia (NH_3) fluxes in conjunction with the graze-rain treatments; we also continued monitoring each system's environmental performance through the following winter and spring, relying on over-winter nitrate (NO_3^-) leaching, and spring NH_3 and GHG fluxes as key environmental performance metrics. We found no significant differences in GHG fluxes between the annual and perennial forages in response to the grazing and rain treatments. However, the perennial exhibited significantly lower post-graze treatment NH_3 volatization in the summer. Overall, the perennial also had significantly lower N_2O-N fluxes than the annual, driven differences in the spring, when annual N_2O-N fluxes exceeded perennial N_2O-N fluxes by an order of magnitude. The perennial also lost less than half as much soil NO_3^- to leaching between October and April compared to the annual. Both annual and perennial organic forages perform well in terms of GHG fluxes in response to graze-rain treatments, but perennial organic forages can provide superior environmental performance in terms of: 1) reduced post-graze summer NH_3 loss, 2) lower cumulative N_2O fluxes, and 3) less over-winter NO_3^- leaching.

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INTRODUCTION

Codified inclusion of diversified crop rotations, cover cropping, and progressive pasture management, as well as generous price premiums make organic agriculture well-poised to provide the environmental and economic services society needs from its future farming systems (Rotz et al. 2009). However, organic production is often criticized for its yield lag (Seufert et al. 2012). Grazed summer annual pastures have been promoted as a means of quickly increasing graze-able organic summer pastures to better meet society's growing demand for organic dairy products by two mechanisms. First, by increasing production relative to perennial pastures in their pasture phase, and, second, by creating rotation space for other high productivity annuals such as corn silage in the subsequent year. Further, by incorporating a C_4 species, summer annual pastures may better tolerate summer weather extremes expected to intensify under future climate scenarios, thus providing potentially superior environmental performance relative to C_3 perennial pastures in the summer (Spierre and Wake 2010).

Despite these proposed benefits of annuals, a shift away from perennials may introduce other challenges. From an agronomic perspective, the yield gap between organic and conventional production is smaller (and not significant) for perennial crops (-5%) compared to annual crops (-25%, Seufert et al. 2012). From an environmental perspective, the winter fallow, spring tillage, and spring fertility inputs in annual systems necessary for establishment of the subsequent silage crop phase could mean that replacing perennial pastures with an annual pasture-crop rotation sacrifices some of environmental services that motivate adoption of organic systems in the first place. Furthermore, grazing itself creates highly concentrated nutrient patches, known hotspots for N_2O fluxes, particularly if exposed to these increasingly-frequent, mid-summer-through-fall, extreme rain events (Allen et al. 1996).

Organic dairy farming contributes to all of the recently articulated goals for future

farming systems: minimize negative environmental impacts, especially GHG emissions; achieve

productivity sufficient to feed and fuel the growing human population; and maximize farmer

incomes (Foley et al. 2011, GHG Working Group 2010). Price premiums of up to 76%[1] or

conventional prices in 2011 and much lower price volatility over the last decade provide strong

economic incentive for dairy farmers to transition to certified organic production, and create an

excellent opportunity for organic dairy production to maximize farmer incomes (Su et al. 2013).

Many practices known to minimize negative environmental impacts of agriculture including

cover cropping, diverse crop rotations, and improved pasture management are core

components of organic dairy systems guaranteed through a system of regulation, inspection and

certification. Unfortunately, the soil organic carbon accrual provided by many of these practices

that has been well-documented in past research does not provide conclusive evidence that

these systems offer net GHG emissions reductions (Li et al. 2005). Even small increases in N_2O

flux can easily offset gains in soil organic carbon due to N_2O's much greater global warming

potential (GWP, Li et al. 2005, Liu and Greaver 2009, Ball et al. 2014). Finally, existing data is

insufficient to support realistic estimates of emissions from these systems (GHG Working Group

2010). This gap is especially pronounced in the Northeast region, where organic dairy expansion

is particularly rapid and already represents a relatively larger fraction of total farmland (GHG

Working Group 2010, USDA-ERS 2013). By providing estimates of GHG emissions from multiple

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[1] 2011 average monthly price premium of CROPP Midwest farm-gate pay price (organic; CROPP)
compared to conventional farm-gate pay price (USDA-ERS) was 45%; monthly premium ranged from 29%-
76% primarily due to price volatility in the conventional market (Su et al. 2013)

points in organic pasture-crop rotations in Pennsylvania, this research contributes to filling this critical gap.

Organic agriculture is a rapidly expanding sector of conservation agriculture. Nationally, certified organic farmland increased rapidly between 1997 and 2011 (123,059 ha yr^{-1}, R^2=0.937, p<0.001, USDA-ERS 2013). With just under 2.2 million hectares of organic farmland in 2011, organic farmland still comprises <2% of U.S. farmland. However, higher land values have driven even higher rates of conversion in the Northeast, where up to 43% of cropland is certified organic in some states, and the portion of certified organic land exceeds the national average in all states (USDA-ERS 2013). Pennsylvania is a regional and national leader in numerous organic sectors, adding organic farmland at about 2000 ha yr^{-1} since 1997 (R^2=0.925, p<0.001, USDA-ERS 2013). PA is the top producer of organic livestock, ranking fifth nationally for organic dairy production (USDA-ERS 2013). It is therefore especially important to understand the environmental impacts of organic dairy systems in PA, as well as the broader category of integrated crop and livestock production systems that also utilize grazing, manure, and legumes. Integrated crop and livestock systems are also understudied in terms of GHG emissions, yet they are often cited for their potential to mitigate N_2O emissions by tightening N cycling in livestock production (Snyder et al. 2014, GHG Working Group 2010). By quantifying CO_2 and N_2O fluxes from certified organic integrated, pasture-crop production systems in Pennsylvania, this study contributes to filling GHG emissions quantification gaps for both understudied groups (GHG Working Group 2010).

While the management practices encompassed by these systems offer some avenues for minimizing the negative environmental impacts of agricultural production, climate plays an

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important role in determining the direction and magnitude of management practices'
environmental impact. Both climate and management impact factors such as soil inorganic
nitrogen concentrations and soil moisture are known to be key controls over GHG fluxes,
especially N_2O, from soil. N_2O fluxes, in general, are highly episodic, spiking when conditions
favor denitrification at water filled pore space (WFPS) >55%, and returning to near zero when
they don't (Mitchell et al. 2013). Denitrification, thought to be the dominant contributor to soil
to atmosphere N_2O flux in agricultural soils, increases when there is sufficient nitrate to fuel it,
and WFPS is high enough to support incomplete denitrification, but not so high as to suppress
gas diffusion out of the soil profile, typically at 60-70% WFPS (Ruser et al. 2006).

In the Northeast, climate and management have changed in the past few decades and is
predicted to continue changing (Spierre and Wake 2010). Climatically, there has been a
significant increase in both the number of extreme precipitation events and the amount of rain
that falls during those events (Spierre and Wake 2010). In Central Pennsylvania, eight of the top
ten rain events between 1941 and 2011 occurred after 1996, with an average of over 96 mm
rain day^{-1} falling during each event (PA State Climatologist 2012). Previous work in Central
Pennsylvania found that a single, 60-mm rain event can significantly increase total annual N_2O
flux relative years without such an event, even when the years have equivalent soil NO_3^- levels
(Adviento-Borbe et al. 2010). These rain events create the wet soil conditions necessary for high
N_2O flux from denitrification, thought to be the dominant process driving N_2O flux (Adviento-
Borbe et al. 2010, Mitchell et al. 2013). Further, N_2O fluxes account for ~40% agriculture's GHG
footprint and N_2O emissions from agriculture are expected to increase 30-60% by 2050 (Herzog

2009, Cavigelli and Parkin 2012). Therefore, understanding potential N_2O fluxes triggered by these rain events is critical to quantifying these systems' potential overall GHG footprint.

We examined possible environmental performance tradeoffs between organic perennial pasture and an organic annual pasture-corn silage rotation. Perennial pastures consisting of a C_3 grass and legume, such as orchardgrass-red clover (*Dactylis glomerata- Trifolium pratense*), typically provide excellent environmental benefits including low NO_3^- leaching and high rates of soil carbon accrual (Conant et al. 2001). They grow fastest in the early spring through early summer, go dormant in mid-summer, and then resume higher growth rates in the fall (Undersander et al. 2014). Environmental performance of annual pastures consisting of a C_4 grass and a legume understory, such as sorghum sudangrass-red clover (*Sorghum bicolor* ssp. *drumondii*), has not been studied; however, their phenology is opposite that of perennial C_3 pastures. Annual C_4 pastures cannot be planted until late spring or early summer when soil temperatures exceed 15.6 °C, and then grow most rapidly through the summer (Undersander et al. 2014). Because actively growing vegetation can mitigate 11-43% of grazing-related N losses, the contrast in growing season between these two vegetation types presents a possibility for divergent performance (Vellinga et al. 2001). Although there are many metrics that could be used to measure an agricultural system's environmental performance; we focused on GHG and NH_3 fluxes, as well as nitrate leaching. Furthermore, because denitrification-driven N_2O fluxes typically dominate the GHG footprint of agricultural soils, creating conditions that favor N_2O production via denitrification was essential to this experiment (Cavigelli and Parkin 2012, Robertson et al. 2000). To explore the performance of these systems under the conditions that would most favor denitrification-driven N_2O fluxes, we designed and imposed grazing followed

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by extreme rain treatments. We applied these treatments in the pasture phase of both vegetation treatments three times per year in two start years, and measured how each performed. We also continued monitoring the following spring in both the perennial pasture and in the establishment phase of the corn silage in the annual treatment. We assessed if (1) there was a vegetation effect, (2) a seasonal effect, (3) a start year effect, or any interaction effects on each of the environmental performance metrics we measured. We hypothesized phenological differences would drive better annual vegetation mitigation of N_2O fluxes in summer, but that the perennial would provide better environmental performance in terms of NO_3^- leaching and GHG fluxes from the late summer and fall through to the subsequent spring.

METHODS
Site Description

We conducted this experiment at The Pennsylvania State University Russell E. Larson Agricultural Research Center (RELARC) in Rock Springs, PA (40° 42'43" N, 77° 56'35" W, elevation 373 m) on land managed organically since 2003 and certified organic since 2006 (~10 years of organic management prior to initiation of this project). We repeated the experiment in 2 "starts", with each "start" implemented on adjacent fields. We initiated Start 1 (S1) in 2012 and followed it through a 2-year rotation ending in 2013. Start 2 (S2) began in 2013 and continued through the same rotation as S1, ending in 2014. About half of the soil in S1 and all of the soil in S2 is a well-drained, Hagerstown silt loam (0-3% slopes, USDA-NRCS 2015). The rest of S1 contains mostly Murrill channery silt loam, (0-3% slopes), with a small area of steeper (3-8% slopes) Hagerstown silt loam (USDA-NRCS 2015). A blocked design was used to capture any variability caused by these minor differences in soil. Soil characteristics including pH, %C, %N,

and %OM were similar across 3 blocks of S1 and all 4 blocks of S2, but block 4 in S1 did have ~50% higher soil carbon, N, and OM than all the other blocks (Table 2-1). Recent (1981-2010) mean annual air temperature and precipitation are 8.7 °C and 1084.2 mm, respectively, for this region (NOAA 2014).

Vegetation Treatments

We used a randomized complete block design to replicate the 6.1 m by 6.1 m plots of two-year vegetation rotation treatments in 4 blocks repeated in each start (S1 and S2, Figure 2-1). We applied grazing and extreme precipitation treatments in the pasture phase of two vegetation rotation treatments: C_3 perennial and C_4 annual. Both treatments were established without irrigation using certified organic seed from King's Agriseed, with the exception of the sorghum sudangrass and corn seed in the annual vegetation treatment, which was untreated (Ronks, PA). Seeding rates were based on standard regional recommendations (Hall 2015). The perennial treatment was a mix of C_3 orchardgrass (*Dactylis glomerata* (L.) 'Niva') and red clover (*Trifolium pratense* (L.) 'Renegade') for the duration of the experiment. We established both S1 and S2 perennial treatments on 22 March 2012 with seeding rates of 6.7 kg orchardgrass seed ha^{-1} and 5.6 kg red clover seed ha^{-1}. Therefore, we applied grazing and rainfall treatments to seeding-year perennial stands in S1, and established one-year-old, perennial stands in S2. During the grazing and rainfall treatments year, the annual vegetation treatment was C_4 sorghum sudangrass (*Sorghum bicolor* (L.) Moench var. 'AS 6402 UT') and red clover, which rotated to corn silage (*Zea mays* [L.] 'Master's Choice 4050') in year 2. We established the annual treatment with seeding rates of 16.8 kg ha^{-1} sorghum sudangrass and 5.6 kg seed ha^{-1} red clover on 08 June 2012 in S1 and on 16 May 2013 in S2. In second year of the annual treatment, we

planted corn silage (76 cm row spacing) on 30 May 2013 in S1 and on 06 June 2014 in S2.

Schrenker et al. (*in review)* report *a*dditional agronomic management details relevant to the

experiment.

Grazing and Extreme Rainfall Treatments

We used a split-plot/repeated-measures design to impose grazing and extreme rainfall

treatments within each of the larger vegetation treatment plots (Figure 2-1). Grazing treatments

consisted of clipping vegetation to <10 cm and applying 0.9 L manure to a 490.9 cm^2 soil area

contained within a 25 cm-diameter, 10 cm-deep polyvinyl chloride (PVC) collar (Figure 2-1). This

manure application rate is less than the rate for pure dung patches used in previous research

(1.2 kg per 20 cm-diameter area, Yamulki et al. 1998, Allen et al. 1996), but greater than the

rate used for pure urine patches (0.2 L per 20 cm-diameter area). We selected this intermediate

rate because the manure material we had access to for this experiment contained both dung

and urine. Because dung and urine patches are often co-located in pastures, our experimentally-

applied grazing treatments are likely a realistic representation of actual N-rich sites common in

grazed pastures (van Groenigen et al. 2005). All manure used in this experiment came from the

same conventionally-managed dairy farm adjacent to RELARC. We subsampled the manure

during grazing treatment application, and stored the subsampled manure at 4°C until

submission to Pennsylvania State University Agricultural Analytical Laboratories (University Park,

PA), where a standard manure analysis was performed on each sample including N, P, and K

content as well as physical characteristics such as % solids (Table 2-2). Calculated actual rates of

manure N application were relatively consistent across all 6 treatment events in this study,

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ranging from 603 to 868 kg N ha^{-1}, and the mean rate of 715 kg N ha^{-1} is similar to N rates

reported in previous grazing manure patch research (Table 2-2, Afzal and Adams 1992).

We applied extreme rainfall treatments (76.2 mm in 24 hours) ~24 h after grazing

treatments, which consisted of 3.75 L water applied within a 24 h period to the same collars

where manure had just been applied. Due to infiltration rates and collar volumes, we split the

applications of the extreme rainfall treatment between a 2 L application and a 1.75 L application

~12 h apart. The volume of our extreme rainfall treatment represents a typical volume for

recent extreme rainfall events in the region (PA State Climatologist 2014). The mean of the top

30 most extreme rainfall events occurring in State College, PA since 1894 is 89 mm, whereas the

mean of more recent top extreme events is 82 mm (n=14, 1988 to 2013, PA State Climatologist

2014). Among the most extreme events since 2000, the size of 24-hr events ranges from 71.8

mm to 128.3 mm (PA State Climatologist 2014).

Each graze-rain treatment event occurred in two collars per vegetation plot– one where

we sampled soil and the other where we monitored soil-to-atmosphere gas fluxes (Figure 2-1).

Growth and availability of forage dictated the exact timing of these paired graze-rain treatments,

but in both years, we applied graze-rain treatments three times: in summer, late summer, and

fall (exact dates reported in supplementary Table 2-S1). Since we could only apply grazing

treatments when both vegetation treatments were in their pasture phase, we applied graze-rain

treatments in S1 in 2012 and in S2 in 2013.

Baseline and control measurements

Before each graze-rain treatment event, we collected baseline soil and gas flux

measurements. Soil measurements included soil NO_3^-, NH_4^+, and gravimetric water content. Gas

fluxes included soil to atmosphere flux of NH_3, N_2O, and CO_2. In 2013, we added control collars to each plot in S2, which we monitored in conjunction with all baseline, treatment event, and follow-up measurements. To enable gas flux monitoring and maintain vegetation heights consistent with the rest of the plot, we clipped vegetation to 10 cm in the control collars, but made no other changes to soil or vegetation in the control collars.

Soil to Atmosphere Gas Fluxes

We measured all soil to atmosphere gas fluxes of NH_3, CO_2, and N_2O following the methods described in Adviento-Borbe et al. (2010) and Mitchell et al. (2013). We lined PVC collars with 0.2 mil PFTE tape (Cole-Parmer Item# EW-08277-12, Vernon Hills, IL) to prevent ammonia gas from adhering to the chamber's surface. We inserted the lined collars ~5 cm into the soil, but measured collar depths in conjunction with each monitoring event in 5 locations per collar to accurately calculate chamber volumes. To enclose the collars and create chambers, we used a vented PVC lid (25 cm diameter by 5 cm height) also lined with PFTE tape on the inside and covered with foil tape on the outside (Nashua 322 Multi-Purpose HVAC Foil Tape, product # 1207792, Home Depot, Atlanta, GA). Total chamber volume was approximately 0.0049 m^3. We monitored NH_3, N_2O, and CO_2 concentrations with an INNOVA 1412 Photoacoustic Field Gas Monitor (INNOVA Air Tech Instruments, Ballerup, Denmark, Adviento-Borbe et al. 2010, Iqbal et al. 2013).

Each monitoring event consisted of 10-15 gas concentration measurements taken every minute for 10-15 minutes and occurred between 0800 and 1400 h to best coincide with 24-h average rates (Adviento-Borbe et al. 2010). For all three gases, we used the SLOPE function in Microsoft Excel to calculate daily flux rates via linear regression from the 10-15 measured

concentrations (Venterea 2013). We then used t-tests to test each slope's significance (Zaiontz 2014). For all significant (p<0.05) slopes, we considered the daily flux rate to be equal to that slope, and for all non-significant (p>0.05) slopes we considered the daily rate to be zero.

We monitored gas fluxes in all 4 replicates of each start 5-7 times per graze-rain treatment event. We timed treatment event monitoring to capture with peak N_2O fluxes and included baseline (pre-treatment), <24 h post-graze treatment, <24 h post-rain treatment, as well as follow-up measurements. We timed follow-up measurements to capture peak, post-treatment, denitrification-driven N_2O fluxes. We expected peak N_2O fluxes would occur 3-10 days post-treatment and quickly disappear as soils dried below the >60-70% WFPS denitrification threshold identified by other workers (Mitchell et al. 2013, Adviento-Borbe et al. 2010, Ruser et al. 2006). Follow-up measurements continued until N_2O fluxes returned to baseline levels and occurred along the following approximate post-treatment timeline: ~3 days later, ~1 week later, ~2 weeks later, and ~1 month later (exact dates reported in supplementary Table 2-S1). In both Starts, spring monitoring in the second year of the rotation occurred ~biweekly and spanned a ~60 day period from late April through late June (exact dates reported in supplementary Table 2-S2).

We calculated cumulative 30-d fluxes by linear interpolation and linear integration for each graze-rain treatment event and for two, 30-d spring periods (May and June) in the second year of the rotation (n=79, due to one missing measurement, for ANOVA and post hoc tests on each gas). In total, we measured fluxes of all three gases 492 times between 07 August 2012 and 24 June 2014.

Soil Measurements

We measured soil temperature, gravimetric water content, NH_4^+, and NO_3^- in conjunction with each gas flux measurement. We measured soil temperature at 5 cm depth in 3 locations adjacent to the PVC chamber during each gas flux measurement with a digital soil thermometer (mfr. model #:HI45-30, Hanna Instruments, Woonsocket, RI, n=492). For gravimetric water content, NH_4^+, and NO_3^-, we sampled soil to a 10 cm depth within the corresponding soil treatment collar in conjunction with gas flux measurement (n=412, 2-5 cores soil treatment collar^{-1}, core dimensions = 1.8 cm diameter by 10 cm length). We stored soil samples at 4°C for transport back to the laboratory, where we weighed, homogenized and subsampled each soil sample to analyze for gravimetric water content, NH_4^+, and NO_3^- within 24 h of sampling. We used ~10 g fresh soil for gravimetric water content determination by drying at 105°C for ~48 h. We extracted ~20 g fresh soil in 2 M KCl (5:1 solution: soil ratio) by shaking for 1h at ~180 rpm (Mitchell et al. 2013). We then immediately filtered and froze soil extracts as described by Mitchell et al. (2013). We determined NO_3-N and NH_4-N by absorbance in microplates using the vanadium (III) chloride reaction and salicylate methods, respectively (Doane and Horwath 2003, Hood-Nowotny et al. 2010, respectively). All extracts were analyzed in triplicate.

In addition to the fast change soil metrics above, which we expected to vary tremendously over short time horizons and therefore measured frequently; we also measured slow change soil variables not expected to change measurably over the time horizon of this experiment, but which could impact the environmental performance metrics we monitored. These metrics, total soil carbon and nitrogen, we measured by combustion on five,

homogenized 20-cm soil cores collected from each plot, in both starts, in the fall of the forage

phase (2012 in S1, and 2013 in S2, respectively).

Potentially Leached Soil NO_3^-

We quantified potentially leached soil nitrate using 10.2 cm-diameter anion resin

capsules buried at ~30 cm depth (Res-Kem, New Britain, CT, 2 per plot, n=16). To capture

potential overwinter leaching, we installed resins in early October 2013 and removed them prior

to tillage on 15 April 2014. Because vegetation was established prior to resin installation, we

took great care to preserve and re-plant vegetation over each resin as we installed the resins.

We dried resins at ~ 30°C for ~ 1 week and removed dry soil by brushing prior to analysis. We

passively extracted each resin in 500 mL 3 M KCl at ~25 C for 1 h. We filtered the extract through

Whatman #42 filter paper, collecting the filtrate in a scintillation vial. Filtrate was diluted 20:1 in

3 M KCl prior to colorimetric analysis using the vanadium (III) reagent.

Data Normality and Transformations

We assessed normality of all variables prior to analysis and performed transformations

as necessary. Among the cumulative 30-d gas fluxes, only CO_2-C fluxes met normality

assumptions. NH_3-N and N_2O-N fluxes required *log(1+x)* and *square root(x)* transformations

prior to analysis to meet variance homogeneity and normality assumptions.

Statistical Analyses

We used a multi-tiered approach to understand variance in the gas fluxes. First, we used

ANOVA on the cumulative, 30-d gas fluxes to identify which factors had significant effects and

which treatment means were significantly different. To assess treatment effect significance

across start years, we utilized an expanded split-plot/repeated measures ANOVA model to

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accommodate a start year factor, a vegetation factor, a time period factor, as well as the block

factor (ssp.plot function, 'agricolae' package, Mendiburu 2014, R Core Team 2014). Fixed effects

were year, vegetation, 30- d period and their interactions; and block was a random effect. We

determined significance of differences between treatment means by Tukey's honestly significant

difference (HSD) method from the 'agricolae' package in R (Mendiburu 2014, R Core Team 2014).

We also used randomized complete block ANOVA procedure from the 'agricolae' package in R to

assess vegetation system effect on potentially leached soil NO_3^- (Mendiburu 2014, R Core Team

2014).

Our second objective was to understand which other measured variables best explained

the variations in gas fluxes we observed between treatments. To address this objective, we used

the soil inorganic N, moisture and temperature variables that we had also measured on a daily

time step in concert with the daily flux measurements. To determine which variables

significantly predicted the daily gas fluxes, and how much each variable contributed; we used

stepwise linear regression, followed by calculation of bootstrapped confidence intervals for

significant predictors, and finally a calculation of significant variables' relative importance. We

conducted these analyses with functions available from the 'MASS' package in R version 3.1.2

(Venables and Ripley 2002, R Core Team 2014). Relative importance of significant predictors was

calculated using the average R^2 contribution of each predictor when added first, second or last

during model construction (Lindeman et al. 1980, Chevan and Sutherland 1991).

RESULTS

Heat and Drought Create Challenging Conditions for Testing Environmental Performance

The weather throughout the duration of this experiment included extreme heat and drought conditions, which provides important context for all environmental performance results we report (Table 2-3). Spring of 2012 (March-June) was the 10th droughtiest since 1948 (NOAA 2014). The period of C_4 summer annual establishment in S1, May 2012, was particularly hot, ranking as the 5th warmest May since 1948, and nearly 5°C warmer than the 1981-2010 mean for May in the region (NOAA 2014). Overall, monthly mean temperatures during the experiment were 1-2°C warmer than 1981-2010 means (Table 2-3). Precipitation was more variable, but Late Summer in S2 was particularly noteworthy, receiving just ~50% of precipitation typical for that time period (Table 2-3). S1 experienced the most extreme drought conditions in Summer and the following Spring, and mean Palmer Drought Severity Index (PDSI) in S1 indicates it was droughtier than S2 overall. However, mean PDSI conditions during both Starts were droughtier than the long-term (1981-2010) mean for the region (Table 2-3). The higher than normal heat and drought conditions during this study challenged crop plants to establish and grow, let alone provide environmental services such as mediation of N losses and GHG fluxes from their respective systems.

Cumulative 30-day N_2O-N Flux

Timing of the 30-d period impacted N_2O-N fluxes, with highest fluxes in the summer and lowest fluxes in the early spring (Figures 2-2a and 2-2b, ANOVA, $p<0.001$). Vegetation also affected cumulative 30-d N_2O-N fluxes (Table 2-4, ANOVA, $p<0.002$, Figure 2-2c). Monthly N_2O-N fluxes were about 31% higher the annual system than in the perennial pasture (30-d cumulative fluxes, Table 2-4, Tukey HSD test, $p<0.05$, Figure 2-2c). Late summer fluxes were greater than

early spring fluxes, but fall and late spring fluxes did not differ significantly from each other, late summer, or early spring fluxes (Tukey HSD test). Start year did not significantly affect 30-d N_2O-N fluxes (ANOVA, p=0.30). Across start years, the effect of vegetation on 30-d N_2O-N flux varied depending on the time period (vegetation x, 30-d time period interaction effect, p<0.02, Figure 2-2). The only significant pairwise difference between annual and perennial N_2O-N fluxes within any given 30-day period occurred in the late spring during corn establishment in the annual system (Figure 2-2c). However, absolute mean N_2O-N fluxes were higher than their perennial counterparts for all five 30-d measurement periods (Figure 2-2c). The effect of time period also depended on the start year (Table 2-4, ANOVA start year x time period interaction effect, p <0.01, Fig. 2-2a vs. Fig. 2-2b). There was no block effect (p=0.34), nor was there a three-way interaction effect between start year, vegetation, and time period (p=0.12).

Cumulative 30-day CO_2-C Flux

Vegetation and timing (30-day period) also significantly affected CO_2-C flux (Table 2-4, ANOVA, p<0.005 and p<0.001, respectively). Vegetation's effect on CO_2-C flux was opposite of its effect on N_2O-N flux, with greater fluxes from the perennial than the annual (Figure 2-3, Tukey HSD p<0.005). However, the seasonal timing effect was similar across N_2O-N and CO_2-C flux, with the largest fluxes in the summer[a] , lower fluxes in the late summer[b] and fall[b] and lowest fluxes in the late spring[c] (lowercase letter superscripts indicate significantly different 30-day period means, p<0.05, determined by Tukey HSD method). Neither start year, nor block significantly affected cumulative CO_2-C fluxes (Table 2-4). However, the seasonal effect and the strength of the vegetation effect did vary significantly year-to-year (Table 2-4, significant start year x time period interaction effect and significant start year x vegetation interaction effect).

There was a significant difference in CO_2-C fluxes between annual and perennial vegetation in the 2012 start year, but not in the 2013 start year (Table 2-4, significant start year x vegetation interaction effect, Tukey HSD means comparison test, $p<0.05$). Likewise, CO_2-C fluxes were significantly higher following the late summer graze-rain treatments in 2012 than in 2013 (Table 2-4, significant start year x 30-day period effect, Tukey HSD means comparison test, $p<0.05$). While overall, perennial vegetation had higher CO_2-C fluxes than annual vegetation the strength of that effect did vary somewhat by season (Table 2-4, vegetation x time period interaction effect, $p=0.08$). Perennial CO_2-C fluxes were not significantly different than annual fluxes for all time periods except late spring, when the perennial fluxes were significantly higher (Figure 2-3).

Cumulative 30-day NH_3-N Losses Following Graze-Rain Treatments

Of all the factors evaluated, time period had the largest effect on NH_3-N fluxes with significant differences between the means of all three time periods (Table 2-4, ANOVA 30-day period effect significance $p<0.001$, Tukey HSD means comparison, $p<0.05$). Fluxes were highest in the summer, lower in late summer and lowest in the fall (Figure 2-4, Tukey HSD means comparison, $p<0.05$). Start year, vegetation and block did not significantly affect NH_3-N fluxes on their own (Table 2-4). However, in the summer, vegetation did moderate the effects of time period on NH_3-N fluxes, with significantly higher NH_3-N fluxes from soil under annual vegetation in than soil under perennial vegetation (Figure 2-4).

Factors Influencing Soil-to-Atmosphere N_2O Flux

We explored which metrics best explained soil-to-atmosphere N_2O flux in this experiment with a multiple linear regression. Metrics we evaluated were gravimetric soil moisture, soil NO_3^-, soil NH_4^+, and soil temperature (n=412). Stepwise linear regression (function

stepAIC, package MASS, Venables and Ripley 2002, R Core Team 2014) was used to determine optimal model structure, which identified soil NO_3^-, soil moisture, and soil temperature as significant model factors. The model including soil NO_3^-, moisture and temperature explained 17% of variation in observed soil-to-atmosphere N_2O fluxes (multiple R^2, model p<0.001). Amongst the three significant predictors, highest relative importance was similar between soil NO_3^- (p<0.01) and soil moisture (p<0.01), which accounted for 48.12% and 41.74%, respectively, whereas soil temperature (p=0.01) accounted for only 8.14% of model explained variation in N_2O flux.

Equation 1. *Significant (R^2=0.1699, p<0.001) multiple regression of N_2O flux (mg N_2O-N m^{-2} day^{-1}) predicted by soil nitrate (mg NO_3 kg^{-1} soil), moisture (g H_2O g^{-1} soil) and temperature (°C).*

$$N_2\text{O-N (mg } m^{-2} \, day^{-1}) = \text{-2.6574} + 0.0587 \times (\text{Soil Temp,°C}) + 0.041 \times (\text{Soil } NO_3^-, \text{ ppm})$$

$$+ 11.904 \times (\text{Soil } H_2O, \text{ g } H_2O g^{-1} \text{ soil})$$

Factors Influencing Soil-to-Atmosphere CO_2 Flux

We also assessed the predictability of the soil to atmosphere CO_2 flux using the same four predictor variables: soil NO_3^-, NH_4^+, moisture, and temperature. Stepwise linear regression identified all four variables in optimal model structure. The model (p < 2.2 x 10^{-16}) including all variables explained 26% of observed variation in CO_2 flux, with bootstrapped confidence intervals suggesting soil NH_4^+ concentration was most informative, accounting for 45% (p<0.0001) of explained variability in CO_2 flux. Soil moisture (26% of explained variation p<0.0001) and temperature (25% of explained variation, p<0.0001) were both less important than NH_4^+, but more important than NO_3^- (4% of explained variation, p=0.0004).

Equation 2. *Significant (R^2=0.2641, p<0.001) multiple regression of CO_2 flux (g CO_2-C m^{-2} day^{-1}) predicted by soil NH_4^+ (mg NH_4 kg^{-1} soil), temperature (°C), moisture (g H_2O g^{-1} soil) and NO_3^-.*

$$CO_2 - C(g\ m^{-2}\ day^{-1}) = -4.7239 + 0.0656 \times (\text{Soil } NH_4^+, \text{ppm}) +$$

$$0.2902 \times (\text{Soil Temp,°C}) + 21.235 \times (\text{Soil } H_2O, \text{g } H_2O g^{-1} \text{ soil}) -$$

$$0.0432 \times (\text{Soil } NO_3^-, \text{ppm})$$

Over-winter Potentially Leached Soil NO_3^- (October 2013- April 2014)

The anion resins buried at 30 cm showed the annual system allowed about twice as much NO_3^- (63±15 kg NO_3-N ha^{-1}) to leach to 30 cm soil depth compared to the perennial (33±5 kg NO_3-N ha^{-1}, mean± SE, n=8 resins per vegetation treatment). We excluded one perennial resin from this reported mean and further analyses because it had a NO_3^- concentration >11 SE greater than the mean. This anomalous resin ripped during removal from the soil, and was likely contaminated. Randomized complete block ANOVA of log-transformed potentially leached soil NO_3^- confirmed potential October through April NO_3^- leaching may have been (significant at p<0.1) higher under the annual system than under the perennial system (ANOVA, vegetation effect, p<0.09). There were no within-field spatial effects on NO_3^- leaching, as block did not have a significant effect (ANOVA, Block effect, p=0.50).

DISCUSSION
Nitrification, not Denitrification, is Key Driver of N_2O-N Loss in Organic N-based Systems

This study was designed to focus on denitrification-derived N_2O because numerous previous reviews of GHG emissions in general and N_2O emissions in particular have identified denitrification as the dominant source of agricultural GHG impact from soils (Cavigelli and Parkin 2012, Venterea 2007). However, our data does not suggest denitrification was the process that

differentiated the two systems in terms of N_2O flux. Despite the divergent phenology between the annual and perennial pasture vegetation in the summer and fall, and the exceptionally large inputs of N and water associated with the graze-rain treatment events applied during these time periods, the biggest difference in N_2O-N fluxes between the systems occurred in the spring through late spring. In late spring, average annual system N_2O-N fluxes were 9-times higher than their perennial system counterparts. These extremely large annual N_2O-N fluxes occurred during corn establishment in the annual system, from tilled soil that was actually dryer than the untilled soil in the perennial pastures. However, these high spring fluxes from the annual soils occurred after incorporation of red clover and manure applied at ~56 kg N ha^{-1}. These high organic-N and NH_4-N conditions in conjunction with tillage and relatively dry, presumably aerobic soils suggest that nitrification, not denitrification, drove the high spring N_2O-N fluxes that differentiated these systems (Cavigelli and Parkin 2012, Ruser et al. 2006). These results have applicability to future work to quantify GHG emissions from organic systems. The increased importance of nitrification as an N_2O source in these systems implies N_2O sampling schedules should focus less on WFPS as a proxy for when to sample, and more on higher frequency sampling after organic N and NH_4-N fertilization events for up to 2 months or more.

Soil CO_2 Fluxes are not a Clear Indicator of Agroecosystem GWP

Both annual and perennial systems are most vulnerable to high graze-rain treatment-stimulated CO_2-C fluxes in the summer, when soil temperatures are higher. Potential for high fluxes drops rapidly in the late summer and fall, with some year-to-year variability introduced by inter-annual variability in soil and air temperature trends. Higher rates of root respiration or root C exudate-fueled heterotrophic respiration from the perennial vegetation could explain the

overall higher fluxes we observed from perennial soils relative to the soils in the annual plots. However, we had expected to observe higher fluxes from the annual soils relative to the perennial in the spring and late spring, driven by tillage and fertility inputs in the annual system that were not applied in the perennial system. We were surprised the annual system CO_2-C fluxes we measured averaged only 56% of fluxes from the perennial system in that late spring time period, despite manure and cover crop incorporation by relatively deep (25 cm) inversion tillage in the annual system and no tillage or manure amendment in the perennial system. Tillage typically results in CO_2-C fluxes 4-14 times greater than fluxes observed from untilled soil, with relative CO_2-C loss ranging from 8-10 times for tillage depths similar to the ones used in this study (Reicosky 1997, Reicosky and Archer 2007). However, the largest CO_2-C flux increase occurs within 24 h of the tillage event, which was not captured in the timing of our measurements (Reicosky and Archer 2007). While this may be the simplest explanation for our surprising CO_2-C flux results, there are at least two additional potential explanations for these surprising results observed in the spring period.

Comparing our results with other studies contrasting CO_2-C fluxes from tilled and untilled soil revealed an important difference between the treatments in our study and those in typical tilled versus untilled experiments. Typically, other studies comparing tilled versus untilled soil CO_2-C flux make their comparisons between un-vegetated soils. In contrast, the comparison in this study is between untilled soil under actively growing perennial vegetation, and largely un-vegetated tilled soil under establishing annual corn silage. This important difference between ours and others suggests the contrast in biology between our annual and perennial systems in the spring could partially explain the difference in CO_2-C flux we observed. Paustian et al. (2000)

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note perennial grasses, like the orchardgrass in this study, have high rates of belowground

carbon allocation, which often drive high rates of soil CO_2 efflux. But, they also note that most of

the CO_2-C emitted from soil under perennial vegetation derives from C fixed by the perennial

vegetation itself in that same year: either directly via autotrophic root respiration, or indirectly

from root exudate-fueled heterotrophic respiration (Paustian et al. 2000). So, the elevated CO_2-

C flux we observed from our untilled soil may have biological significance reflective of real

increases in soil respiration directly and indirectly fueled by the actively growing perennial

vegetation, but still not indicate any net increase in GWP.

Despite the plausible biological explanation for these results, differences between

annual and perennial soil structure revealed through frustrations in the mechanics of collecting

these data in the field offer another potential, measurement-error-derived explanation for the

spring results. The tilled soil in the annual plots dried rapidly after tillage, developing an

extremely hard blocky structure, which persisted through further soil preparation and

cultivation events and complicated insertion of our chambers. Tilled annual soil in S2 best

exemplified this poor soil structure issue. In 2014, tilled soil in S2 annual plots displayed crusting

that was so bad it largely inhibited corn emergence, despite repeated attempts to facilitate

emergence with rotary hoe passes, and after several weeks these plots had to be completely re-

planted. From a soil gas flux monitoring perspective, the extensive cracking and fissures

between the hard blocks of soil made it impossible to create good soil-atmosphere seals with

the collars in the annual plots. Without tillage and with continuous vegetative cover for more

than a year at the time of spring measurements, soil in the perennial plots facilitated excellent

soil-to-chamber seals. Therefore, we suspect unavoidable measurement error may have

contributed to an underestimation of spring and late spring fluxes from the annual plots,

beyond that stemming from measurement timing (or lack thereof in the first 24-hr after tillage).

This suspected leakage from the chambers in the annual plots would make both the CO_2-C and

N_2O-N fluxes we measured in spring and late spring underestimates of actual fluxes.

   While it is impossible to comment on the actual relative importance of these three

factors in explaining our results, they do raise several important limitations to both the

conclusions we can draw related to the original objectives of this study, as well as to the broader

generalizations we can support with these results. First, the potential biological mechanism at

play in elevating measured soil-to-atmosphere CO_2-C fluxes from the soils under perennial

relative to the annual vegetation served as a valuable reminder of the limited role for flux

chamber techniques in elucidating ecosystem carbon balances and GWP (Cavigelli and Parkin

2012). A key objective driving this research was to compare environmental performance of

these agroecosystems, using various metrics related to GHG emissions to differentiate

environmental impact between the two systems by quantifying their GWP. However, for carbon,

our flux chamber measurements only captured the soil outputs dimension of net ecosystem

exchange in these systems. So, in addition to the basic measurement uncertainty issues we

raised, and other problematic artifacts of chamber-based flux measurements raised by others, it

is important to acknowledge the limits inherent to these measurements, namely that they are

just a measure of soil output, and do not account for C inputs to the ecosystem via

photosynthesis (Cavigelli and Parkin 2012, Pautian et al. 2000). The attention our results draw

to photosynthesis as an important underlying driver of increasing soil CO_2-C flux is an important

reminder that summing chamber-based GHG flux measurements on a CO_2 equivalent basis as a

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value of GWP is an inaccurate and misleading approach to comparing GWP across any systems (Cavigelli and Parkin 2012). Cavigelli and Parkin (2012) also note the prevalence of this approach in recent studies of agricultural systems, and reiterate that while GWP is a valuable metric of agroecosystem environmental performance, chamber-based measurements are not and cannot be a comprehensive GWP metric.

Despite the challenges and limits to inference related to the carbon portion of this systems comparison, this study does offer robust support for the broader conclusion that organic perennial systems display superior environmental performance relative to annuals. Even from a GHG perspective, across all the time periods and both starts included in this study the N_2O-N flux results demonstrate consistently better performance, as well as significantly lower cumulative fluxes overall from the perennial system compared to the annual system. These N_2O results alone constitute valuable and relatively thorough assessment of the systems' potential impact on global warming. This strength stems from several factors. First, N_2O is the leading GHG of concern in agriculture, accounting for about two-thirds of agriculture's GWP, and essentially all of the GHG contributions of non-flooded agricultural soils (Cavigelli and Parkin 2012). Secondly, agriculture accounts for >60% of global N_2O emissions, with recent models showing N_2O production increases of 30-60% by 2030 (Barker et. al 2007). Third, while our sampling schedule may have missed some key CO_2 fluxes, it was specifically designed to capture key N_2O flux events by increasing sampling frequency when soil moisture and nitrogen conditions were optimal for N_2O production via denitrification or nitrification. Furthermore, even though the duration of this study was too short to capture any changes in soil carbon that could be used to reasonably infer superior environmental performance from the perennial

system in terms of NEE, numerous other studies have documented consistently superior

environmental performance in terms of carbon storage from perennials (Paustian et al. 2000).

Therefore, this study provides substantive additional data to support the conclusion that

perennial systems confer greater environmental benefits in terms of lower GHG emissions than

annual forages.

Beyond, the GHG benefits, the perennial also appears to confer additional

environmental benefits in terms of less NO_3^- leaching than the annual system, even when an

overwintering red clover cover crop was included in the annual system. We were initially

surprised to observe these relatively large rates of potential overwinter NO_3^- leaching under the

red clover cover crop, and were concerned the single winter represented by these data might

not be reflective of the broader nitrate leaching performance of winter legume cover crops in

our region. However, other researchers monitoring potentially leached soil NO_3^- under an array

of winter legume cover crops including red clover across Pennsylvania have also measured

similarly high rates of NO_3^- leaching in multiple years (C. White and D. Finney, *pers. comm.*).

Their research suggests overwintering legume cover crops in this region offer poor performance

in terms of nitrogen retention and may even facilitate greater nitrate loss via mineralization of

nitrogen fixed by the legume cover crops relative to bare fallow controls. While the perennial

system in this experiment also included overwintering red clover, we posit it owes its superior

performance to its orchardgrass component, an excellent NO_3^- scavenger as both a grass and a

deep-rooted perennial.

Unlike the other environmental performance metrics, where vegetation had an effect,

vegetation's lack of an effect on NH_3-N flux suggested vegetation played a relatively minor role

in this prominent N loss pathway. This was surprising given the markedly higher ratio of post-graze residual vegetation to bare soil in the perennial system compared to the annual system in this study, and the long-standing evidence that plant leaves absorb substantial portions (6-11%, or even as high as 30%) of NH_3-N fluxes (Harper et al. 1996, Porter et al. 1972, Schlesinger 1997). However, season or timing of the grazing treatment did have a significant effect on this flux, highlighting the dominant role of higher temperatures in driving higher NH_3-N fluxes (Krauter et al. 2007). This also re-focuses attention on the summer period, as a key time when manure-N is most vulnerable to loss via NH_3-N volatization due to high temperatures and dry soils (Schlesinger 1997). It is also the time when the highest rates of NH_3 absorption by plants and thus greatest potential plant-derived mitigation of NH_3-N loss has been observed (Harper et al. 1996). Harper and colleagues (1996) found summer plant NH_3-N absorption rates can be nearly double absorption rates in cooler seasons. Summer was also the only period in this study when we observed a significant difference in NH_3-N flux between the two vegetation types (Figure 2-4). The annual system's cumulative mean NH_3-N flux was nearly double that of the perennial in the summer period (Figure 2-4). This difference suggests the greater post-graze residual biomass and leaf area of the perennial vegetation in the summer provided higher rates NH_3-N absorption, thus providing enhanced mitigation of NH_3-N volatization from manure in this key time period. As the red clover understory became better established in the annual system through the late summer and fall, differences in NH_3-N mitigation between annual and perennial vegetation vanished. These results suggest that even in the summer, when we had hypothesized the phenological differences between the annuals and perennials would give the annuals an edge in

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NH$_3$-N mitigation, the perennials actually outperformed the annuals likely due to greater post-graze residual vegetation leaf area.

While the short-term treatments included in this experiment provided a wealth of new knowledge about the performance of multiple organic systems on annual to sub-annual timescales, this study's siting on land under long-term organic management (9-11 years) forces us to consider the role of that legacy in shaping these results. This long-term organic management legacy included frequent (6 total in ~10 years) heavy manure applications and frequent cover cropping to support an organic reduced tillage experiment. We had thought positive soil changes engendered by this long legacy of organic management might mask any treatment differences in this short-term study. However, heavy perennial weed pressure was the most obvious legacy, which in conjunction with our management of those weeds, revealed additional environmental challenges remain with organic production. The perennial weed-hampered productivity in S1 inspired a false-seedbedding approach (Brainard et al. 2013) consisting of repeated summer tillage in S2 to manage that perennial weed legacy, prior to the initiation of the annual vegetation in S2. The damaging effects of that repeated summer tillage prior to this study's initiation, as well as the tillage and cultivation events associated with establishment of this study's annual crops, were clearly evident in the damaged soil structure of the annual plots in S2. In addition to hampering gas flux data collection, this battered soil also hampered productivity and environmental performance of the annual system, highlighting the ongoing struggle to balance weed and soil management in annual organic systems, and a need for further research aimed at improving this balance. This study indicates greater incorporation of perennials is one way to improve this balance and achieve greater environmental

performance, as the perennial system suffered neither from excessive weed pressure, nor from poor soil structure damaged by too much tillage.

Lastly, despite these remaining challenges to organic production, the environmental performance of these organic systems compares favorably to published performance of conventional systems. Since this study was conducted on certified organic land, it was not possible to include conventionally-managed systems for comparison. However, Adviento-Borbe and colleagues (2010) measured the same GHG and NH_3 fluxes from long-term, conventionally-managed corn systems also located at RELARC. The corn crop Adviento-Borbe and colleagues (2010) studied was grown in one of four conventional cropping systems defined by crossing two cropping sequences (continuous corn or four-year corn followed by four-year alfalfa) with two fertility regimes (manure as main N source or NH_4NO_3 as main N source). In addition to the difference in crop and system management between our study and theirs, there was also 1) a difference in annual monitoring period duration (their monitoring began at the end of March vs. our spring monitoring began at the end of April), and 2) a difference in the number of N fertilization events per year (theirs only included a single spring N fertilization event compared to this study's three events through summer and fall). Despite these differences, 2-year mean cumulative N_2O-N flux was higher in 3 out of 4 of their systems than both organic systems in this study (Adviento-Borbe et al. 2010). The NH_4NO_3-fertilized continuous corn in their study did have a lower 2-year mean cumulative N_2O-N flux than the annual system in this study, but its flux was still higher than the perennial in this study, which had the lowest cumulative N_2O-N flux of all systems in both studies (Adviento-Borbe et al. 2010).

This preliminary comparison is encouraging for the potential relative benefits of organic systems from an N_2O-dominated GHG perspective, especially given the fact that there were 3 times as many N fertilization events in the organic systems, and much higher rates (3-9x higher) of N applied in each of those events (Table 2-2 vs. Adviento-Borbe et al. 2010). This comparison also provides an interesting contrast between the effects of manure on N_2O flux depending on system. Cavigelli and Parkin (2012) reported that manures are often thought to increase N_2O emissions, due to a stimulatory effect of the C in manures on heterotrophic activity, a thought supported by the findings of Adviento-Borbe and colleagues (2010). In contrast, the lower fluxes in this study provide greater support for a different relationship between manure inputs, system management, and resultant N_2O flux. This study offers more support to the hypothesis that management that increases $C:NO_3$ ratios reduces the proportion of denitrification end products lost as N_2O because the higher organic matter serves to increase complete reduction of NO_3^- to N_2 during denitrification. The combination of manure additions with high OM settings, such as in the perennial pastures under grazing treatments in this study, may reduce N_2O fluxes via increased $C:NO_3$ ratio, emphasizing the importance of context in predicting N source effects on N_2O emissions.

Conclusions

This is the first study to investigate environmental performance with an emphasis on GHG fluxes in grazed organic systems' coupled with extreme rain events, the foremost weather event predicted to increase with climate change on this region. It also represents the region's first field study to quantify the impact of an annual legume cover crop, red clover, on N_2O emissions (Cavigelli and Parkin 2012). As such, this study contributes to filling a gap in our

knowledge of the performance of both currently understudied systems, and future systems

defined by climate-management interactions likely with climate change and organic market

growth trajectories. Experimental data from treatments designed to create future conditions

likely under climate by management scenarios show these organic systems perform similarly in

the wake of field-applied grazing and extreme precipitation treatments, with the one exception

that the perennial system appeared to better mitigate grazing treatment NH_3-N flux in the

summer. Furthermore, as documented by nitrate leaching and N_2O flux data, that superior

perennial system performance extends through the subsequent winter and spring. But, these

benefits aren't without caveats and motivate many more research questions requiring future

investigation to develop future farming systems that provide the best environmental

performance.

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TABLES

Table 2-1. Mean Soil Characteristics by Start

	Location	pH	% C	% N	% OM**
S1 (2012-2013)*	*Blocks 1-3*	7.1	1.442	0.176	2.639
	Block 4		2.252	0.261	4.118
S2 (2013-2014)	*All Blocks*	6.9	1.319	0.168	2.414

*Soil %C and %N was significantly higher in Block 4 of S1.

**Estimated for S1 using % C measurements and the formula %OM=%C*1.83

Table 2-2. Manure[Ɨ] Characteristics by Grazing Treatment Event

Start and Season of Grazing Treatment	% Solids	C:N[‡]	%C[‡]	%N[‡]	Manure N Applied[§] (g m^{-2})			
					total N	NH_4-N	NO_3-N[‡]	Organic-N
S1 - Summer	10.00				64.39	22.90		41.49
S1 - Late Summer	14.06	33.4	15.82	0.473	86.83	22.16	0.37	64.30
S1 - Fall	6.60				65.49	27.20		38.28
S2 - Summer	15.10	15.4	6.57	0.427	78.22	10.72	0.09	67.41
S2 - Late Summer	5.60				60.27	22.90		37.37
S2 - Fall	6.62				74.10	27.66		46.44

Ɨ All manure was dairy manure obtained from neighboring Kocher's Dairy Farm.

‡ Only measured on manure samples from manure applied in Late Summer in S1 and in Summer in S2

§ Analytical lab reported an assumed manure density of 999 g L^{-1}, calculated N applied based on manure density and manure application rate of ~0.9 L manure per 490.9 cm^2 for each grazing treatment.

Table 2-3. Regional[§] Climatic Variables by Start (S) and 30-day Period*

	Summer	Late Summer	Fall	Spring	Late Spring
	Palmer Drought Severity Index (values<0 indicate drought)				
1981-2010 mean	*0.14*	*0.12*	*0.35*	*-0.01*	*0.29*
S1 (2012-2013)	-1.58	0.44	1.00	-1.33	0.45
S2 (2013-2014)	0.62	-0.67	-0.98	0.70	1.03
	Mean Daily Temperature (°C)				
1981-2010 mean	*19.9*	*15.8*	*9.7*	*13.7*	*18.4*
S1 (2012-2013)	20.2	16.0	10.9	14.3	18.9
S2 (2013-2014)	21.8	17.2	11.2	14.4	19.1
	Sum of Monthly Precipitation (mm)				
1981-2010 mean	*98.5*	*99.8*	*84.0*	*101.0*	*113.9*
S1 (2012-2013)	83.8	114.8	116.3	84.1	81.5
S2 (2013-2014)	122.2	57.7	78.2	83.3	156.7

[§]All climate data obtained from NOAA records for the Pennsylvania, region 7, Central Mountains (NOAA 2014)

*Fall, Spring, and Late Spring 30-d measurement periods aligned with October, May, and June for both Starts, so data in this table are the averages for those months. For S1, Summer and Late Summer refer to August and September 2012, but for S2, Summer refers to July 2013 and Late Summer included half of August and half of September, so Late Summer is the mean of August and September 2013.

Table 2-4. ANOVA: 30-day Cumulative Fluxes

	P values		
Factors	**N_2O-$N^‡$**	**CO_2-$C^‡$**	**NH_3-$N^§$**
Start Year	0.47	0.15	0.20
Block	0.70	0.25	0.85
Vegetation	**<0.002**	**<0.005**	0.25
Start Year x Vegetation	0.37	0.58	*0.08*
30-d Period	**<0.001**	**<0.001**	**<0.001**
Start Year x 30-day Period	**<0.003**	**<0.001**	*0.09*
Vegetation x 30-day Period	**0.02**	*0.08*	**0.02**
Start Year x Vegetation x 30-day Period	0.12	0.56	0.27

N_2O-N and NH_3-N ANOVAs were on transformed data, *square root(x)* and *log(x+1)*, respectively.

[‡] We monitored N_2O-N and CO_2-C fluxes following all graze-rain treatments, as well as during two consecutive 30-day periods from late April through late June (n=80).

[§] We did not monitor for spring NH_3-N fluxes, so these are just the fluxes following the three graze-rain treatments applied in both start years (n=48).
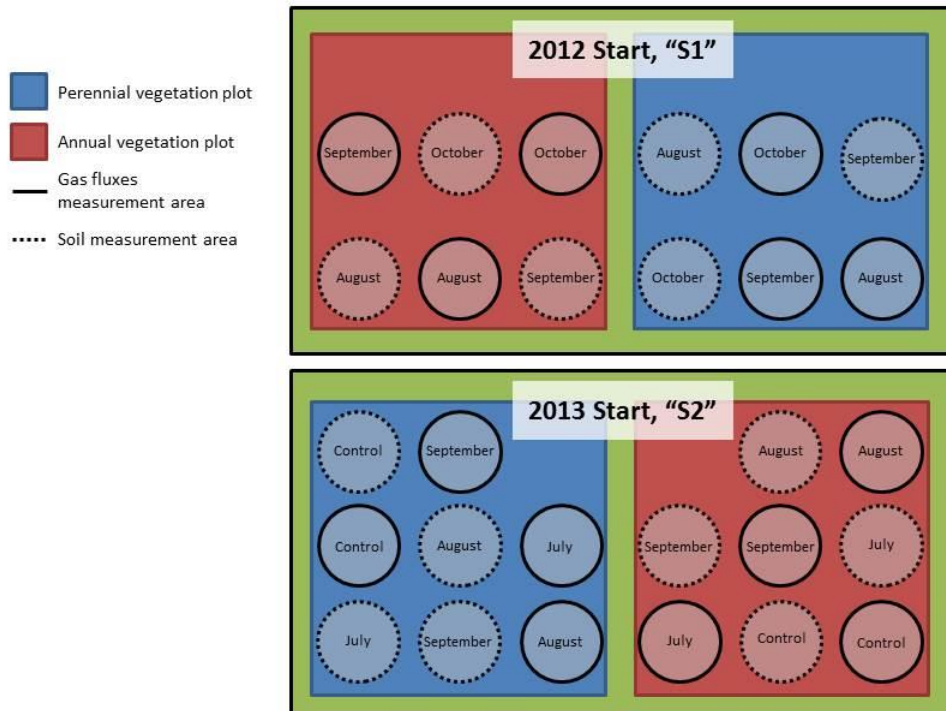
Figure 2-3. *Schematic Diagram of Experimental Design.* Above is a simplified depiction of one block of each Start's plot and monitoring collar layout for the first year of the experiment, when we applied grazing and rainfall treatments (not to scale). S2 included control collars in each plot. PVC rings (25 cm diameter) denoted soil sampling areas in S2, while flagging delineated soil sampling areas in S1. All collars/ sampling areas were randomly arranged within a 1.5 m by 5m area along one edge of each of the 6.1m x 6.1m plots, at least 0.5 m from the plot edge to avoid edge effects.
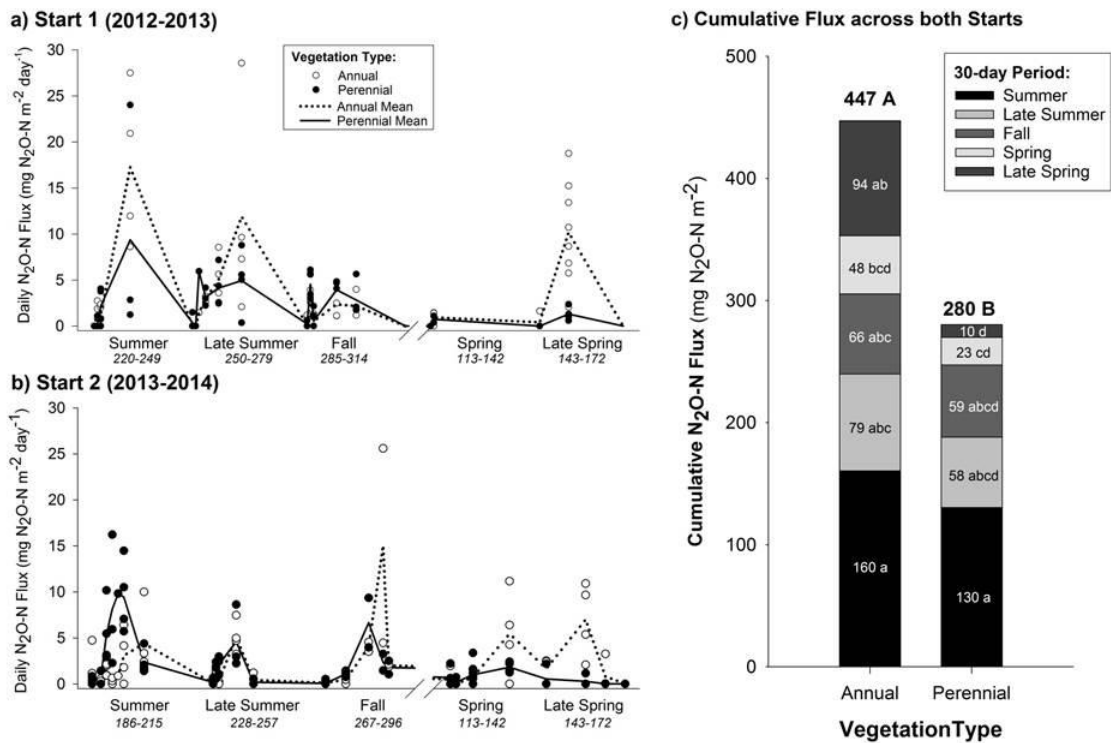
Figure 2-2. *Daily and Cumulative N$_2$O-N Fluxes by System.* Overall, N$_2$O-N fluxes were higher from soils under annual vegetation than under soils under perennial vegetation (c, p<0.002). The biggest difference in N$_2$O-N flux between vegetation types occurred in the spring during the corn establishment phase in the annual. This difference was consistent across Starts (**a** and **b**, above), although Late Spring was the only 30-day period where annual N$_2$O-N fluxes were significantly greater than perennial fluxes (**c**, Tukey's HSD, p<0.05). Grazing and extreme-precipitation treatments stimulated similar N$_2$O-N fluxes from soils under both annual and perennial forages (**a** and **b**, Summer, Late Summer, and Fall periods). However, the highest absolute fluxes came from the annuals (**a** – Summer and Late Summer, **b** – Fall), and mean annual fluxes were higher (though not significantly so) for all five 30-day periods (**c**). Italic numbering under the x-axis in **a** and **b** denotes the Julian Day range of each 30-day period for which we performed a linear integration to calculate a cumulative flux (**c**), or areas under the curves depicted in **a** and **b**. Axes in **a** and **b** are to scale, with breaks representing the winter period, during which we collected no data.

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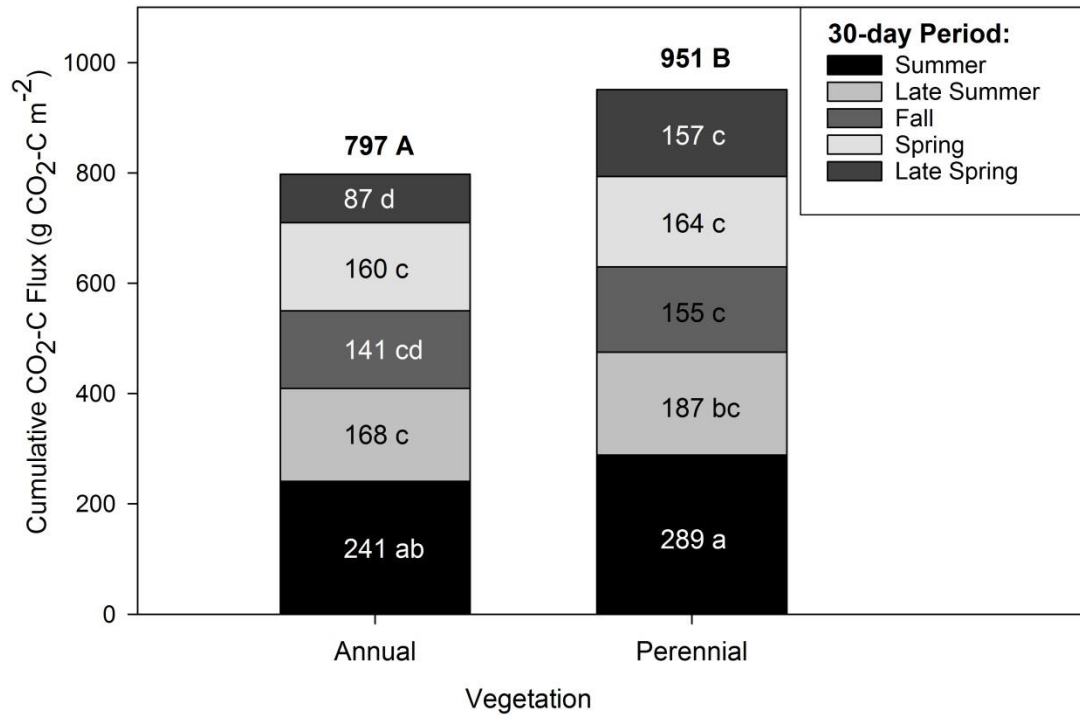
Figure 2-3. *Cumulative CO_2-C Flux.* Over all five, 30-day measurement periods across both start years, CO_2-C fluxes were significantly higher in perennial compared to annual vegetation treatments (uppercase letters, n=40 for vegetation means, $p<0.005$). However, significant, within-30-day period differences between vegetation only occurred in late spring (lowercase letters, n=8 for each vegetation x 30-day period treatment mean, Tukey HSD means comparison $p<0.05$).
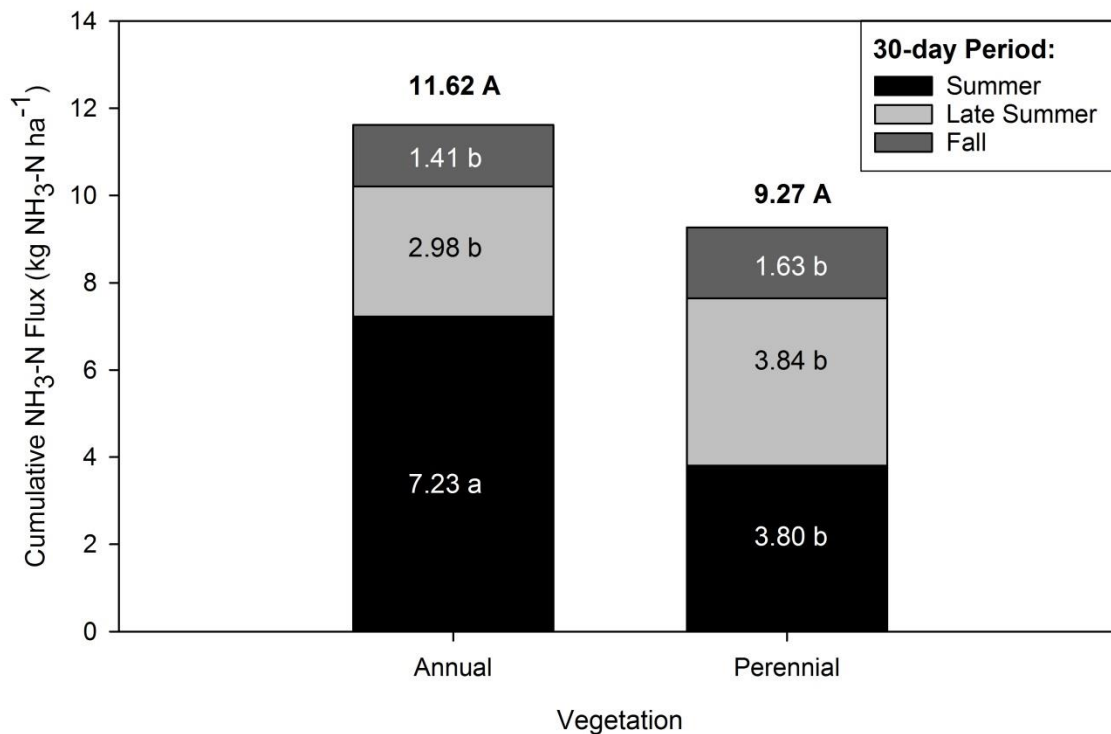
Figure 2-4. *Cumulative NH$_3$-N losses induced by grazing and rain treatments*. Summer annual NH$_3$-N losses were significantly greater than summer perennial NH$_3$-N losses, as well as losses from the annual and perennial following both late summer and fall graze-rain treatments (lowercase letters, n=8 for each vegetation x 30-day period treatment mean, p<0.05). Overall there was not a significant difference between annual and perennial vegetation in graze-rain treatment-induced NH$_3$-N loss (uppercase letters, n=24 for each vegetation mean, p>0.05).

Table 2-S1. Grazing and Extreme Rainfall Treatment Monitoring

	Start	Baseline	Post-Graze	Post-76mm rain	Post-treatment monitoring →			
Graze-Rain 1	*S1 (2012)*	07 Aug.	08 Aug.	09 Aug.	18 Aug.	07 Sept.	12 Oct.	
	S2 (2013)	05, 08 July	10 July	12 July	14 July	16 July	23 July	16 Aug.
Graze-Rain 2	*S1 (2012)*	06 Sept.	07 Sept.	08, 10 Sept.	14 Sept.	21 Sept.	11 Oct.	
	S2 (2013)	15 Aug.	17 Aug.	18 Aug.	24 Aug.	30 Aug.		
Graze-Rain 3	*S1 (2012)*	11 Oct.	12 Oct.	13 Oct.	20 Oct.	26 Oct.		
	S2 (2013)	24 Sept.	1 Oct.	2 Oct.	9 Oct.	14, 16 Oct.		

Table 2-S2. Spring Monitoring in Rotation Year 2

Start Year	Early Spring→			Late Spring→				
S1 (2013)	02, 03 May[2]			05 June[3]		14 June[3]		
S2 (2014)	23, 25 April[2]	01 May[2]	14 May[3]	27 May[3]	10 June[3]	17 June[3]	24 June[3]	

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[2] Monitored "Graze-Rain 3" and control (S2 only) collars

[3] Post-tillage monitoring, included 2 collars plot -1 in S1 (2013), 1 in-row and 1 between-row. Only 1 collar plot^{-1} in S2 (2014), post-tillage

Chapter 3
Low Soil Inorganic Nitrogen: Not So Yield-Limiting in Organic Systems?

ABSTRACT

Yield lags in organic systems are frequently attributed to low soil inorganic nitrogen (N) availability. Within a 2-year organic forage systems trial conducted on 2 sites that had been under long-term organic management, we compared two alternative N provisioning strategies, legume-based and manure based. We monitored crop yields and soil inorganic nitrogen availability in throughout forage and silage phases of 3 manure-based annual systems and 4 red clover (RC, *Trifolium pratense* L.*)*-based annual systems. Annual systems consisted of annual forage followed by corn silage (CS, *Zea mays* L.), whereas a perennial forage system remained in orchardgrass (OG, *Dactylis glomerata* L.*)*-RC throughout the experiment, and served as a reference control. Annual forage yields were inversely correlated with soil inorganic N availability, and, from a groundwater quality perspective, manure-based N management resulted in concerning quantities of end-of-season soil nitrate. The relationship between early season nitrate availability and yields was also weaker than typically reported for conventionally managed systems and suggested lower levels might be more appropriate for both manure- and legume-based systems with a long history of organic management. Across both phases, these results imply low soil test inorganic N may not be so yield-limiting, and even that higher soil inorganic N can depress yields under certain circumstances. Our results contradict the oft-cited notion that organic crop yields are limited by soil inorganic N and suggest that high soil inorganic N can depress crop yields as a result of indirect effects from increased weed competitive ability.

INTRODUCTION

More than a century after development of the Haber-Bosch process, and with more than 210 Tg yr^{-1} inorganic nitrogen (N) fertilizer fixed by the process annually, N is still one of the primary limits to crop yields globally (Fowler et al. 2013, Mueller et al. 2013). And, while the critical importance of increasing global yields to meet increasing global food demand remains as one of agriculture's "grand challenges", further increasing soil inorganic N availability is a proposition fraught with serious economic and environmental consequences (Foley et al. 2011, Sutton et al. 2011). Furthermore, even in the U.S., where inorganic N fertilizers are readily accessible, N yield limitation still persists under certain circumstances. In organic agriculture, a U.S. agricultural sector expanding at a rate of 20-25% annually that prohibits use of synthetic N fertilizers, yields lag 8-25% behind conventionally managed crops (USDA-ERS 2015, Badgley et al. 2007, de Ponti et al. 2012, Seufert et al. 2012, Ponisio et al. 2015). In organic production, N is routinely cited as being both the most limiting macronutrient, and as being a root cause of the organic yield lag (Clark et al. 1999, Badgley et al. 2007, de Ponti et al. 2012, Seufert et al. 2012, Ponisio et al. 2015). These issues coalesce into a single N-constrained goal for organic agriculture: to meet rapidly expanding demand for organic food, while not only avoiding reductions in the global food supply, but also continuing to increase global yields.

To achieve this goal, organic producers have two primary N provisioning strategies to choose between: legume-based and manure-based. Legume-based and manure-based N management strategies each offer their own set of benefits and drawbacks. Despite these differences, both strategies may be evaluated using the same two agricultural N management goals: (1) providing sufficient plant-available N during the growing season while (2) minimizing

polluting N losses. However, timely N provisioning must also be evaluated within the context of how well the N management strategy supports high yields, the primary service sought from agricultural systems. Our objective was to assess how well each of these alternative N management strategies (manure N vs. legume N) performed in terms of achieving each of these three inter-related goals: (1) providing inorganic N, both early in the season, and then throughout the growing season; (2) maximizing crop yields; and (3) minimizing the amount of end-of-season inorganic N (nitrate) likely to be lost via leaching over the subsequent winter.

Manure-based N management is not viable as a global strategy for N provisioning due to both simple mass balance-related supply constraints, as well as due to physical and economic supply challenges caused by spatial and temporal discontinuities between manure supply and crop demand (Keplinger and Hauck 2006). However, these very same factors make manure-based N management viable and even attractive in regions with high densities of animal production, and correspondingly large quantities of available manure (Keplinger and Hauck 2006). The Mid-Atlantic region of the U.S. is one such region. The states in the Mid-Atlantic region are among the top 5 nationally in terms of dairy, poultry, and egg production; making it also home to a tremendous concentration of especially N-rich manure (USDA-NASS 2012). Manure-based N management is attractive to organic producers because it alleviates the amount of rotation space they would otherwise have to dedicate to legume cultivation. Manure-based N management facilitates rotating from one high-yielding grass or grain to another, without having to take a season or a year out to grow the requisite N with a legume crop or cover crop. This manure-facilitated rotation flexibility is especially attractive in the mid-Atlantic, where shorter growing season length complicates or even precludes effective winter-

annual legume establishment after any long-season crops that are not harvested until after about mid-August.

Despite these considerable potential agronomic advantages of manure-based N management for organic producers in the Mid-Atlantic, relying too heavily on manure to achieve N fertility can result in both short- and long-term agronomic and environmental costs. Short-term drawbacks of manure-based N management include agronomic costs associated with manure storage and application, as well as environmental costs in the form of ammonia volatilization, nitrous oxide emissions from both nitrification and denitrification, and nitrate leaching associated with high rates of manure application. Manure stoichiometry and greater P and K retention relative to N, means that longer-term reliance on a manure-based N management strategy leads to P and K excesses in the soil, which, combined with the right transport factors, leads to P-driven eutrophication of freshwater aquatic systems (Sharpley et al. 1994, Sims et al. 1998, Kleinman et al. 2011). The long history of animal husbandry and manure applications to soil has already led to excessive P levels in many of the region's soils, contributing to eutrophication of regional waterways and the Chesapeake Bay (Sharpley et al. 1994, Sims et al. 1998, Kleinman et al. 2011). Together, these factors motivate further evaluation of the agronomic and environmental benefits and drawbacks of exclusive reliance on manure for N management in organic systems of the Mid-Atlantic region.

Globally, legume-based N management is much more tractable as a scalable strategy for N provisioning for all types of producers, regardless of proximity to animal production. Legume-based N provisioning can be particularly attractive to producers in regions where historic manure applications have already created excessive levels of soil P, and/or in regions where

legume cultivation is feasible during a traditionally fallow period. Potential to cultivate the

legume in this previously fallow period reduces opportunity costs associated with cultivating a

legume cover crop when a cash crop would have been or could have been produced. This

opportunity is limited across much of the U.S. where current crop diversity is limited to summer

annual crops: corn (*Zea mays L.*) and soybean (*Gycine max* (L.) Merr.). A critical obstacle to

achieving greater legume N provisioning on these lands is attaining adequate legume

establishment without interfering with cash crop cultivation, although some new work on cover

crop inter-seeding in crop rows shows potential in corn and soybean (Dillon et al. 2012).

Strategies available to producers to facilitate winter annual legume establishment require

switching to shorter season varieties, or switching crops entirely, from corn to oats, for example,

to create more space and time at the end of the growing season to establish the legume.

However, these strategies fail to address the overarching "grand challenge" of increasing the

size of the global bread basket, as these strategies result in net reductions in system yield, due

to the lower yield potential of both shorter season varieties as well as shorter season crops like

oats, relative to corn (Foley et al. 2011). These strategies also require additional management

time from producers to establish the legume, which is often not feasible for the vast majority of

time-limited producers who earn most of their income from off-farm work (USDA-ERS 2015).

To overcome both the limited establishment window and the management time

barriers, researchers and innovative producers have experimented with other legume

establishment strategies (SARE 2007). Those strategies include hydro-seeding or aerial seeding

of legumes into corn and modifying equipment to seed the legume in conjunction with other

necessary summer annual management (i.e. in conjunction with last cultivation in organic

systems, or with side-dress N application, and/or post-emergent herbicide application in conventional systems, SARE 2007). Like these other strategies that strive to establish the legume within the summer annual crop, producers' whose rotations include summer annual forages have yet another opportunity for legume integration into their systems. RC, a short-lived perennial legume often managed as a winter annual, can be seeded with summer annual grasses, such as sorghum sudangrass (SSG, *Sorghum bicolor* (L.) Moench subsp. *drummondii* (Nees ex Steud.) de Wet & Harlan) and/or teff (*Eragrostis tef* (Zucc.) Trotter), as a forage mix (SARE 2007). Using this strategy, producers can plant both the summer annual grass and the legume (RC) in the same operation. Producers can then choose to either graze livestock on the resulting vegetation through the summer and fall, or harvest it mechanically as forage. After the summer annual grass senesces, the RC may persist through to the following spring, accumulating biomass and N until termination immediately prior to CS establishment.

Previous work pairing RC with winter annual grasses showed that growing RC with grasses can increase N fixation by the clover (Schipanski and Drinkwater 2011). In this way, the total amount of N provided by the RC can remain the same or even increase, due to the grass-related competition that increased both the rate of N fixation and legume biomass (Schipanski and Drinkwater 2011). If warm season grasses also exert this stimulatory effect on RC N fixation, due to their ability to draw down inorganic N availability in the soil, then RC N provisioning may be similar across all RC-containing treatments, making growing RC-summer annual grass forage systems a potential strategy for increasing overall RC-related N provisioning (Schipanski and Drinkwater 2012). This strategy could thus contribute to achieving the overarching goal of

increasing N provisioning to ultimately increase yields from agricultural systems overall, and, from organic systems in particular.

Diversifying CS and perennial forage rotations with summer annual grasses has additional appeal to organic livestock producers after implementation of the National Organic Program's 2010 Pasture Rule (USDA-AMS 2011). This rule stipulates that, during the grazing season, organic livestock must acquire more than 30% of their daily dry matter intake from grazed forage (USDA-AMS 2011). For organic producers in the Mid-Atlantic region, where hot, dry mid-summer conditions typically decrease cool season perennial forage production for a period dubbed the "mid-summer slump", there is additional demand for alternative warm-season forages that could provide complementary productivity and facilitate compliance with the Pasture Rule throughout the summer. This confluence of factors resulting in the need for additional research on the agronomic viability of summer annual forages to fill organic livestock producer's mid-summer slump, also created a fortuitous opportunity to quantify the relative benefits and drawbacks of manure-based versus legume-based N provisioning strategies within these systems featuring organic summer annual grazed forages rotated to CS.

METHODS

We assessed relative performance of manure-based versus RC-based N management strategies in terms of their impacts on soil inorganic N availability and yields in a 2012 through 2014 organic forage systems experiment conducted in Central Pennsylvania. At the time of this experiment, the land we used at Pennsylvania State University's Russell E. Larson Agricultural Research Center at Rock Springs had been under certified organic management for 9 to 11 years. We repeated the experiment on two adjacent sites: the first site ran from 2012 through 2013 ('2012 Start'), and the second site ran from 2013 through 2014('2013 Start'). The experiment included eight, 2-year forage system treatments: 7 annual forage-to-CS rotation treatments; and 1 perennial, grass-legume forage control treatment (Table 3-1). In the first year of the experiment, or 'Forage Phase', the annual systems consisted of: 3 grasses-only systems (manure-based N management strategy), 3 grass-RC mixtures (RC-based N management, var. 'Renegade'), and 1 RC monoculture (RC-based N management, var. 'Renegade'). Annual grasses included SSG (var. 'AS 6402 UT') and teff (var. 'Velvet'). All annual systems rotated to CS (var. 'Master's Choice 4050') in the second year, or 'Silage Phase' of the experiment, but the perennial system remained in OG (var. 'Niva')-RC (var. 'Renegade') throughout the experiment.

Between the 2 N management strategies, RC-based and manure-based, actual N management practices employed varied both between Forage and Silage phases as well as between systems under RC-based management in the Silage phase (Table 3-1). RC (var. 'Renegade') was the primary N source in all RC-based systems, planted at 13.45 kg ha^{-1} in the RC-CS system, 5.6 kg ha^{-1} in the bi-cultures (RC+ 1 grass), and 4.48 kg ha^{-1} in the tri-culture (RC+ both grasses). Grass seeding rates were as follows (monocultures, bi-cultures and tri-cultures,

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respectively): teff (5.6 kg ha^{-1}, 3.4 kg ha^{-1}, and 2.2 kg ha^{-1}), SSG (34 kg ha^{-1}, 17 kg ha^{-1}, and 11 kg

ha^{-1}), OG (6.7 kg ha^{-1}, bi-culture only). During the forage phase, RC was the only N input in

systems containing RC (4 annual systems and the perennial system). In contrast, for the manure-

based annual systems in the forage phase, manure was the only N input, added at a rate of 56

kg available-N ha^{-1} after the second forage cutting (11 September 2012 in Start 1 and 16 August

2013 in Start 2). The standard definition for manure available N in this region includes 80% of

ammonium-N (NH$_4$$^+$-N) and 35% of organic N present in the manure, when manure is

incorporated on the day of application(Beegle 2013). In the silage phase, the manure-based

systems all received a second manure application at 56 kg ha^{-1} available-N in early May 8-18

days prior to CS planting (03 May 2013 in Start 1 and 12 May 2014 in Start 2). Therefore, during

the silage phase in the manure-based systems, the primary N sources for the corn were 22-39 kg

ha^{-1} residual N from the previous manure applications, plus 56 kg ha^{-1} available N from the

spring pre-plant manure application, for a total of 78-95 kg ha^{-1}available manure-derived N

(Beegle and Wolf 2002). For the RC-based systems in the silage phase, there were two N

management strategies employed. For the RC monoculture rotating to CS, the RC was the only N

source added prior to silage establishment, estimated to supply 101 kg N ha^{-1}(Beegle

2015).However, for the RC-grass mixtures, RC was still the primary N source estimated to supply

67-101 kg N ha^{-1}, but supplemental manure N was also applied at 56 kg available-N ha^{-1} in

conjunction with RC plow-down in early May (03 May 2013 in Start 1 and 12 May 2014 in Start 2,

Beegle 2015). Additional detailed descriptions of agronomic management in each system are

available in Schrenker et al. (*in review*) and Table 3-1.

We relied on 5 seasonal soil N metrics to assess the efficacy of manure-based N provisioning compared to RC-based N provisioning in providing enough plant-available soil N to support yields, while minimizing the amount of N lost. Those metrics were: 1) early season soil nitrate-N (NO_3^--N), 2) growing season soil NO_3^--N, 3) growing season soil NH_4^+-N, 4) growing season inorganic N ($NH_4^+-N + NO_3^--N$), and 5) late season soil NO_3^--N. We standardized growing season measures to correspond with the length of the growing season in each phase of the experiment (weighted average, or interpolated sum divided by length of the growing season). The growing season in the first year of the rotation, or 'Forage Phase', was a 136-day period extending from 1 June to 15 October, which roughly encompassed the time from forage establishment through the last of the forage harvests in both the 2012 and 2013 Starts. The metrics for the second year of the rotation, or 'silage phase', were standardized to a 92-day growing season, extending from CS planting in mid-May to silage maturity in mid-August. We calculated growing season metrics by weighting daily measures of soil nitrate, ammonium and combined soil inorganic N, by the portion of the growing season represented by each measurement (Equation 1). We sampled soil monthly in the forage phase, and twice monthly in the silage phase. While exact timing of soil sampling within phases varied somewhat start-to-start, sampling in both phases of both starts began prior to N inputs and continued until the last month of the growing season (October in the forage phase, and August in the silage phase). In total, we collected 538 soil samples across all forage system plots to measure soil water and inorganic nitrogen content 8 times in the 2012 Start (August-October 2012 and May-August 2013), and 15 times in the 2013 start (June-October 2013 and April-August 2014).

Equation 1. Calculation of weighted growing season means

Growing Season Weighted Daily Mean

$$= [(\% \ grow.seas.up \ to \ meas.day \ 1, inclusive)$$

$$\times (amt.avail.on \ meas.day \ 1)]$$

$$+ \left[(\% \ grow.seas.meas.day \ 1 \ to \ 2)\right.$$

$$\times \left(\frac{amt.avail.meas.day \ 1 + amt \ avail.meas \ day \ 2}{2}\right)\bigg] \dots$$

$$+ \left[(\% \ grow.seas.meas.day \ n \ to \ meas.day \ n + 1)\right.$$

$$\times \left(\frac{amt.avail.meas.day \ n + amt.avail.meas \ day \ n + 1}{2}\right)\bigg]$$

$$+ [(\% \ grow.seas.from \ meas.day \ n + 1 \ to \ end \ of \ grow.seas., inclusive)$$

$$\times (amt.avail.on \ meas.day \ n + 1)]$$

Each soil sample consisted of 5 cores to 20-cm depth (450-500 g fresh weight). Immediately following soil collection into sealed plastic bags, we transferred all soil samples to ice chests for storage at ~4°C until analysis. For each sample, within 24 h of collection, we dried a ~10 g subsample at 60°C to determine gravimetric water content (GWC), and extracted inorganic N from a ~20 g subsample in 2 M KCl (5:1 solution: soil ratio) by shaking for 1h at ~180 rpm (Mitchell et al. 2013). We determined soil rock content by wet sieving each sub-sample to 2 mm after drying or KCl extraction. We then immediately filtered and froze soil KCl extracts as described by Mitchell et al. (2013). We determined soil NO_3-N and NH_4-N by absorbance in microplates using the vanadium (III) chloride reaction and salicylate methods, respectively (Doane and Horwath 2003, and Hood-Nowotny et al. 2010). We analyzed all soil KCl extracts in triplicate. We calculated daily plot concentrations of extract nitrate and ammonium from triplicate means, and converted to units of PPM using the dry mass of extracted soil (mg NO_3-N

or NH_4-N kg^{-1} dry soil). We summed daily soil nitrate-N and ammonium-N to calculate daily soil inorganic N by plot.

For the early season soil nitrate metric, we used soil nitrate concentrations (in parts per million, PPM) from a single measurement date. We chose this metric as an analog to the standard pre-side-dress soil nitrate test (PSNT). In the silage phase, we selected the soil nitrate measurement that was within the 35-40 days after emergence window specified for PSNT sampling to facilitate comparison to the established 21 PPM NO_3^--N threshold for corn in our region (Beegle 2013). Our early season soil nitrate sampling depth was only 20 cm, compared to the 25 cm specified for the PSNT, so our values may have been slightly higher than a true PSNT assessment. In the forage phase, we selected the soil nitrate measurement from the first date when we sampled soil in all plots, which, in both Starts, was too late to compare directly to the PSNT threshold for warm season annual grasses (80% of the corn threshold, or 16.8 PPM, Ketterings et al. 2003). However, while soil sampling occurred especially late relative to standard PSNT sampling in the forage phase of the 2012 Start, it still occurred prior to any manure application in the grass-only plots or legume incorporation in the other treatments. We managed manure inputs differently in the forage phase of the 2013 start, applying manure prior to grass-only forage establishment, which justified an earlier measure of soil nitrate to best quantify early season soil nitrate. Therefore, while exact timing of this measure differed from Start-to-Start, so did the timing of manure N application. Thus, the relative timing of the measurement to early season N availability was similar Start-to-Start, making this early season soil nitrate metric still potentially useful as a relative metric of early season soil N availability in the forage phase across both Starts.

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The other soil N metrics we utilized were all calculated on a kg N ha^{-1} basis to a 20 cm soil depth. To derive these metrics, we first transformed our daily measurements in PPM to daily measurements in kg N ha^{-1} using Start-specific 0-20 cm bulk densities. To calculate Start-specific bulk densities, we used estimates derived from our samples' mass and area. We weighed each soil sample (n=538) prior to subsampling for KCl extractions and GWC; which in conjunction with known core volumes, and subsequent measurements of soil moisture and rock content; allowed us to compute an estimate of rock-free, dry bulk density of each soil sample. While the relatively narrow diameter of our soil cores may have decreased the accuracy of any one estimate, the grand means of all bulk density measurements from each trial (1.32 g cm^{-3} for the 2012 Start, n=178; and 1.44 g cm^{-3} for the 2013 Start n=360) bracket the Web Soil Survey-reported bulk density for this soil (1.37 g cm^{-3}, USDA-NRCS 2015). The difference between our Start-specific bulk density estimates is consistent with the difference in soil structure we observed between Starts. The 2012 Start, where we measured a slightly lower bulk density 1.32 g cm^{-3}, exhibited a looser soil structure compared to the 2013 Start, which had a mean measured bulk density of 1.44 g cm^{-3}, and had a structure so dense as to interfere with seed emergence, requiring re-planting to achieve sufficient CS stand densities. These observations and bulk density estimate of 1.44 g cm^{-3} for the 2013 Start are in agreement with the threshold of 1.40 g cm^{-3} as the soil bulk density above which plant rooting and productivity decreases (USDA-NRCS 2015). Therefore, we used these Start-specific mean bulk densities for calculating all metrics requiring soil bulk density.

We calculated the other 3 seasonal soil nitrogen metrics -growing season soil NO_3^-N, growing season soil NH_4^+-N, and growing season inorganic N - from daily nitrogen

measurements following the procedure described above in Equation 1. In addition to soil

nitrogen availability, we also monitored soil water availability as GWC to 20 cm, which we

transformed into soil water to 20 cm using bulk density, and then to a "plant-available water" by

subtracting soil water content at permanent wilting point (14.2% water-filled pore space for

these soils) from total soil water (USDA-NRCS 2015). We then calculated a growing season

average plant-available water metric, using Equation 1 and daily, plot-level measurements of

plant-available water.

To determine efficacy of legume-based versus manure-based N management strategies

in achieving maximum yields, we strove to link soil metrics to yield. However, before delving

deeper into impacts of actual values of each soil metric on yield, we first assessed how yields

and soil metrics varied by planned experimental factors.

We used an expanded randomized complete block ANOVA procedure to discern effects

of the 3 planned experimental factors: 1) Start, 2) Rotation Treatment, and 3) Phase of the

Rotation; as well as interactions between those factors on yield and each soil N and water

availability metric (Table 3-2). In our analyses, we expanded the standard randomized complete

block ANOVA procedure to include Phase of the Rotation as a repeated measures factor (levels

were 'forage phase', otherwise known as rotation year 1, and 'silage phase', or year 2 of the

rotation), nested within each rotation treatment plot. We then treated Rotation Treatment as

the standard within-blocks factor, but also added the Start factor as a between blocks factor.

We built these models of yield and soil N and H_2O availability metrics using ANOVA procedures

available from the agricolae package in R (R Core Team 2014, Mendiburu 2014). For factors with

significant effects, we assessed differences between levels of the factor with Tukey's HSD post

hoc tests available in agricolae (R Core Team 2014, Mendiburu 2014).

Then, we used a linear regression approach available in SigmaPlot version 13.0 (Systat

Software, San Jose, CA) to directly test the relationships between each soil metric and yield

Sigma Plot. To insure the same spatial and temporal-scale between soil metrics and yield for the

regression analyses, we aimed to have all metrics represent the same standardized plot-level

spatial scale and growing-season temporal scale. We used plot-level growing season crop yield

by Phase, in conjunction with plot-level soil metrics: the 3 integrated seasonal soil N measures,

the single integrated seasonal plant available water metric, and the early and late season soil

nitrate metric. We assessed correlations by phase of the rotation yield and each N and season

average plant-available water metric across both Starts. To ensure similar yield potentials across

regression analyses within each phase of the rotation, and because the perennial had an

inherently different yield potential than the annual treatment in either the forage or silage

phase, we excluded data from the perennial treatment from all regression analyses (n=56 for all

regression analyses).

We compared mechanically-harvested yields and soil metrics between manure-

management treatments (n=12 per Start, n=24 combined) and RC-based N management

treatments (n=16 per Start, n=32 combined) by calculating means and standard errors by

strategy (both within Starts, and then across starts), for each metric. Then, we used t-tests on

the soil N metric most strongly correlated with yields in each phase of the rotation (SigmaPlot,

Systat Software, San Jose, CA). For datasets that did not pass the Shapiro-Wilk normality test,

we used Mann-Whitney Rank Sum Tests in place of t-tests to assess whether water availability,

N availability, or yield differed between plots under manure-based N management compared to those under RC-based N management (SigmaPlot, Systat Software, San Jose, CA). We repeated these analyses for each phase of the experiment.

RESULTS AND DISCUSSION
Multiple Factors and Factor Interactions Significantly Impacted Yields and Soil N Availability

All factors and factor interactions significantly affected soil nitrate and overall soil inorganic N concentrations, which was not surprising given the importance of both management (i.e. rotation treatment, and the phase of the rotation) and weather conditions specific to each growing season (i.e. Start x Phase interaction effect) in determining both early season and total season N availability (Table 3-2, Figure 3-1). In contrast to N, soil water availability was primarily affected by growing season (Start effect, $p < 0.05$). Yields, the result of the combined effects of N availability, water availability, crop present, and other variables such as weed competition, were also significantly impacted by all experimental factors and factor interactions except block ($p = 0.58$) and an interaction between Start and Rotation Treatment ($p = 0.09$). The lack of a significant Start x Rotation Treatment Effect indicates that the Rotation Treatment effect did not vary by Start, even though Start itself did have a significant effect on yields ($p < 0.05$), likely because of the significant difference in Water Availability ($p < 0.05$) and all N metrics except ammonium ($p < 0.01$) between Starts .

Yield Limitations Unrelated to Management: Early Season Drought and Water Availability

Water availability likely explains year-to-year yield variability. Higher soil water availability may have been an important driver of higher yields in 2013 compared to 2012. Similarities between factor effects on yield compared to factor impacts on soil metrics elucidate

when each soil metric was most important in determining yields (Figure 3-1). Yields were

significantly higher in the 2013 Start compared to the 2012 Start, and soil water availability was

also higher in the 2013 Start compared to the 2012 Start (Figure 3-1a and 3-1b). This difference

was consistent within each phase of the experiment, with significantly less plant available water

in both the forage and silage phases of the 2012 Start compared to the 2013 Start (Mann-

Whitney Rank Sum Tests: forage phase, Figure 3-2a, $p<0.0001$; silage phase, $p<0.0001$; data not

shown). However, mean growing season plant available water was significantly correlated with

forage yields only (Figure 3-2c, linear regression, slope significant at $p<0.0001$).

Water (precipitation) distribution may have been as important as total amount of water

in influencing yields. Despite silage phase mean plant available water and silage phase yields

also being significantly lower in the 2012 Start compared to the 2013 Start, silage phase yields

were not significantly correlated with plant available water ($p=0.43$ for slope of linear regression,

data not shown). Rather than suggesting that forages are more sensitive than silage to water

limitation, these results suggest that differences in the distribution of water availability within

the forage versus silage growing seasons may have been critical. Even though growing season

mean water availability was similar in the 2012 Start forage phase and silage phases, silage

phase precipitation was much more evenly distributed throughout the growing season. In

contrast to the 2012 Start silage phase, the 2012 forage phase was characterized by early season

drought, followed by two extremely large rain events in August and September, which increased

mean growing season plant-available water in the soil, but came too late to increase forage

yields.

Annual county-average CS yields 2012-2014 also reflect impacts of the 2012 drought, when Centre county silage yields were only ~75% of either 2013 or 2014 silage yields (USDA-NASS 2015). County average CS yields were the same in 2013 and 2014, further suggesting that in terms of yield-reducing drought, only 2012 was the only year among the three years included in this study where timing of drought suppressed CS performance (USDA-NASS 2015).

Water Availability- Forage Yield Relationship

Because crops in each phase of the rotation have different yield potentials, we examined the relationship between soil water availability and yield in each phase of the rotation separately. In the forage phase, we observed a larger spread and contrast in soil water availability and in yields between 2012 and 2013, compared to the silage phase, when soil water availability was similar across both starts. 2012 Forage water availability and 2012 forage yields were 75% and 43% of 2013 forage yields and water availability, respectively (Figure 3-2a and 3-2b). Growing season mean plant available water was positively correlated (p<0.0001) with forage yields (n=64, Figure 3-2c). Each 1-cm increase in growing season mean soil water availability corresponded with more than 3600 kg ha^{-1} increase in forage yields, and growing season mean soil water availability explained 43% of the variability in forage yields (Figure 3-2c).

Soil N Availability Also Limited Forage Phase Yields, Likely by Increasing Weed Pressure

Soil N availability was also correlated with forage yields, however not in the way we expected (Figure 3-3a). Soil N availability was negatively correlated with forage yields; with growing season mean soil inorganic N availability explaining another 24% of forage yield variability (p<0.0001, yields decreased 242 kg ha^{-1} for each 1 kg growing season mean inorganic N ha^{-1}). This relationship suggests that under the dry growing conditions that so strongly

suppressed forage productivity in 2012, higher soil N availability suppressed yields. While limited forage growth, reduced inorganic N uptake by crops, and low leaching may partially explain the higher soil inorganic N availability we observed in 2012 compared to 2013, other factors likely influenced the significant inverse relationship we observed between soil inorganic N availability and forage yields (Figure 3-3a).

As organic systems rely on a suite of biological mechanisms, including good establishment of a highly competitive crop, to manage weed-crop competition; organic systems are more vulnerable to weed competition when crop establishment is poor than in herbicide-managed systems (Figure 3-4). A plausible biological explanation for these results is that crop-weed-N availability feedbacks stemming from poor forage crop establishment resulted in this inverse relationship between N availability and forage yields (Figure 3-4, scenario #2). Poor crop establishment allowed weed establishment and inorganic N accumulation in the soil. Increased N availability further encouraged weed growth, and through this increased weed competition *indirectly* negatively impacted forage yields (Figure 3-4). Although weed biomass data is not available for 2012 when we observed both the higher soil inorganic N availability and the poor forage performance driving this relationship, we did observe extremely dense stands of Canada thistle (*Cirsium arvense* (L.) Scop.) in the 2012 forage plots. In fact, Canada thistle stands were so dense at the first 2012 forage harvest that we were not able to harvest any forage from the Teff x SSG and Teff x SSG x RC treatments, although there were harvests from those treatments at the second and third harvests of 2012 (Figure 3-4, T+SSG and Canada thistle in 2012*)*.

Shortcomings of N Management in the Forage Phase and Opportunities for Improvement

Forage phase yield and soil N contrast between N management strategies reveal some important short-comings in N management that have implications for N loss from organic forage systems, and reveal some key opportunities for improvement. These interpretations stem from comparisons between yield and 3 key N metrics: early season soil nitrate levels, growing season mean soil inorganic N availability, late season soil nitrate. Differences in each of these N metrics between Starts translate into 3 different morals of N management for the forage phase of this experiment.

Moral #1: Forages Needed More Early Season N.

Despite being an analog to the early season soil nitrate test, a well-established metric for assessing N availability in forage systems, early season soil nitrate was not significantly correlated with forage phase yields in this experiment (either across starts or within each start, p>0.05). However, Ketterings et al. (2003) report 16.8 ppm NO_3-N the PSNT threshold for warm season annual grasses at which additional N is unlikely to increase yields. The timing of our measurement was too late to compare directly with the PSNT threshold in either Start, but both the mean (2.3 ppm, 13.7% of threshold, n=64) and the maximum (8.6 ppm, 51% of threshold, 5 July 2013) early season soil nitrate level we measured in the forage phase across both Starts and all rotation treatments suggest early season N availability limited forage phase productivity (Ketterings et al. 2003). Despite this PSNT-indication that N should have been highly limiting across all rotation treatments, there were differences between the grass-only treatments and the annual RC-containing treatments that that suggest N was less limiting in the plots containing RC, despite also having very low early season nitrate. This was surprising given that the RC represented <20% biomass in all RC-containing treatments, and that, at least in the 2013 Start,

the grass-only plots had received manure estimated to provide 56 kg N ha^{-1} prior to the early

season soil nitrate measurement. Mean forage phase yields were 886 kg ha^{-1} and 203 kg ha^{-1}

higher, although not significantly, in RC-containing annual treatments compared to grass-only

forage treatments in 2012 (3584 vs. 2699 kg ha^{-1}, t-test p=0.11) and in 2013(7231 vs 7029 kg ha^{-1},

t-test p=0.34, Figure 3-3b). Including the perennial RC-containing treatment in this analysis does

not change the difference in yields between RC-containing and grass only treatments in 2012,

but including the perennial treatment in the mean of red-clover containing treatments does

increase the difference in 2013 to 589 kg ha^{-1} (t-test, p=0.11). This is likely because the 2013

Start forage phase perennial orchardgrass-RC stand was in its 2nd year, and was benefiting from

higher N availability from the established RC in the stand. Across both Starts, early season soil

nitrate was 39% higher in the annual forage treatments that contained RC compared to the

grass only treatments (data not shown). This difference was especially marked in the 2013 Start,

when early season soil nitrate was 125% higher in the RC-containing annual forage plots. Overall,

these results were all lower than the established PSNT threshold for summer annual forages,

suggesting that both RC-containing and grass-only annual forages yields could increase with

additional early season manure or other N inputs, but that the RC-containing plots would

require less than the grass-only plots, even when the portion of RC in the forage stands is

minimal (8-25%).

Take-Home # 2: Assess Weed Abundance & Modify N Management Accordingly.

Increasing growing season soil inorganic N availability is not guaranteed to increase crop

yields. In fact, as evidenced by the inverse relationship between growing season mean soil

inorganic N availability and forage yields we observed, more soil inorganic N may even decrease

forage yields. We hypothesize this effect was driven by an indirect mechanism, wherein higher

soil inorganic N availability increased weed-crop competition, thereby reducing forage yields

(Figure 3-4). This potential soil inorganic N – weed pressure – forage yield relationship

encourages a conservative approach to N management, especially in organic cropping systems

where weed exploitation of available soil N cannot be corrected with herbicides. To achieve

higher yields in organic annual forages producers must be especially cognizant of this balance,

and in situations where weed populations are high and crop establishment is low, such as in our

2012 forages, electing against further N additions may be the best option to avoid further

stimulating the weeds. In general, assessing weed populations and communities, and, where

weed abundance is high, managing N at lower than optimal rates may be the best option.

"Optimal" N management, or managing to achieve N levels recommended for conventional

system can increase weed-crop competition, possibly causing even greater weed competition-

related reductions in forage yield than would have been caused by N limitation alone.

Take-Home #3: Apply Early or Not at All.

During the forage phase, grass-only forage mixes in both Starts received manure at 56

kg available-N ha^{-1}. However, the timing of manure application varied Start-to-Start. In the 2012

Start, manure was not applied until early September, immediately after the second harvest and

about 6 weeks prior to the final harvest and first frost of the year. In contrast, manure

application in the 2013 Start occurred immediately prior to establishment. By mid-October,

when late-season soil nitrate measurements were taken in both Starts there were huge

differences (Figure 3d). In 2012, late season soil nitrate in the grass-only treatments averaged

35-45 kg NO$_3^-$-N ha^{-1} compared to <5 kg NO$_3^-$-N ha^{-1} in those same treatments in the 2013 Start.

These late-fall soil nitrate levels in 2012 likely contributed to concerning rates of over-winter leaching losses of nitrate from those grass-only treatments where frost had already killed the summer annual grasses and there was no over-wintering cover crop. These results suggest current forage N management recommendations that call for a manure application in early September can result in concerning quantities of end-of-season soil nitrate, likely to be lost via leaching over the subsequent winter (Craig 2015). This accumulation probably occurred because warm season annual forages begin senescing earlier than the cool season perennials for which these recommendations were likely developed, and thus are less effective at mitigating high soil inorganic N resulting from late season N applications. Including RC in the mixes could mitigate this NO_3^- leaching risk by several mechanisms. First, it would reduce the demand for external N inputs in the forage phase. Second, as a winter hardy legume that accumulates most of its biomass (and N) in the spring, its fall through winter biomass represents a very low NO_3^- leaching risk, having very little biomass and therefore N, and retaining most of the N it does have in non-leachable organic forms (Schipanski and Drinkwater 2011). Overall, the main conclusion is that late summer is too late to apply manure to summer annual grasses. Late manure applications were neither agronomically nor environmentally beneficial. Agronomically, they conferred no yield benefits, and, depending on the circumstances (i.e. crop vs. weed establishment, and/or low soil water availability), may actually lower forage yields by increasing weed pressure. Environmentally, they pose a nitrate leaching hazard by resulting in high late season soil nitrate levels. Applying manure to annual forages in spring, or relying on RC or another legume to supply N are strategies that appear to avoid all of these issues.

RC-based N Management Increases Soil Inorganic N Availability and Silage Yields

Yield and soil N availability benefits of including RC in the annual rotation treatments were clearer in the silage phase. Because the perennial system is inherently different from CS in terms of yield potential and yield-N relationship, and because our objective was to compare the efficacy of manure-based versus RC-based N management strategies in **annual** forage systems, we excluded the perennial treatment from these silage phase analyses. Water availability in the silage phase had no significant relationship with yield, and did not differ between Starts (linear regression R^2=0.006, p=0.43). Without water limitation hindering silage establishment and completely inverting the impact of N availability on yield, all metrics of N availability, except growing season mean soil NH_4^+-N availability, were significantly positively correlated with silage yields across starts (linear regressions, yield vs. early season soil nitrate, growing season soil nitrate, and growing season inorganic N, R^2=0.12-0.18, slopes significant at p<0.0001 for all). Early season soil nitrate (ppm) was most strongly correlated with CS yields, with early season soil nitrate explaining 18% of variability in CS yields (linear regression, slope=215 kg ha^{-1} yield increase per 1 ppm NO_3^--N increase, p<0.001), so we selected it as our primary metric of silage phase N availability (Figure 3-5a). However, compared to typical correlations reported between early season soil nitrate availability and yield on N-responsive sites (R^2=0.76-0.98), the early season nitrate availability-yield relationship we observed was relatively weak (Heckman et al. 1996).

To understand how N management strategy impacted N availability and CS yields, we compared N availability and yield results from RC-containing annual treatments to grass-only treatments that had received two, 56 kg NH_4^+-N ha^{-1} manure applications, once in the forage phase and again prior to silage establishment. Annual rotation treatments that included RC had

higher levels of all soil N availability metrics except growing season NH_4^+-N, as well as higher CS

yields compared to grass only rotation treatments that relied on manure applications for N

provisioning (Figure 3-5b and 3-5c). Despite the higher soil N availability and correspondingly

higher yields in the RC-containing treatments compared to the grass-only treatments, N

availability, even in the RC containing treatments, was still low compared to the established

early season soil N availability PSNT critical level of 21 ppm (Figure 3-5b). However, assuming 45%

DM, we did observe many yields that compared favorably to the Web Soil Survey rating for well

managed CS on these soils (24-27 tons acre^{-1}, or ~12,000-14,000 kg DM ha^{-1}) as well as to the

county average silage yields for 2013 and 2014, (23-24 tons acre^{-1} or ~12,000 kg DM ha^{-1}, USDA-

NRCS 2015, USDA-NASS 2015, Figure 3-5a).

These higher-yielding CS plots within this experiment offer insights into how to further

increase organic CS yields. Plots with yields in the top quartile had yields ranging from 14320-

17709 kg ha^{-1}. Three-quarters of these top-quartile yielding plots were managed with RC-based

N strategies. And, among the 12 plots yielding >15,000 kg ha-1 CS, mean ± S.E. early season soil

nitrate was still only 15.01 ± 1.73 ppm, much lower than the 21 ppm threshold established for

CS, especially considering early season soil nitrate ranged all the way down to 5 ppm in these

high yielding corn silage plots. This suggests that the 21 PPM early season soil nitrate threshold

may be too high for organically-managed CS, for one of three reasons. First, N cycling may be

enhanced on these sites, such total growing season inorganic N availability is similar between

these higher-yielding sites with ~15 PPM early season nitrate and other sites that have >21 ppm

early season nitrate. Alternatively, growing season nitrate N may indeed be lower in these

organic systems, but there may be other plant-available N pools that compensate, supporting

similar levels of N nutrition as sites with higher nitrate N availability. Or, lastly, this could be related to high early season soil nitrate (or higher overall growing season inorganic N availability) increasing weed competition and resulting in a net yield reduction (i.e. Figure 3-3c, Figure 3-4).

Evidence from this study and previous research provides support for the explanation that either or both current year management and long-term management legacies contribute to the phenomenon of relatively-low early season soil nitrate coupled with high yields (Heckman et al. 2011, Heckman et al. 1996). The short-term legacy of the RC may be a longer release of soil N throughout the season, reflected by the persistence of higher soil nitrate availability through the CS maturity in August (data not shown). Research that compared soil inorganic N levels and yields in sweet corn following large organic N inputs also found that sweet corn yields were much higher that would have been predicted via PSNT testing, further suggesting that short-term changes induced by plant-based organic N inputs could reduce the effectiveness of PSNT testing (Heckman et al. 2011). Grain corn following alfalfa and 10-year grass sod crops were also characterized by PSNT levels 5-7 ppm below the PSNT critical level, yet displayed no response to sidedress N rates ranging from 0-224 kg N ha-1 (Heckman et al. 1996). Short-term change explanations could be increased rates and durations of cycling organic N to inorganic N (Drinkwater et al. 2011). Or, the benefits in N availability could stem from a non-nitrate pool of plant available N, either ammonium or even amino acids (e.g. Heckman et al. 2011). These mechanisms and/or pools could also explain N availability changes stemming from long-term changes induced by the experimental sites' long (10-11 years) history of organic management (Drinkwater et al. 2011). While soil organic matter was not anomalously high in either Start 1 or Start 2, it could be that this long legacy of organic management has developed microbial

communities that cycle organic matter to plant available N more quickly (Grantham et al. *in prep,* Drinkwater et al. 2011). Regardless, these results suggest further calibration of the PSNT may be necessary for organic systems, especially for CS following a legume cover crop like RC, and/or for sites with a long history of organic management.

Conclusions

Despite the prevailing perspective that low soil inorganic N is the main N problem in organic systems, we observed a relatively weak to inverse relationships between soil test inorganic N availability and CS and annual forage yields, respectively. These initially surprising and counter-intuitive observations suggest there may be important differences in the structure and function of organic systems, especially those with a long legacy of organic management, compared to systems managed primarily with inorganic N and herbicides (Figure 3-4). These results also point to several opportunities to improve N management and increase yields in organic systems. In the forage phase, increasing N provisioning via higher rates of early season manure in both systems has potential to improve yields, while reducing or even eliminating later season manure applications can reduce nitrate loss potential while potentially improving yields even further. In the silage phase, RC-based N management appeared to provide sufficient N to support high CS yields, regardless of whether spring ground cover was 50% or 90%, and despite actual levels of inorganic N being less than thresholds established systems under conventional management. In contrast, doubling the silage phase manure applications would be necessary to achieve inorganic N availability similar to that provided by the RC alone. Thus, for CS, using higher rates of manure N than used in this study, but still far lower than regularly recommended based on the PSNT is likely warranted to further improve organic CS yields under manure-based

N management. However, across both starts, phases, and N management strategies; these data

all suggest that practices to increase early crop densities and/or vigor may ultimately support

greater yield increases in organic systems than increasing N provisioning via either legumes or

manure alone. Our results highlight the crucial role of a competitive crop in organic systems and

emphasize the importance of a judicious approach to inorganic N management in organic

systems, where too much inorganic N availability can actually decrease yields.

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TABLES

Table 3-1. N Management by system and phase of the rotation

System	N Management Strategy	Forage Phase N Supplied (kg ha^{-1})		Silage Phase N Supplied (kg ha^{-1})	
		Manure	Legume	Manure	Legume
OG+RC	RC-only	0	*	NA	NA
RC >CS	RC-only	0	**	0	101
SSG+RC > CS	RC-based	0	***	56	45-67
Teff+RC > CS	RC-based	0	***	56	45-67
SSG+Teff+RC > CS	RC-based	0	***	56	45-67
SSG> CS	Manure-only	56	0	78-95	0
Teff > CS	Manure-only	56	0	78-95	0
SSG+Teff > CS	Manure-only	56	0	78-95	0

*RC made up ~48% of the stand, and thus likely provided sufficient N to meet the N needs of the OG (Schrenker et al. *in review)*

**RC assumed to meet its own N needs via symbiotic N fixation

***RC made up <20% of the stand and thus likely contributed little to the N requirement of the grass(es).

Abbreviations: RC= red clover, CS= corn silage, OG = orchardgrass, SSG=sorghum sudangrass

Table 3-2. Effects of Experimental Factors on Annual Yield and Annual Soil Nitrogen and Water Metrics *(results of ANOVAs)*

Factor or Factor Interaction *(Meaning of significant factor interactions)*	Metric						
	Yield	Plant Avail. Water	Early Season Nitrate	Soil Inorganic N	Soil Nitrate	Soil NH_4^+-N	Late Season Soil Nitrate N
	$kg\ ha^{-1}\ yr^{-1}$	cm	ppm NO_3^--N	kg Inorganic $N\ ha^{-1}$ (growing season daily mean)	$kg\ NO_3^-$-$N\ ha^{-1}$ (growing season daily mean)	$kg\ NH_4^+$-$N\ ha^{-1}$ (growing season daily mean)	$kg\ NO_3^-$-$N\ ha^{-1}$ (measure at crop maturity/end of season)
Start	**p<0.05**	**p<0.05**	**p<0.01**	**p<0.001**	**p<0.01**	p=0.53	**p<0.01**
Block	p=0.54	p=0.83	**p<0.01**	**p<0.01**	**p<0.05**	p=0.40	**p<0.05**
Rotation Treatment	**p<0.05**	p=0.59	**p<0.001**	**p<0.001**	**p<0.001**	**p<0.05**	**p<0.01**
Start x Rotation Treatment *(Rotation trt. effect differed between the 2012 Start &2013 Start)*	p=0.09	p=0.87	**p<0.05**	**p<0.05**	**p<0.05**	p=0.17	**p<0.05**
Phase of Rotation	**p<0.01**	p=0.74	**p<0.001**	**p<0.001**	**p<0.001**	**p<0.01**	**p<0.001**
Phase of Rotation x Start *(effect of specific growing season)*	**p<0.01**	**p<0.05**	**p<0.001**	**p<0.001**	**p<0.001**	p=0.58	**p<0.001**
Rotation Trt. x Phase *(Rotation treatment effect differs between Forage Phase & Silage Phase)*	**p<0.01**	p=0.17	**p<0.001**	**p<0.001**	**p<0.001**	p=0.12	**p<0.05**
Start x Rotation Treatment x Phase *(Rotation trt. effect is growing season-specific)*	**p<0.01**	p=0.68	**p<0.05**	**p<0.05**	**p<0.001**	p=0.74	p=0.44

FIGURES
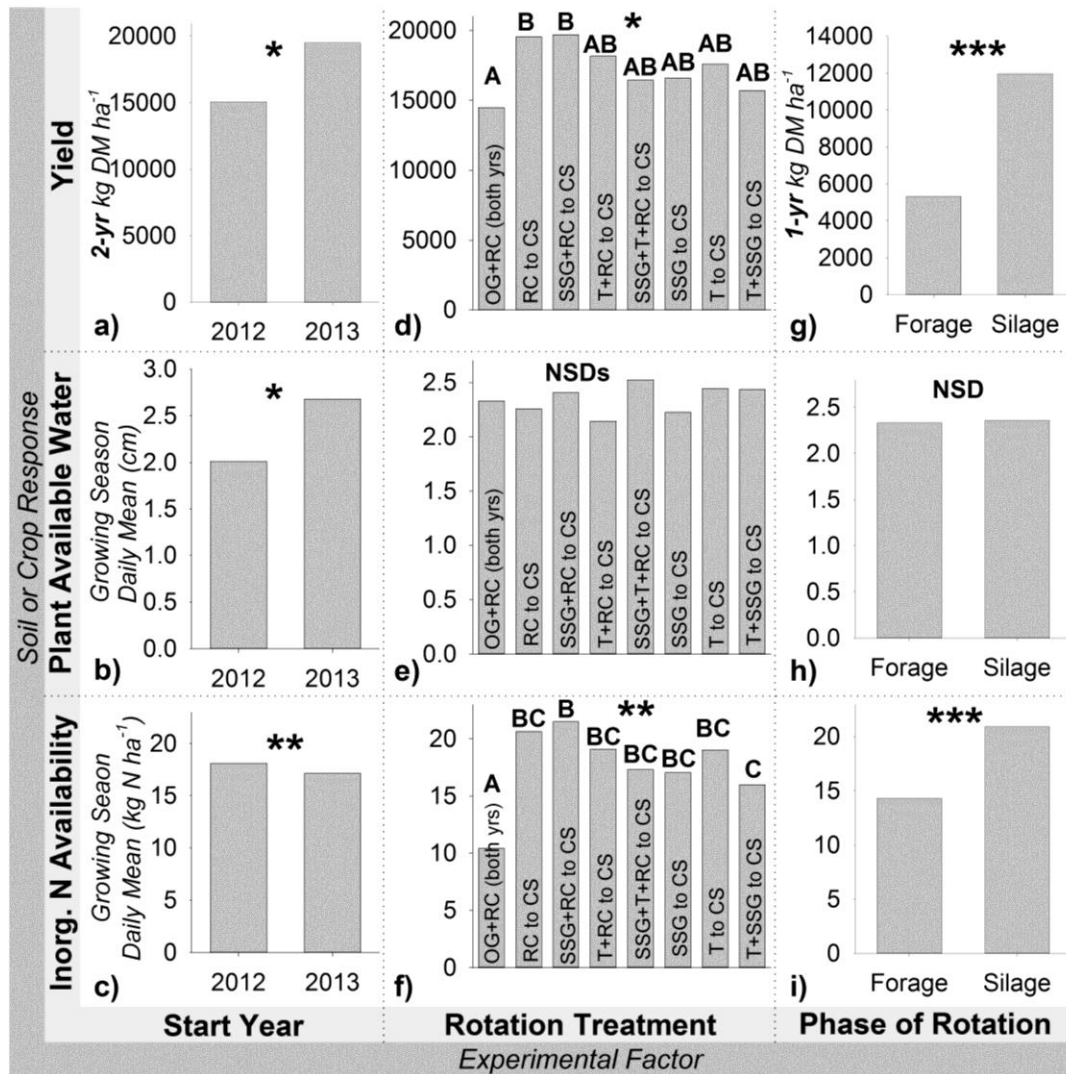
Figure 3-1. *Experimental Factors' Main Effects on Crop Yields, Plant Available Water, and Soil Inorganic N Availability as determined by ANOVAs.* Asterisks indicate significance level of factor effect for all factors (* indicates $p<0.05$, ** indicates $p<0.01$, and ***indicates $p<0.0001$). For the factors with just two levels, Start and Phase of Rotation, astericks also indicate that there is a significant difference between the two means (n=32 for each bar). For mean differences between Rotation Treatments, different capital letters indicate significant differences, as detected by Tukey's HSD post hoc tests, between Rotation Treatment means ($p<0.05$, n=8 for each). Crop abbreviations in the rotation treatments are: OG= orchardgrass, RC=red clover, CS=corn silage, SSG=sorghum sudangrass, T=Teff.
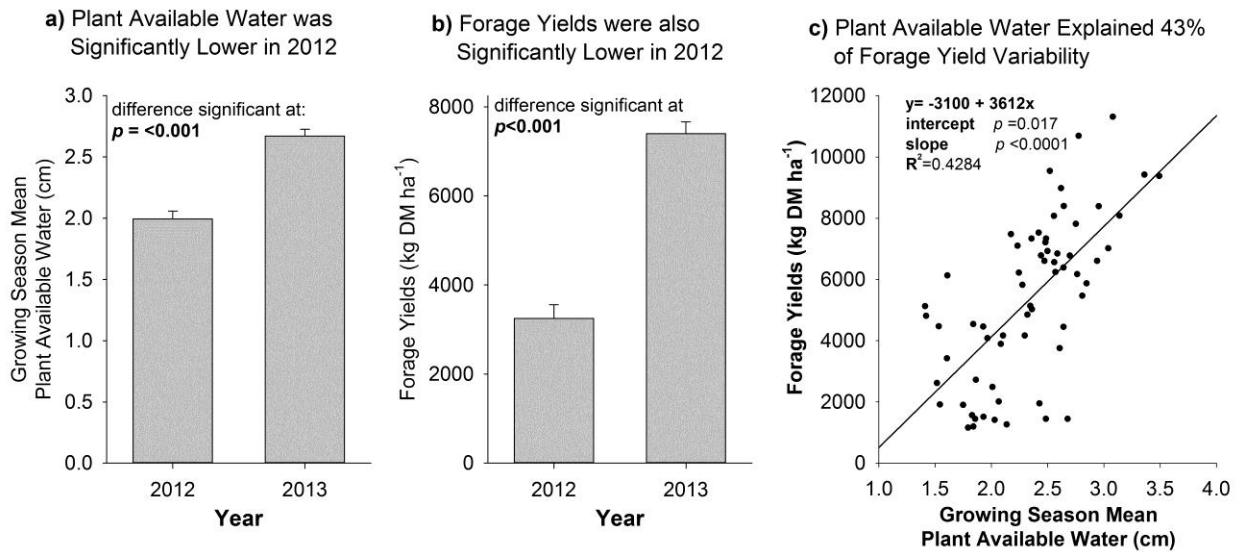
Figure 3-2. *Water availability impacted forage yields.* Mean growing season plant available water and forage yields were both lower in 2012 and higher in 2013 (a and b, respectively). Plant available water data passed normality (Shapiro-Wilk) and equal variance tests (Brown-Forsythe), therefore significant differences between years was determined by a t-test (p<0.001). Yield data failed the Shapiro-Wilk normality test; therefore a Mann-Whitney Rank Sum Test was used to determine significant year-to-year differences in forage yields. Over both years, plant available water significantly correlated with forage yields (c).

a) Growing Season N Availability

b) Annual Forage Yields

c) Relationship between Growing Season N Availability and Yield

$y = 8391 - 223 x$
intercept p<0.0001
slope p=0.0003
R^2=0.22

d) Risk of Post-Season Nitrate Leaching
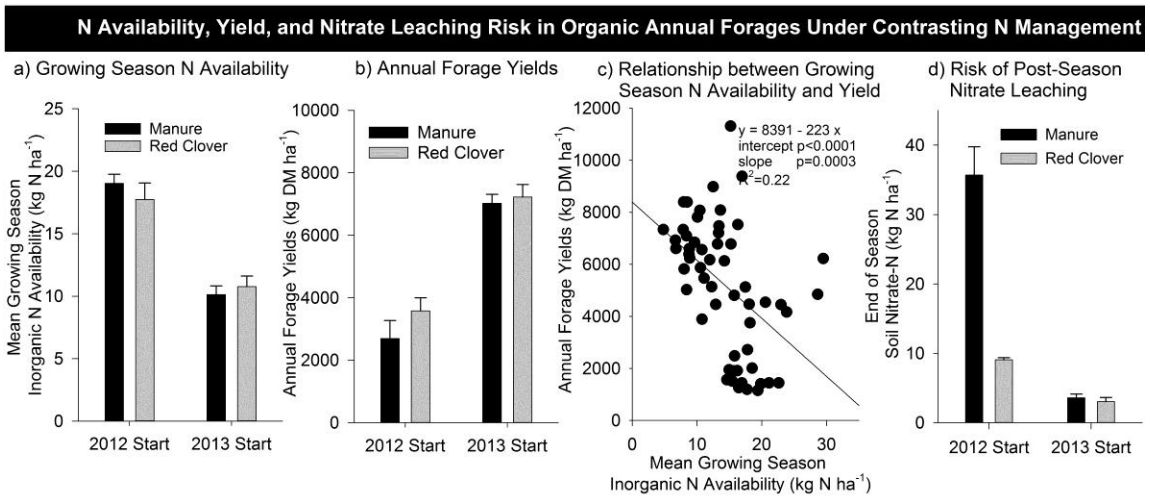
Figure 3-3. *Impacts of N Management Strategy during the Forage Phase.* Both red clover-based and manure-based N management strategies resulted in similar growing season N availability and yields in the annual forages (a, b; bars denote means and standard errors; n=12 for manure bars and n=16 for red clover bars). However, growing season daily mean soil inorganic N availability was negatively correlated with annual forage yields (c). Lastly, while growing season inorganic N availability was similar across both N management strategies within each Start (a), in 2012 there was about 4 times as much end-of-season soil nitrate left in annual plots under manure-based N management than in the plots under red clover-based management (d). The lack of this difference in 2013 (d), suggests that differences in timing of manure management between 2012 and 2013 were at the root of this contrast rather than anything inherent to manure-based management itself.
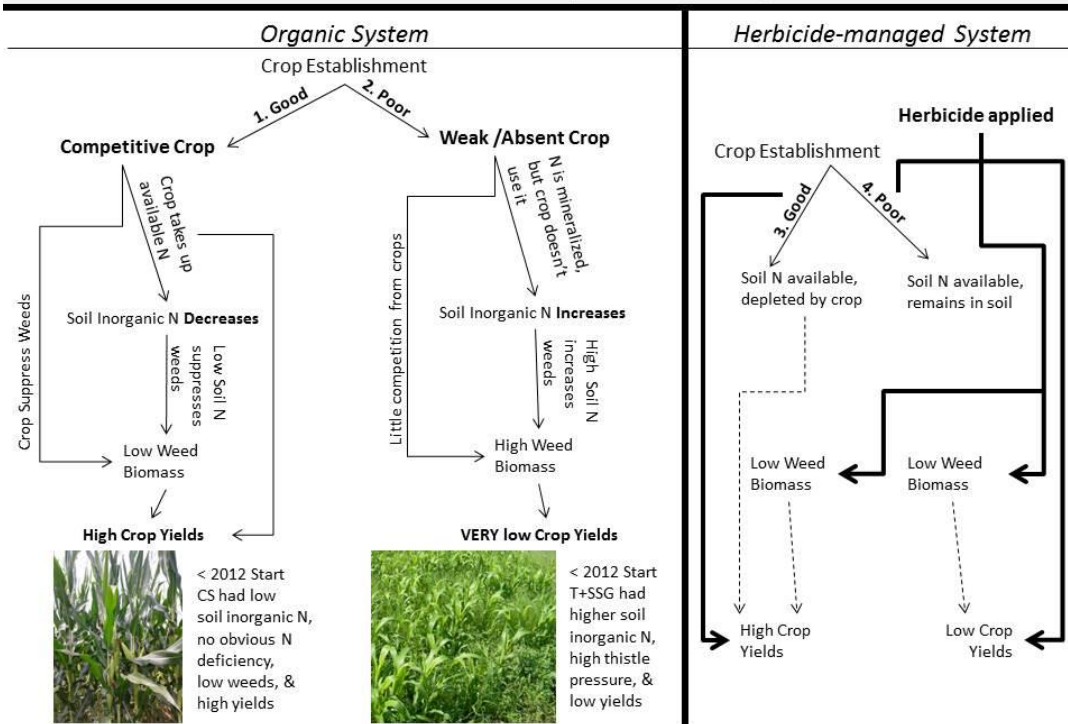
Figure 3-4. *Positive Feedbacks between Crop Establishment, N Availability and Weeds Have a Larger Role in Determining Organic System Crop Yields.* This hypothesized conceptual model shows crop establishment impacts subsequent yields in organic systems via direct and indirect impacts on soil inorganic N availability and weed biomass. Crop establishment in herbicide-managed systems also impacts yields, although in those systems its role is largely direct. In herbicide-managed systems, crop establishment impacts on weed biomass are largely eliminated via near-complete herbicide control, which also limits weed impacts on subsequent crop yields. Thick arrows indicate dominant processes or relationships, whereas dashed arrows indicate relationships with limited effect on yield relative to crop establishment, and assume high herbicide efficacy and sufficient N availability. Abbreviations are CS=corn silage, T+SSG=teff and sorghum Sudangrass.

Red Clover-based N Management Increased Silage Phase N Availability and Corn Silage Yields

a) Spring Pre-plowdown Red Clover Groundcover was Significantly Higher in the 2012 Start (*p*<0.0001)

b) Red Clover-based N Management resulted in Significantly Higher corn Silage Yields in the 2013 Start

c) Red Clover-based N Management resulted in Significantly Higher corn Silage Yields in the 2013 Start

d) Corn Silage Yields were Significantly Positively-correlated with Early Season Soil Nitrate

Figure 3-5. *Effects of N Management Strategy on N Availability and Yield in the Silage Phase.* Despite start-to-start differences in red clover performance **(a),** mean early season soil nitrate availability **(b)** and yields **(c)** were higher in plots under red clover-based N management compared to plots that received manure applications (56 kg ammonium-N ha^{-1} yr^{-1} in both years). Corn silage yields were significantly positively-correlated with early season soil nitrate **(d).** Within each Start, n=12 for systems under manure management, and n=16 for systems under red clover management. For the linear regression (d), n=56. Due to differences in sample size, we compared means by N management strategy both within each start, and then across Starts (n=24 for manure treatments, and n=32 for red clover treatments) using t-tests for all data sets that passed Shapiro-Wilk and Brown-Forsythe tests of normality and equal variance, respectively. Only the red clover % cover data, and the across Start early season soil nitrate data sets failed normality tests, so Mann Whitney Rank Sum tests were used to determine whether significant differences existed between those groups (a and c).

Chapter 4

A Tale of Two Tracers: Quantum Dots as an Alternative to Stable Isotopes for Tracking Plant Nitrogen Nutrition

ABSTRACT

Recent research increasingly demonstrates that amino acids (AAs) play a much larger and more direct role in plant N nutrition than previously believed. However, methodological limitations of traditional isotopic tracer methods have complicated and limited study of the AA uptake pathway, especially at lower field-relevant AA concentrations. Quantum dots (QDs) are an alternative tracer method, effective for quantitatively tracking plant uptake of AAs at moderate concentrations. We compared QD-labeling to isotope labeling (^{13}C and/or ^{15}N) for tracking uptake of glycine (Gly), serine (Ser), arginine (Arg), asparagine (Asp), and NH_4NO_3 by 4 agricultural plant species: orchardgrass (OG, *Dacylis glomerata* L.), red clover (RC, *Trifolium pratense* L.), sorghum sudangrass (SSG, *Sorghum bicolor* ssp. *drummondii*), and corn (Zea mays L.). We assessed maximum possible performance and actual performance of each label in terms of detection in plant shoot tissue of each species. Our results suggest QDs offer a promising alternative for tracking AA and NH_4^+ uptake in shoot tissue of multiple plant species, and that QDs offer greater detectability than ^{13}C, but less than ^{15}N in plant shoot tissue.

INTRODUCTION

Recent work on plant nitrogen (N) nutrition has challenged long-prevailing paradigms

around the exclusivity of mineral N nutrition for plants and spawned renewed interest in

tracking plant uptake of various organic N compounds alone and relative to inorganic N

compounds (Neff et al. 2003, Nasholm et al. 2009, Puangfoo-Lonhienne et al. 2012). Research is

rapidly expanding beyond documenting organic N uptake by mycorrhizal plant species from

inorganic N-limited ecosystems to include direct uptake by non-mycorrhizal plant species, and

even by crop plant species (Chapin et al. 1993, Kielland1994, Nasholm et al. 2000, Weigelt et al.

2005, Puangfoo-Lonhienne et al. 2008, Whiteside et al. 2009, Puangfoo-Lonhienne et al. 2010,

Whiteside et al. 2012). As basic plant uptake abilities are more widely documented, the new

research questions around this seemingly broadly occurring phenomenon increasingly shift to

focus on ecological and biogeochemical interactions governing plant uptake of divergent N

forms. These new questions challenge the limits of established isotope tracer techniques and

inspired this search for an alternative method (Jones et al. 2005b).

Despite accumulating evidence that direct plant uptake of organic and especially AA-N

may be an important N-uptake pathway, Jones and colleagues (2005b) questioned the relevance

of this pathway by documenting several important shortcomings of established stable or

radioactive isotope methods, upon which almost all of previous plant organic N uptake

literature is based. While dual-labeled isotopic tracers applied at 1-8mM are capable of

documenting intact AA uptake, those rates are far in excess of measured soil AA concentrations

(Jones et al. 2005a). Jones and colleagues (2005a) found that plant AA uptake (in terms of %

applied) increased up to 25-fold between AA concentrations of 0.1-10 μM, and AA

concentrations of 1-10 mM; suggesting that plant AA uptake rates are dependent on the

concentrations of labeled tracer used. Furthermore, they argued that these AA uptake rates of

5-25%, reported when isotopically-labeled AAs are applied at >1mM concentrations, may simply

be a relic of the high concentration tracer methodology, rather than a result with potentially

important ecological and biogeochemical ramifications (Jones et al. 2005b). These higher AA

uptake rates may also have little relevance from an agricultural plant N-nutrition standpoint,

Jones et al. (2005a) argued, reporting that growing season mean concentrations of individual

AAs in agricultural soil ranged from only 0.03 µM (Methionine) to 9.78 µM (Alanine), and

averaged 2.53 µM (mean of individual concentration all AAs), or 3 orders of magnitude less than

concentrations typically used in isotope tracer studies (i.e. Nasholm et al. 2000, Wiegelt et al.

2005). Even total soil AA concentrations (sum of all individual AAs) for any of the months of the

growing season are orders of magnitude lower than ranges of AA concentrations relied on for

isotope tracer studies (23.5-58.1 µM, Jones et al. 2005a). Far *less* monitoring of soil AA

concentrations, especially in agricultural soils, has occurred relative to monitoring of soil

inorganic N, but in Nasholm et al.'s (2009) review, they asserted average agricultural soil AA

availability is likely equal to or greater than 10 µM. Therefore, tracer % uptake values are likely

most relevant to actual direct contributions of AA N to plant N nutrition, especially in

agricultural settings, when the traceable AA is applied at 2.5-25 µM (Jones et al. 2005a, Nasholm

et al. 2009). Lastly, although stable and radioactive isotopes have frequently been used to trace

plant uptake of various N compounds, the disposal challenges associated with radioactive

isotope work, the cost of both radioactive and stable isotope-labeled compounds, and the costs

associated with isotope analysis in general motivated this search for an alternative tracer.

Whiteside and colleagues (2009) recently proposed QDs as an alternative method, successfully utilizing them at to qualitatively track AA uptake by plants. QDs are a fluorescent label, much like the green fluorescing proteins (GFPs) used by Puangfoo-Lonhienne and colleagues to track plant uptake of intact proteins and even whole bacteria and yeast (2008 and 2010). Confocal laser scanning microscopy (CLSM) allows tracking of both QDs and GFPs, supporting direct resolution of the movement and locations of these N compounds within intact plant tissues, even resolving the precise location of these N compounds inside of plant cells (Puangfoo-Lonhienne et al. 2008, Whiteside et al. 2009, Puangfoo-Lonhienne et al. 2010). For ~6 nm diameter QDs, the resolution of CLSM allows detection of as few as 4 QDs (M. Whiteside, *pers. comm.*). However, unlike GFPs, QDs are commercially available in a wide array of colors to facilitate labeling of multiple N compounds to theoretically support simultaneous tracking of multiple N compounds within a single plant. Additionally, unlike GFPs, QDs are available in both and amine- and carboxyl-terminal forms to enable labeling of sub-protein N compounds. Carboxyl-terminal QDs offer even greater potential utility than amine-terminal QDs, as they could even theoretically be used to label NH_4^+ in a basic solution. In this process, NH_4^+-derived NH_2 would replace the OH^- group on carboxyl terminal QDs, yielding a QD coated with $CO-NH_2$ (M. Bootman, Crystalplex Corp., *pers. comm.)*. Utilizing amine-terminal QDs to label AAs or carboxyl-terminal QDs to label AAs and NH_3 derivatives constitutes a more flexible method compared to the GFP method, which is restricted to larger proteins. Given AA's and NH_4^+'s smaller size, a method useful for tracking these smaller compounds likely to be more accessible to plants, and therefore play a larger role in and thus be more relevant to plant N nutrition makes QDs an attractive alternative to GFP (Nasholm et al. 2009). However, while CLSM paired

with QDs makes it possible to detect plant uptake of such extremely small quantities of QD-AA

compounds (as few as ~100-120 AAs) useful for documenting rare phenomena, for the purposes

of quantifying whether plant uptake of AAs constitutes a meaningful portion of plant N nutrition

another non-CLSM-based quantification method is necessary.

To expand QD-tracer utility from documenting rare phenomena to quantifying

contributions of QD-labeled AAs to plant N nutrition, Whiteside and colleagues' (2012) used a

fluorescence microplate reader-approach rather than CLSM to quantify QD-labeled AA uptake.

This pioneering work established QD-labeling as a tracer method capable of tracking 0.8 µM

QD-AA (26.4 µM AA) uptake by 45-day old Sudan grass seedlings (*Sorghum bicolor*, Whiteside et

al. 2012). This work suggested QD-labeling detected by fluorescence microplate readers offers

an alternative method to dual-labeled, isotope-based techniques for quantifying intact AA

uptake. However, Whiteside and colleagues' (2012) work did not include a QD-inorganic N

treatment, nor did it compare QD performance to performance of standard stable isotope (^{15}N

and ^{13}C) labeling methods. These gaps illustrated the opportunity for further validation of

fluorescence plate-based quantification of QD-labeling as an alternative to stable isotope-based

labeling.

As we are aware of no work that directly compared detectability of stable isotopes (^{13}C

and ^{15}N) to QDs detected via microplate reader in plant material, the primary objective of this

work was to compare these two alternative labeling strategies. We made this comparison by: 1)

assessing potential tracer performance(maximum possible signal: noise) of field-relevant

concentrations of ^{15}N, ^{13}C and QD-labeled N compounds applied to four agricultural plant

species, and then by 2) assessing agreement between N compound uptake or tracer recovery

estimates derived from ^{15}N, ^{13}C and fluorescence (from QDs) signatures in plant shoot tissue.

METHODS

We carried out all comparisons of potential and actual performance of these 3 tracers by the utilizing subsets of data obtained from a large, greenhouse experiment conducted in two phases: a growth phase followed by tracer introduction, and tracer uptake phases. Plant species (4 species) and N regime of the rooting zone (3 "N environments") were the only factors present in the growth phase of the experiment (Table 4-1). Data on control plants' biomass characteristics (mass, root to shoot ratios, isotopic signatures, and fluorescence signatures) from the growth phase was used in conjunction with data on maximum tracer signal strength to assess the potential performance of each tracer.

In the tracer phase, we further explored efficacy of these three tracer types (^{13}C, ^{15}N, and QDs) by crossing a labeling strategy factor (stable isotope only versus QD-stable isotope) and a labeled compound factor to create 12 unique "tracer-phase labeling treatments" (Table 4-2) applied within each of the 12 unique "growth-phase treatments" (4 plant species x 3 N environments). In total, this resulted in 144 unique treatments, each of which was replicated on 4 different plants for a total sample size of 576 individual plants. Based on mortality rates we observed in a preliminary run of this experiment, we also included 8 extra plants in each growth phase treatment, to insure that even if several plants died during the growth phase there would still be enough plants for complete implementation of the tracer phase of the experiment. We randomized implementation of each factor within the experimental manifold and re-randomized plants within their growth-phase treatment every 3 days to avoid any location-specific effects on growth. We also re-randomized plants within each growth phase prior to application of the tracer phase treatments.

We initiated the growth phase of the experiment on 12 March 2014, implementing the tracer phase on 3 April 2014 in corn and on 11 April 2014 in the other 3 plant species. We assessed tracer performance in terms of both possible and actual tracer accrual in the shoots after 4-5 days of exposure to tracer-labeled N compounds (4 d for RC, 4.5 d for corn, and 5 d for SSG and OG). We calculated possible tracer performance as maximum possible tracer signal (at 100% uptake) given characteristics of tracer-labeled solutions (isotopic enrichment, fluorescence) relative to inherent noise characterized from measurements of control plants' shoots (fluorescence and isotopic signatures, biomass, N content, and C content, See Appendix: Equations and Definitions). This maximum possible tracer performance, or potential detectability, is also known as "Peak Signal-to-Noise", or PSNR. Overall, the experiment's objective was to assess these agricultural plant species' capacity to take up inorganic versus organic forms of N, and assess whether rooting zone chemistry impacted the plants' uptake abilities over the short growth timeframe encapsulated by this experiment (~30±5 d, depending on plant species). However, the objectives of this work are to determine:

1)   Potential detectability of these 3 tracers (PSNR) when used to track these 5 N compounds in shoot tissue of 4 plant species, grown in 3 N environments; and

2)   Actual tracer recovery in shoots measured via isotopic and/or fluorescence enrichment in plants exposed to QD+isotope-labeled N compounds versus those exposed to N compounds labeled only with stable isotopes.

Steps Taken to Minimize the Presence of Microbes and the Potential for N Mineralization

We aimed to assess the direct role of each of these N compounds in plant N nutrition or plant N uptake abilities and in the absence of any microbial intervention. Therefore we took several methodological precautions to minimize the presence of microbes and/or microbial mineralization. First, we conducted the experiment in a greenhouse, where we were able to create a highly controlled rooting zone, where the form of N was the only variable that differed between treatments, and we minimized the microbial and physical dimensions of rooting zoneal variability as much as possible. To do this, we used sand as the growth substrate for the plants, which has both a low water holding and a low cation exchange capacity, to prevent retention of any N compounds (intact or mineralized) media. We also sterilized the sand via autoclave (121 °C, 103.4 kPa for 2 hr) immediately prior to initiating the experiment to kill any microbes present and minimize microbial populations. Additionally, we used a 1:5 NaClO(bleach): reverse-osmosis (RO) water solution to sterilize the entire experimental manifold, soaking all cone-tainers in it for >20 min and pumping it through the fertilizer reservoirs, irrigation pumps, and irrigation tubing for more than 20 min. After sterilization, RO water was pumped through the entire system for 1 hr. All seeds were also sterilized in 5% bleach solutions for 10 minutes prior to germination to reduce microbial contamination on the seed surfaces by ~90% (Caetano-Anolles et al. 1990). Throughout the experiment, we used two strategies to minimize potential for mineralization and mineralized N contamination of organic N treatments. First, every 24-72 hrs, we mixed fresh fertilizer solutions, disposed of any fertilizer solutions remaining in the reservoirs, pumped pure RO water through the reservoirs for 1 min (until clear water was freely draining through the conetainers), and then re-filled fertilizer reservoirs with fresh solutions. The second tactic, frequent flushing of the conetainers with the fertilizer solutions for 1 min,

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occurred every 2 hr 40 min throughout the 30-40 d duration of the experiment, and served to flush any N that may have been mineralized in the sand in the intervening 2hr 40 min out of the conetainers, and insure the plants were primarily exposed to fresh fertilizer solution of known composition (Table 1). Together, while these tactics may not have completely eliminated microbes from the plants' rooting zone, they should have minimized the contribution of microbial mineralization to our results.

Overall Experiment Description – Growth Phase Factors and Treatments

We conducted the experiment in the greenhouse utilizing an automated irrigation system connected to an experimental manifold, which supported all treatments listed in Table 4-1. Greenhouse temperatures fluctuated between a minimum of 21 °C at night and a maximum of 25 °C during the day, throughout the experiment. We included four plant species: two C_4 grasses; corn and sorghum sudangrass ("SSG"); one C_3 grass, orchardgrass ("OG"); and one legume, red clover ("RC", Table 1). We created three hydroponic rooting zones differing only in the source of N: AA-N only ("organic"), a 50:50 mixture of AA-N and NH_4NO_3-N ("Mix"), and NH_4NO_3-N only ("Inorganic", Table 1). To ensure rooting zones were otherwise uniform, we supplied the balance of all plant nutrient needs except calcium with commercially-available Cornell No N fertilizer, applied at 0.5 g L^{-1} (Greencare Fertilizers, Kankakee, IL). We supplied calcium at 90 mg L^{-1} as calcium phosphate monobasic monohydrate, and adjusted the pH of all solutions to 6.8 to 7.0 using CaOH. In all the fertilizer solutions, we supplied N at 150 mg L^{-1} (10.7 mM N), varying only the form of the N.

For the regimes where we supplied N as AAs, the AA-N was composed of equal parts arginine (Arg), asparagine (Asp), glycine (Gly), and serine (Ser) to represent an array of AA sizes

and chemical traits (C:N ratio, charge) thought to influence plant availability (Whiteside et al. 2012). We included both large (Arg and Asp) and small (Gly and Ser) AAs, as previous work has hypothesized smaller AAs may be more plant available (Whiteside et al. 2012). Within each size category, we included both a relatively N-rich AA (Arg and Gly had lower C:N), as well as a relatively N-poor AA (Asp and Ser, which had higher C:N). Arginine was positively charged, but the rest were neutral, and all were soluble. Absolute C:N ratios ranged from low (Arg, 3:2), to intermediate (Gly and Asp, both 2:1), to high (Ser, 3:1). All AAs used in this experiment have been demonstrated to be plant-accessible to one or more plant species in one or more recent experiments (Nasholm et al. 2000, Ohlund and Nasholm 2001, Weigelt et al. 2005, Forsum et al. 2008, Gioseffi et al. 2012, Gruffman et al. 2012, Whiteside et al. 2012).

Preliminary Experiment Shortcomings Guide Tracer Phase Design

Design of the tracer phase was heavily influenced both by a preliminary experiment as well as by information available from the literature. The preliminary experiment, which used QD-labeling exclusively, consisted of conjugating 2.5 μM of each N compound with 0.1 μM of a different color of carboxyl-terminal QD (Trilite™ 5 Colors - Carboxyl CdSeS Core Nanocrystals, Crystalplex| Biology Division, Pittsburgh, PA). These conjugations followed a manufacturer-recommended 25:1 ratio of AA:QD, and followed established conjugation procedures (Crystalplex 2009, Hermanson 2008). Furthermore, although no previously published research has attempted this, we used a basic (OH$^-$-enriched) QD conjugation solution to strip H$^+$ ions from NH$_4^+$, allowing carboxyl-terminal QDs to bind N compounds as small and simple as NH$_2$ (M. Bootman, Crystalplex Corporation, *pers. comm.*). In this way, we attached "inorganic N" to QDs and facilitated QD-based comparisons of plant uptake of both long-accepted inorganic sources

of plant N nutrition (albeit in an organic conglomerate with 24 other NH_2s also bound to the

carboxyl-terminal QD) and more-recently posited organic N sources of plant N nutrition. In the

preliminary experiment, we used the same 0.1 μM QD – 2.5 μM N compound concentration

used by Whiteside and colleagues (2009) used in their initial qualitative experiments. After

conjugation, we combined the uniquely-labeled compounds in 3 solutions consisting of: 1)

inorganic N only, 2) 50:50 inorganic N to an even mixture of all 4 AAs (actual ratio of 4:1:1:1:1),

and 3) an even mixture of all 4 AAs only. We also included 2 control solutions: 1) an

unconjugated QD control consisting of a mix of all 5 colors of activated QDs, each at 0.1 μM, and

2) a deionized water control. These QD activation and conjugations did result in detectable

fluorescing solutions, albeit at much lower levels than expected (data not shown). Furthermore,

we had expected no variability between the various colors of QDs in terms of fluorescence (just

peaks in different location on the spectrum). However, we observed potential fluorescence

between the different colors of QDs differed by 3 orders of magnitude (data not shown).

Shortcomings of these QD-tracer solutions extended to problems with QD-fluorescence

detection in plant tissue. After Whiteside and colleagues (2012) who introduced QD-AA

solutions after 45 d of growth, we introduced the QD solutions after 42-47 days of summer

growth, which amounted to individual plant biomass of 5-25 g (data not shown). 1.5 mL of each

of the 5 treatment /control solutions was added to the sand in each plant's growth conetainer

via an 18-gauge, side-port needle (Whiteside et al. 2012, custom side-port needle source:

Cadence, Inc., Cranston, RI; SC7 Stubby Ray Leach Cone-tainers[TM], Stuewe & Sons, Tangent, OR).

We incubated plants with the solutions for 24 h prior to destructively harvesting by separating

roots from shoots, and then drying at 60 C for 48 h. Our incubation and post-harvest procedure

included washing roots with 1% saline to remove any QDs on the root surfaces, after the

technique described by Whiteside and colleagues (2012). Despite similarities of these

procedures to those utilized by Whiteside et al. (2009) and Whiteside et al. (2012), we did not

detect any QD fluorescence in either root-tissue or shoot-tissue solutions (analyzing 200 µL

solution of 1 mg dry ground plant tissue per 100 µL^{-1} buffer; 4 mg per plant). Based on these

shortcomings in the preliminary experiment, we designed a new approach to evaluate QD tracer

performance using stable isotopes as a back-up tracer validation strategy. We also decreased

the plant growth period from 47 ± 3d to ~30 ± 5d, increased tracer concentrations six-fold for

corn and two-fold for the other 3 plant species, and increased the tracer incubation time from 1

day to 4-5 days.

Tracer Phase Design and Implementation

The main major methodological modification between the preliminary experiment and

this ultimate experiment was the switch from using QDs alone, to verifying QD performance

using stable isotopes. We included 2 separate stable isotope tracer strategies: stable isotope-

only ("Isotope-only") and QD-stable isotope ("QD+Isotope", Table 2). This strategy supported

both direct quantification (fluorescence-based) and indirect quantification (isotope-based) of

QD-uptake by plants. Comparing recovery measured with and without binding the stable

isotope-labeled compounds to QDs enabled testing of the hypothesis that the large size of QDs

interferes with plant uptake of these N compounds, ultimately reducing uptake rates shown by

QDs and/or ^{15}N relative to actual rates of plant uptake of these compounds when the

compounds are free in the soil solution (recovery shown by isotope-only labeling strategy). If

this discrimination occurred, recovery in the QD+Isotope treatments, indicated by fluorescence

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and/or isotopic signatures, would be lower than recovery indicated by isotopic signatures in plants exposed to the Isotope-only tracer solutions.

Another concern with the QD tracer method is that the QD conjugation process may result in higher or lower than intended concentrations of target compound (the N compound to bind to the QD). With the QD-tracer method, the concentration of the target compound is inferred from the concentration of the QDs. So if the success of the conjugation procedure is higher or lower than the expected rate of 25 molecules to each QD, then the tracer solution will not contain the intended concentrations of target compound (or if plants discriminate against the larger QD+isotope labeled compounds compared to the isotope-only labeled compounds). As previous work has shown uptake rates are concentration-dependent, this is concerning (Jones et al. 2005a). To maximize QD-target compound binding and insure that the concentration of target compound is indeed 25 times the concentration of the QD, the conjugation process entails introduction of an excess of target compound, followed by a centrifuging and filtering process meant to remove any unbound molecules (Crystalplex 2009). This process creates the possibility for higher than intended concentrations of the target N compound in the tracer solution, if unbound molecules remain in solution after filtering. If present, these unbound molecules of the target N compound, and the higher concentrations of AA N in the tracer solution they would create, could increase uptake rates beyond what would occur if the concentration of N compound was at the lower target level (i.e. Jones et al. *2005a*). Using stable-isotope labelled molecules as the target N compound allowed cross checking of uptake rates shown by QDs, ^{15}N and ^{13}C within plants. Using this QD+isotope tracer strategy in conjunction with isotope-only labeling strategies, also enabling cross-checking uptake rates

shown by ^{15}N or ^{13}C between plants where uptake was labeled by stable isotopes only versus those where uptake was labeled by stable isotopes (theoretically) bound to QDs.

To increase chances of detection relative to the preliminary experiment, we increased the concentration of QDs, decreased the duration of the growth phase, and increased the duration of the tracer phase in an attempt to increase signal by both increasing absolute signal applied and decreasing signal dilution. We doubled the concentration of N-compound (0.2 μM QD *if applicable* with 5 μM N compound) for all plant species except corn (Table 2). For corn, due to its larger anticipated size – corn was twice the size of SSG and 5-10 times larger than OG and RC in the preliminary experiment– we sextupled the concentration of tracer relative to the preliminary experiment (0.6 μM QD *when applicable* with 15 μM N compound). We still utilized the same 5 N compounds used in the preliminary experiment, and stayed within the bounds of published field-relevant concentrations of tracer-labelled solutions, despite these increases in the concentrations of tracer used in all plant species (Jones et al. 2005a). Prior to tracer introduction, we flushed the sand in each plant's conetainer with RO water to try to minimize the presence of an unlabeled N from the fertilizer solutions used in the growth phase, which might artificially enhance uptake rates, and removed all plants from the growth phase experimental manifold (Jones et al. 2005a). After flushing, we sealed the base of each cone with parafilm to prevent the introduced tracer solution from immediately draining out of the sand. Then, we introduced 1.5 mL of each labeled N/control treatment to the sand in each plant's growth cone via the same type of 18-guage side port needle (Cadence Inc., Cranston, RI). Tracer introduction occurred after 22 days in corn and after 30 days in the other 3 species as opposed to after 47 days in the preliminary experiment, to further reduce any tracer dilution problems

and improve detection. During the incubation period, I monitored the water status of the plants frequently, adding enough deionized water to the surface of the sand via wash bottle to prevent desiccation. The incubation period lasted, 4-5 days, as opposed to 1 day in the preliminary experiment.

Although this incubation time was much longer than the 24 hr period typically used in radioactive tracer experiments, it was still much shorter than the 14 d incubation period used by Whiteside and colleagues (2009). In their time series observations throughout the 14-d incubation, Whiteside and colleagues (2009) found it took ~24 hours for the 25 mL of 0.1 µM QD-ON solution to move from root cells to vascular tissue to mesophyll cells, to finally be detectable by CLSM in the chloroplasts. While CLSM can detect as few as 4 QDs (100 ON molecules), the best optimized fluorescence plate detection limits we observed for 200 uL of QD-plant tissue-buffer solution in the preliminary experiment were about 11 orders of magnitude higher at 0.1 µg mL^{-1} or 1.5 x 10^{-4} µM for the 575 nm emission QD, which was the best performing dot in the preliminary experiment (data not shown). Therefore, despite concerns about potential mineralization prior to uptake, we selected a 4-5 day incubation period to try to maximize the possibility for tracer detection in plant shoot tissue.

Plant Tissue Processing

After the incubation period, we harvested plant shoots and dried them at 60 °C for 48 hr or until they reached a constant mass. After drying, we fully ground and homogenized all shoot tissue from each plant with a SPEX 8000M Mixer/Mill until all shoot tissue became fine powder (SPEX SamplePrep, Metuchen, NJ). To prevent damaging QDs and reducing fluorescence intensity, we used Zirconia vials and beads for all potentially QD-containing samples. For control

samples and samples potentially containing stable isotope labels, we used vials and beads made

of either Zirconia or stainless steel. To prevent cross-contamination, we cleaned vials and beads

between each sample by flushing with pressurized air, shaking with ethanol, rubbing with lint-

free wipes, and flushing again with pressurized air to dry.

Stable Isotope Analyses

We analyzed ~2 mg subsamples of each ground and homogenized plant shoot tissue

sample for total concentrations of carbon (C) and nitrogen (N), as well as characterized shifts in

$\delta^{15}N$ or $\delta^{13}N$ to sub-per mil precision. The Laboratory for Isotopes and Metals in the

Environment (Penn State University, University Park, PA) conducted these analyses using an ECS

4010 CHNSO analyzer (Costech Analytical Technologies, Inc., Valencia, CA) connected to a

ConFlo IV universal continuous flow interface (Thermo Fisher Scientific, Inc., Waltham, MA) that

served as an inlet to a Delta V™ Isotope Ratio Mass Spectrometer (Thermo Fisher Scientific, Inc.,

Waltham, MA).

Removal of Outlier Isotope Data When C_3 to C_4 Cross-Contamination was Suspected

We excluded nine samples (one corn and eight SSG) from isotope analyses due to $^{13}\delta$

that were not consistent with a C_4 plant identity, exhibiting $^{13}\delta$ values less than -19

(Supplemental Table 1). These 8 excluded SSG samples' $^{13}\delta$ values were 23 or more standard

deviations away from the mean of the 122 retained samples. Likewise, the excluded corn

sample was more than 22 standard deviations away from the mean of the 129 retained corn

samples. All C_3 OG and RC samples had $^{13}\delta$ values less -25, so we suspected no C_4 contamination

and removed no OG or RC samples from subsequent isotope analyses.

QD-containing Sample Preparation

We sub-sampled 140-160 mg of ground, homogenized shoot tissue from each plant exposed to QDs; and combined with 14-16 mL 50 mM carbonate-bicarbonate buffer (pH 9.6) to achieve a concentration of 1 mg dry shoot 100 μL^{-1} buffer solution (Whiteside et al. 2012). As fluorescence intensity of these QDs increases with increasing pH, we selected this buffer with pH >8.0 to maximize QD fluorescence intensity and improve detectability in highly autofluorescent plant tissue (M. Bootman, Crystalplex Corp., *pers. comm.*). For each plant exposed to QDs, a total of 6 mg shoot tissue was analyzed via fluorescence plate reader.

Fluorescence Plate Reader Instrument Selection

To further overcome the issue of plant tissue's high auto-fluorescence, and identify the type of fluorescence plate reader best suited to this application (detection of QDs in plant material), we evaluated two types of commercially-available fluorescence microplate readers: a filter-driven FLUOstar Omega and monochrometer-driven Infinite M1000 (BMG LABTECH, Offenburg, Germany; and Tecan, Salzburg, Austria; respectively). Each type offers a unique set of advantages and disadvantages. Filter-driven fluorescence microplate readers offer lower initial instrument purchase costs and greater measurement simplicity. However, the simplicity afforded by their filter-based function also limits their flexibility and precision. For the FLUOstar Omega, filters can only restrict excitation bandwidth to 40 nm and emission bandwidth to 10 nm; ranges too broad to reduce background fluorescence of the plant material sufficiently to facilitate detect the QD signal from concentrations of labeled N compounds that would be relevant from a plant nutrition perspective. Furthermore, even achieving this level of performance from a filter-based instrument would require purchase of a specific set of excitation and emission filters for each unique pairing of QD and plant material to optimize QD

signal to background plant fluorescence noise ratios. In contrast, the monochrometer-driven

instrument allowed both better flexibility in terms of enabling full excitation and emission

spectra to be recorded to identify the optimal excitation/emission combination to maximize QD

signal and minimize plant background fluorescence noise; and also offered greater precision,

facilitating bandwidth restrictions down to 5 nm to further avoid and restrict plant background

fluorescence and improve signal to noise ratios. For these reasons, we selected the

monochometer-driven fluorescence plate reader, the Infinite M1000, as the better option for

this application (Tecan, Salzburg, Austria).

Optimization of Fluorescence Scans for Detection of QDs in Plant Material

To maximize QD detection, we relied on instrument-specific guidance to optimize all

adjustable parameters including: the excitation and emission spectra and bandwidths, the gain,

the Z-position, measurement mode, flash number, and flash frequency (Tecan 2012, Table 4). To

maximize instrument accuracy for our 200 µl well^{-1} sample volume (2 mg shoot well^{-1}), we used

the instrument in "top measurement mode" in conjunction with black-bottomed FLUOTRAC$^{™}$

96-well microplates read from a Z-position of 21374 µm vertically relative to the sample surface

(Greiner Bio-One, product #655077, Kremsmünster, Upper Austria). We also maximized the QD

signal: plant noise by tuning and restricting the excitation and emissions bandwidths using full

excitation and emission spectra characterizations of the 575 nm QD and each type of plant

tissue (Tecan 2012). First, we selected the QD with the highest signal of those evaluated in the

preliminary experiment ("575 nm-QD", which displayed peak fluorescence emissions between

570-575nm), and then we performed excitation scans on standard QD solutions to determine

which excitation wavelengths were associated with maximum emissions from 570-575 nm. This

procedure identified excitation wavelengths 305-315 nm and 365-375 nm as those associated

with maximum fluorescence emissions at 570-575 nm. Then, we performed excitation spectra

scans from 290-380 nm of solutions of control plants from each species to locate which of the

optimal QD-excitation wavelengths resulted in the lowest plant emissions (plant auto-

fluorescence, "AF") in that same 570-575 nm band. For all 4 plant species, regardless of N

fertility regime, excitation at 305 nm minimized plant AF between 570-575 nm. Therefore, we

measured fluorescence of all samples and QD-labelled solutions as the maximum fluorescence

observed in any 1nm-bandwidth between 570-575 nm emissions, as stimulated by 1nm-

bandwidth excitation at 305 nm (Table 4-4). Triplicate means of these maxima in relative

fluorescence units (RFUs) were used for all subsequent interpretations of potential QD

fluorescence per plant (derived from fluorescence of each QD-solution), and actual QD uptake

per plant (% uptake = observed fluorescence per plant - control plant AF/ potential QD

fluorescence per plant; Table 4-5). Because each QD-labeled solution varied in fluorescence, we

used solution-specific fluorescence to derive QD-fluorescence associated with peak signal (PS,

Appendix: Equations and Definitions, Table 4-6).

Calculation of Potential Signal

We defined maximum uptake (100% uptake) as the ratio of QD- or isotope-added per

plant relative to the plant's total biomass, or an uptake equivalent to PS (Appendix: Equations

and Definitions). For isotopic tracers, the units of this PS variable were mass ^{13}C or ^{15}N per mg

plant. For QDs, PS was in units of fluorescence per mg plant (RFU mg^{-1}). This approach assumes

equal distribution of the tracer throughout the plant – no post-uptake fractionation between

roots and shoots. Based on Whiteside et al.'s (2009) observation that intact QD-AA compounds

were translocated to cells in the shoots prior to degradation and within 48 hours of introduction

the growth media, it seems likely that although our proportional approach was relatively

accurate. However, if uptake and redistribution in the plant was slower than this, our estimates

of uptake may be lower than actual. Conversely, if translocation to shoots was more complete,

this would result in estimates of uptake in excess of 100%. As a more conservative alternative to

uptake, we also used % or proportion recovered, which was simply the amount of ^{13}C, ^{15}N or QD

recovered in the shoot, relative to the total amount added to each plant.

For isotope-labeled samples, there is evidence that fractionation of AA-N and -C occurs

in the roots, with AA-derived C retained in the roots or respired from the roots, while AA-

derived N is transported to the shoots (Nasholm et al. 2000, Puangfoo-Lonhienne et al. 2010). If

this was the case, shoot-derived ^{13}C isotope measures of AA uptake may be underestimates of

actual uptake of intact AAs. This root fractionation process could also greatly diminish the utility

of QD labeling for shoot-based quantification of plant N uptake. Fractionation would separate

the N molecules from the QDs in the roots, making the N compound untraceable via ^{13}C or QD

after translocation to shoots (but still trace-able via ^{15}N). However, if mineralization of isotope-

labeled AAs occurred in the sand prior to uptake, then shoot ^{15}N-derived estimates of uptake

would be overestimates. In essence, detection of either ^{13}C-enrichment or QD-enrichment in the

shoots provides strong evidence of intact uptake. But, lack of detection of ^{13}C-enrichment

and/or QD-enrichment in the shoots does not necessarily mean the AAs were not taken up

intact: intact uptake followed by root fractionation is one alternative explanation.

Calculation of Potential Noise and Potential Tracer Detectability

We defined noise as isotopic and fluorescence signature dispersion in control plant shoots (plants un-exposed to tracers) in units of % uptake (Appendix: Equations and Definitons, Figures 4-5, 4-7, 4-8). We then compared this absolute noise to the maximum possible signal from tracer-labeled compounds in plant shoots, which assumed all added tracer was taken up and distributed evenly through the plant (Appendix: Equations and Definitions). We used the ratio of maximum potential tracer signal to inherent control plant noise as PSNR, which we also refer to as "potential signal: noise" (Appendix: Equations and Definitions). Mean PSNR values greater than 1 indicate positive potential detectability, whereas mean PSNR values that are less than one, or not significantly greater than one indicate poor to negligible potential detectability of tracer in plant tissue. We chose this metric because it incorporates information about both signal dilution and inherent background signal variation. By combining them, this ratio provides a good estimate of how plant biomass characteristics produced by factors manipulated in the Growth Phase of this experiment (Plant Species, N Environment) interacted with factors manipulated in the Tracer Phase of the experiment (Tracer Type, Labeled Compound). To summarize impacts of these factors and their interactions, we analyzed potential detectability for all combinations of treatments in this experiment using ANOVA with 4 factors and their interactions, paired with Tukey's HSD post hoc testing to identify significant differences indicated by factor or factor interaction significance. The four factors were: Plant Species (corn, SSG, OG, RC), N Environment (inorganic, mixed, organic), Tracer Type (^{15}N, ^{13}C, and QD), and Labeled Compound (NH_4NO_3, Arg, Asp, Gly, Ser), which combined to create 168 unique analytical treatments replicated 4 times.

Tracer Performance

With PSNR as a gauge of tracer reliability and expected performance in the context of this experiment, we then calculated actual rates of uptake shown by each of the tracers. We calculated tracer enrichment two ways: % recovery in shoots and % uptake. We calculated proportion tracer recovery in shoots or "% recovery" as shoot enrichment relative to the control relative to the amount of tracer added. Because this metric makes no assumptions about the distribution of the tracer through the plant, or adjustments in maximum potential enrichment using the proportion of biomass contained in the shoot, it serves as a more conservative (theoretical absolute maximum of 100%) and coarse value, potentially resulting in lower than actual estimates of recovery for plants that had higher root: shoot ratios.

To account for systematic differences in root to shoot ratio we also calculated a % uptake metric. We defined maximum enrichment differently for % uptake, assuming the maximum enrichment was proportional to the amount of biomass contained in the shoot:

$$Maximum\ tracer\ concentration\ (\frac{tracer}{mg\ biomass})$$

$$= (shoot\ biomass \div total\ biomass) \times (\frac{tracer\ added}{total\ biomass})$$

If distribution of tracer throughout the plant was not constant, % uptake is vulnerable to both under and overestimations. If tracer re-distribution to the shoots occurred, resulting in tracer concentration, then % uptake estimates can exceed 100%, depending on the ratio of shoot to total biomass by up to 100%.

Both % Recovery and % Uptake calculations resulted in multivariate datasets (^{13}C-derived, ^{15}N derived, and QD-derived), with both % Uptake and % Recovery variables for each of the 3 tracers. For isotopically-labeled compounds, factors included in ANOVA analyses included:

plant species (corn, SSG, OG, RC), N environment (inorganic, mixed, organic), labeling strategy (QD+isotope vs. Isotope only) and labeled compound. For QD-quantified uptake/recovery, only 3 factors were included (Plant Species, N environment and Labeled Compound).

Data Analysis

The factorial design used in both phases of this experiment and for both potential and actual tracer performance datasets facilitated similar analytical approaches to all datasets: ANOVA followed by Tukey Post Hoc Testing conducted in SigmaPlot or R (SigmaPlot Version 13.0, Systat Software, San Jose, CA; R Core Team 2014). Two-way ANOVAs tested for plant species effects, N environment effects, and species by environment interaction effects on all measured and derived raw variables that met normality and equal variance assumptions from the growth phase of the experiment. The Shapiro-Wilk method was used to test normality, the Bonferroni Outlier Test was used to identify outliers, and the Brown-Forsythe method was used to test for equal variance (Fox and Weisberg 2011, R Core Team 2014). Standard log, log(x+1), square root, and cube root transformations were used to transform raw variables characterized by violations of normality and/or equal variance assumptions to facilitate ANOVA methods. However, if these transformations were ineffective, we used a Kruskal-Wallis ANOVA on ranks to determine significance of factor main and/or interaction effects (SigmaPlot Version 13.0). When ANOVA results indicated significant effects of factors or factor interactions that included more than 2 treatments, Tukey's Honestly Significant Difference was used as a post-hoc test to determine which treatments were significantly different from one another (at $\alpha=0.05$). In R, all analyses were performed using functions available from the core "stats" package or from the companion to applied regression package, "car" (Fox and Weisberg 2011, R Core Team 2014).

RESULTS AND DISCUSSION

Potential Tracer Detectability

^{15}N and QDs Potential is Superior to ^{13}C.

Tracer type itself; ^{13}C, ^{15}N, or QD; was the single biggest factor affecting PSNR (Figure 4-1, Table 4-7). The three tracers displayed fundamentally different PSNR (Figure 4-2, Table 4-7). Overall, ^{15}N offered the greatest potential tracer performance, with double the PSNR of QDs and 9 times the PSNR of ^{13}C (Figure4-2). However, other factors, primarily plant species, also affected the magnitude and directions of between-tracer differences (Figure 4-1, Figure 4-3). Plant species primarily impacted the difference in potential detectability between ^{15}N and QDs, as ^{15}N potential detectability varied widely (and significantly) between species, but ^{13}C and QD potential detectability did not (Figure 4-3a). ^{15}N offered highest potential detectability for some species (corn and RC), but for OG there was no significant difference in potential detectability between ^{15}N and QDs, and for SSG QDs offered superior potential detectability relative to ^{15}N (Figure 4-3b). Unlike ^{15}N and QDs, ^{13}C consistently had the lowest potential detectability, regardless of plant species (Figure 4-3b). Rooting zone, which did have a small effect on plant biomass and thus tracer dilution/potential signal, did not significantly affect potential ^{15}N or QD detectability within any plant species (Figure 4-4). However, Corn and SSG plants grown in the mixed N environment were smaller than plants grown in either inorganic N or organic N environments, which resulted in higher potential ^{13}C tracer detectability (due to less dilution) in corn (Figure 4-4a). The difference was not significant in SSG because lower concentrations of tracer were used in SSG, and thus the relative improvement was less (Table 4-6, Figure 4-4b).

Natural ^{13}C Variability (Noise) Exceeded Potential ^{13}C Enrichment (Signal).

Potential for detecting ^{13}C enrichment related to AA uptake was low for all plant species, regardless of rooting zone chemistry (Figure 4-2, Figure 4-3). While the grasses were roughly twice the size of RC, potential detectability of ^{13}C in RC did not differ significantly from potential detectability in either corn or SSG (Figure 4-3a). Differences in potential signal caused by differences in ^{13}C-enrichment among the AAs illustrated how the same amount of background variability inherent to each plant translates into more or less noise depending on the ^{13}C signal size of each AA (Figure 4-5). AA ^{13}C enrichment varied 3-fold, from Arg, which contains 6 ^{13}C per molecule, to Gly which contains only 2 ^{13}Cs per molecule (Table 4-6, Figure 4-5). Therefore, there is more variation within each plant species between AAs in potential ^{13}C detectability, than between plant species, despite using 3 times the rates of tracers in corn than the other 3 species (Figure 4-3, Figure 4-5). However, both corn and OG displayed skewed variability in control plant ^{13}C, with control corn and OG plants having a tendency toward ^{13}C enrichment (Figure4- 5a and 4-5c). Thus extreme ^{13}C signatures tended to be greater than the mean (more ^{13}C enriched) rather than less enriched than the control plant mean (Figure 4-5a and 4-5c). Natural variability in ^{13}C of the control plants implied up to 1600% uptake of the least ^{13}C-enriched AA, Gly (Figure 4-5a and 4-5c). This skew could be carried through calculations of actual uptake based on control means, resulting in positive uptake and/or recovery estimates due to an inherent skew, even if the actual tracer's signal is undetectable in the plants δ^{13}C. Even for Arg, the most ^{13}C-enriched AA, background variability in ^{13}C enrichment exceeded potential signal at 100% uptake for all plant species except RC (Figure 4-6). Thus, with the possible exception of Arg in RC, these potential detectability analyses indicate that the potential ^{13}C tracer enrichment from any of these AAs was not detectable due to dilution and background variability in ^{13}C. Furthermore,

estimates of uptake, especially for corn and OG may be biased in the positive direction due to a skewed distribution in $\delta^{13}C$ signatures of corn and OG (Figure 4-5).

Superior Potential of ^{15}N relative to ^{13}C.

Despite all AAs being 2-3 times more ^{13}C enriched than ^{15}N enriched (Table 4-6), potential AA detectability was 2-30 times greater with ^{15}N relative to ^{13}C, thanks to less dilution of the AA-N in plant biomass (Figure 4-7a). Greater dilution of ^{13}C relative to ^{15}N was the primary driver of these differences in potential detectability of these isotopic tracers, as plants have 8-30 times more C than N (Figure 4-7b). However, differences in ^{13}C background variability also influenced relative differences in potential ^{13}C versus ^{15}N AA detectability within species (Figure 4-7c and 4-7d). Contrasts between corn and SSG best exemplify this interaction between dilution and background variability in impacting ultimate potential detectability (Figure 4-7c). While both corn and SSG were similar in size (data not shown), SSG had greater inherent C dilution than corn (Figure 4-7b), as well as less signal added (Table 4-6), but far less background noise in ^{13}C (Figure 4-7c). SSG's extremely low ^{13}C noise (Figure 4-7c) ultimately resulted in greater potential ^{13}C AA detectability compared to corn, even though ^{13}C AAs were applied to corn at triple the rate (Figures 4-5a and 4-5b, Table 4-6). Since background variability in ^{15}N was similar in corn and SSG (Figure 4-7d), as was total ^{15}N content (data not shown), but triple the rate of ^{15}N-AAs were used in corn, ^{15}N had greater potential detectability in corn (Figure 4-8a, Figure 4-8b-d).

Across the 5 N compounds labeled with ^{15}N, all compound's potential ^{15}N signal exceeded variability in control plant ^{15}N across all plant species, with the exception of ^{15}N-

labeled Gly and Ser in SSG (Figure 4-8). This was driven both by characteristics of SSG and by

characteristics of Gly and Ser. On the signal side, both Gly and Ser contain just a one ^{15}N per

molecule, translating into lower potential signal, relative to the other more N-enriched AAs and

ammonium nitrate (Table 4-6). But, it was also SSG's greater biomass (more signal dilution) that

ultimately resulted in SSG's ^{15}N noise exceeding potential ^{15}N-signature from Gly and Ser by

about 50% (Figure 4-8b). Although corn was similar in size to SSG, it had roughly three times the

potential tracer enrichment (15 μM applied vs 5 μM applied) of SSG (Table 4-6). This made

potential detectability of ^{15}N-labeled compounds in corn similar to detectability of ^{15}N-labeled

compounds in red clover, which was 30-50% the size of corn, but also had 1/3 of the potential

tracer enrichment (5 μM, Figure 4-3a, Figure 4-8a vs 4-8d). Overall, potential for detecting of

^{15}N-labeled AAs in all plants was high.

QD Potential was high for all species except corn.

Potential signal to noise ratios of QDs were consistent across plant species, with all 4

plant species exhibiting similar potential for detection of QD-labeled compounds (Figure 4-3a).

While overall potential QD-labeling performance was roughly half of that of potential ^{15}N-

labeling (Figure 4-2), for SSG, potential detectability was actually greater for all labeled

compounds with QDs than with ^{15}N (Figure 4-3b). For orchardgrass, potential detectability was

similar for QDs and ^{15}N, whereas for red clover and corn, potential ^{15}N detectability was better

(Figure 4-3b). For red clover, despite potential detectability being worse with QDs than with ^{15}N,

both potential signal from all labeled compounds from both QD and ^{15}N tracers exceeded

control plant noise in either fluorescence or ^{15}N signature (Figures 4-8d and 4-9d). Corn showed

the least potential for detection of QD-labeled compounds, largely because of the inferior

fluorescence exhibited by the QD-labeled solutions used for corn (Table 4-6, Figure 4-9a).

QD solution fluorescence, rather than inherent compound chemistry, was a main driver

of potential QD performance (Table 4-6, Figure 4-9). While we had expected uniform

fluorescence across QD-labeled solutions, we observed considerable variability in solution

fluorescence (Table 4-6). Also unlike the isotope tracers, where the higher concentration used

for corn conferred a potential detectability benefit, there was no increased signal benefit of

using the higher concentration of QDs for the corn QD-tracer solutions, with corn's 0.6 µM QD-

Asp and QD-Gly solutions actually exhibiting less fluorescence signal than their respective 0.2

µM QD solutions used for the other 3 plant species (much less for QD-Asp, Table 4-6).

Like isotopic tracers, tracer dilution in biomass was one of the main limitations to

potential QD detectability. But, unlike isotopic tracers, noise stemmed from shoot

autofluorescence (Table 4-5). Shoot autofluorescence varied significantly by species, with red

clover emitting significantly more autofluorescence than all of the grass species, which did not

vary significantly from one another (Figure 4-10, Kruskal-Wallis one-way analysis of variance on

ranks). Within species, N environment significantly affected shoot autofluorescence in red clover

and corn, but N environment did not significantly affect shoot autofluorescence in SSG or

orchardgrass (Table 4-5, Tukey Post Hoc Tests p<0.05 and p>0.05, respectively). Therefore, for

corn and red clover, we used N environment-specific as well as species-specific

autofluorescence values to calculate potential and actual QD-related fluorescence; whereas for

both orchardgrass and SSG we used grand means of control plant autofluorescence across all 3

N environments (Table 4-5). With the exception of QD-labeled Asp applied to corn, potential QD

signal exceeded fluorescence noise in all species, suggesting QDs may be an excellent alternative to ^{13}C for tracking intact uptake of AAs, at concentrations well below those necessary for ^{13}C detectability (Figure 4-10).

Actual Tracer Performance – Did QDs actually perform as well as ^{15}N?

QD Performance Indicated by Fluorescence.

Of the 4 species exposed to QD-labeled N compounds, only SSG and RC displayed direct evidence of QD-uptake (Figure 4-11a). Across all plant species, N environment did not affect the plants' uptake of QD-labeled compounds (Figure 4-11b). Lastly, across all species, the only compound-to-compound differences in QD-indicated uptake we observed were between QDs conjugated with NH_4NO_3 and QDs conjugated with Asp (Figure 4-11c).

Within SSG and RC, QD-indicated uptake was not affected by N environment (p=0.94 for SSG and p=0.95 for RC, 2-way ANOVAs by species), or by N compound (p=0.15 for RC and p=0.08 for SSG, Figure 4-12a). However, means of compound uptake hinted at differences in compound uptake across broader categories (Figure 4-12a). While ANOVAs of QD-indicated uptake for each species did not reveal any pairwise differences between N compounds in RC, in SSG compound recovery means did suggest that QD-serine uptake was greater than uptake of free QDs (Figure 4-12a). Heteroscedastic t-tests comparing mean uptake of unbound QDs to mean uptake of QDs conjugated with N compounds indicated that SSG took up conjugated QDs at a significantly greater rate than the unbound QDs (Figure 4-12b). However, RC took up unbound QDs and QDs conjugated with N compounds at similar rates (Figure 4-12b). There were also differences between QD-NH_2 uptake and QD-AA uptake, but the difference was only apparent in RC not SSG

(Figure 4-12c). Overall, these results suggest that while detection of QD-labeled compounds was theoretically possible for all plant species (Figure4- 3a), only SSG and RC actually exhibited direct fluorescence evidence of QD-uptake in shoots (Figure 4-11).

QD- and ^{15}N-indicated Uptake Agreement

Among plants exposed to N compounds labeled by both isotopes and QDs (n=60 for each plant species), linear regressions of mean QD-indicated uptake and mean ^{15}N-indicated uptake revealed support for the QD-only based results (Figure 4-13). In SSG and RC, the two species that showed QD-uptake, ^{15}N-indicated uptake was positively significantly correlated with QD-indicated uptake (Figures 4-13b and 4-13d). This suggests that the QDs were taken up with the ^{15}N-labeled N compounds. In RC, the linear regression indicated that ^{15}N label was recovered at about 20% of the rate of QD label recovery in shoots (Figure 4-13d). A possible explanation for this could be that QD labeling was incomplete, and that rather than 25 N compounds bound to each QD there were only an average of 5 N compounds bound to each QD.

The regression in SSG revealed a very different relationship, with 45 times more ^{15}N enrichment than expected (Figure 4-13b). This suggests that there were unbound ^{15}N-labeled molecules present in addition to the QD-bound ^{15}N-labeled molecules. This could have been caused by incomplete filtration during the conjugation process. Conjugation of QDs with N compounds requires introduction of an excess of target compound, whatever needs to be bound to the QD, followed by a centrifuge and filtration process meant to remove the excess molecules that were not bound to the QDs. These data suggest that this process was not complete in the QD+isotope solutions introduced to SSG.

Corn exposed to QD+isotope labeled compounds showed no consistent evidence of [15]N

enrichment, and fluorescence was actually consistently lower than the control plants that were

not exposed to QD+isotope solutions (Figure 4-13a). This suggests that the QD+isotope solutions

used in corn were not accessible to the corn, perhaps due to the large size of the QDs, or

perhaps due to some other characteristic of the solutions, which impeded uptake. We observed

QD clumping and precipitation in the corn solutions due to a drop in pH during the conjugation

process (Figure 4-S1). Despite correcting the pH, these solutions exhibited equal or inferior

fluorescence to the QD+isotope solutions prepared for the other 3 species, 5-30% of expected

fluorescence, based on the performance of the 0.2µM solutions. All of this together suggests the

corn QD+isotope solutions were not equivalent quality to the others, and that the corn

definitely didn't take up any of the QDs, and, lastly, that exposure to the solutions themselves

may have damaged the corn shoot tissue to reduce its autofluorescence.

OG showed altogether different patterns in [15]N- and QD-indicated uptake (Figure 4-13d).

Although there was not a significant relationship between QD-indicated and ^{15}N-indicated

recovery in OG, one-third of the treatments did exhibit both positive mean uptake, both when

uptake was estimated by QD and by [15]N. Most of the remaining treatment means, 60% of all OG

treatment means, showed positive ^{15}N-indicated recovery, but no QD recovery (Figure 4-13c).

Since OG showed good potential for QD detection (similar to SSG and better than RC), the most

likely explanation for these results is that the ^{15}N-labeled compounds were cleaved from the

QDs prior to ^{15}N transfer to the shoots. Other workers have found that when plants take up

organic molecules, like these carboxyl-terminal QDs, the C portion of the molecule is retained in

the roots and the N portion is transferred to the shoots (Nasholm et al. 2000, Weigelt et al. 2005,

Puangfoo-Lonhienne et al. 2010). This cleavage and transfer can happen on timelines as short as 24 hours, so the 5-day incubation period we used in this experiment was long enough to have accommodated this process. While SSG seems to transfer intact QD-N compound conglomerates to its shoots, it may be that organic N nutrition in OG is more similar to that exhibited in other C_3 grasses that cleave organic N molecules in their roots (Whiteside et al. 2009, Whiteside et al. 2012).

QD+isotope vs. Isotope Only Performance Indicated by ^{15}N-Enrichment. We sought to determine whether QDs impeded recovery of ^{15}N -labeled compounds, as others have suggested that QD's relatively large size may serve as a barrier to uptake. We also sought to verify that our attempts to bind NH4 to carboxyl-terminal QDs were successful. To evaluate this hypothesis and objective, we compared ^{15}N-indicated uptake shown by samples exposed to isotope-only labeled compounds versus those exposed to QD+isotope-labeled compounds, with labeling strategy – either isotope-only or QD+isotope – as a factor (Figure 4-14, ANOVA). We found that for the species that displayed evidence of QD-uptake – either directly or in the form of ^{15}N enrichment – SSG, OG and RC, ^{15}N-indicated uptake was greater in QD+isotope-exposed SSG, no different in OG and greater in isotope-only exposed RC (Figure 4-14a). The over-enrichment in the QD+isotope exposed SSG was present in all 5 N compounds (Figure 4-14b). However for both OG and RC, ^{15}N -indicated was similar regardless of labeling strategy for all four AAs, suggesting QDs to not impede AA N uptake (Figure 4-15c and 4-15d). But, ^{15}N-indicated recovery for NH_4NO_3 was significantly greater in both OG and RC plants exposed to $^{15}N_2$-NH_4NO_3 compared to those exposed to QD conjugated with $^{15}N_2$-NH_4NO_3 (QD-NH_2, Figures 4-15c and 4-15d). The isotope-only recovery was almost exactly double the recovery of the QD+isotope treatment, strongly

suggesting that the conjugation of $^{15}NH_4$ with QDs was successful at approximately the same rate as the AA-QD conjugations (Figure 4-14c and 4-14d).

Conclusions

QDs are a promising alternative to stable isotope tracers for tracking N uptake in plants, even when used at very low, field soil-relevant rates. QD detection in shoot tissue was less and more variable than expected based on our sensitivity analyses. Furthermore, differences between uptake shown by ^{15}N and uptake shown by the QDs suggest that similar in-plant fractionation may occur, as has been observed with ^{13}C and ^{15}N in other studies. This suggests that QD detection may be improved in future studies by analyzing the roots instead of the shoots as carboxyl-terminal dots may behave more like ^{13}C. However, they are much more detectable than ^{13}C, even when used in a plate-based method. Therefore they constitute an ideal alternative method for tracking plant uptake of intact organic N molecules, but species must be screened for autofluorescence to best select QDs with emissions outside the plant's autofluorescence.

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TABLES

Table 4-1. *Experimental Factors and Levels for Analysis of Potential and Actual Performance of Each Tracer*

		Factor	Levels
Tracer Phase Factors	Growth Phase Factors	Plant Species[†]	Corn (*Zea mays* [L.] 'Master's Choice 4050')
			SSG (*Sorghum bicolor* [L.] Moench cultivar 'AS 6402 UT')
			OG (*Dactylis glomerata* [L.] 'Niva')
			RC (*Trifolium pratense* [L.] 'Renegade')
		N Environment[†]	Inorganic (NH_4NO_3-N only)
			Mix (50:50 NH_4NO_3-N: amino acid-N)
			Organic (amino acid-N only)
		Labeling Strategy[‡]	Isotope only (^{13}C and/or ^{15}N)
			Isotope+QDs[§] (stable isotope labeled compounds bound to QDs)
		Labeled Compound[‡]	NH_4NO_3
			Arg (Arginine)
			Asp (Asparagine)
			Gly (Glycine)
			Ser (Serine)

[†] Experimentally-imposed factor (derived from "control" plant data for potential performance/signal:noise analyses; also impacts potential signal as higher concentrations of tracer were used for corn)

[‡] **Theoretically-imposed** factor for potential performance analyses including 3 levels **^{13}C**, **^{15}N**, and **QD**; **Experimentally-imposed** for assessments of actual tracer performance

[§] Levels derived from actual measurements of QD solution fluorescence

Table 4-2. *Tracer-labeled inorganic and organic nitrogen compound treatments.* 575-nm Emission QDs were used for all QD-containing solutions in this experiment. Each QD bound ~25 target N compounds, so concentrations are calculated assuming a QD:N compound ratio of 25:1.

Tracer Strategy	Labeled N Compound	Corn N Compound	Corn QD	Other 3 Plant Species N Compound	Other 3 Plant Species QD
		Concentrations of Tracer Solutions (applied 1.5 ml plant^{-1})			
Isotope-only	$^{15}N_2$-NH$_4$NO$_3$	15 µM	none	5 µM	none
	$^{13}C_6^{15}N_4$-Arg	15 µM	none	5 µM	none
	$^{13}C_4^{15}N_2$-Asp	15 µM	none	5 µM	none
	$^{13}C_2^{15}N$-Gly	15 µM	none	5 µM	none
	$^{13}C_3^{15}N$-Ser	15 µM	none	5 µM	none
Isotope+QD	$^{15}N_2$-NH$_4$NO$_3$	15 µM	0.6 µM	5 µM	0.2 µM
	$^{13}C_6^{15}N_4$-Arg	15 µM	0.6 µM	5 µM	0.2 µM
	$^{13}C_4^{15}N_2$-Asp	15 µM	0.6 µM	5 µM	0.2 µM
	$^{13}C_2^{15}N$-Gly	15 µM	0.6 µM	5 µM	0.2 µM
	$^{13}C_3^{15}N$-Ser	15 µM	0.6 µM	5 µM	0.2 µM
N/A - Controls	None – QD control	none	0.6 µM	none	0.2 µM
	None – d.i. H$_2$O only	none	none	none	none

Table 4-3. *Plants Exposed to/analyzed for Each Type of Tracer* (sample sizes)

	Type of Label(s) Applied						
	Isotope Only		*QD+isotope*			*None - Controls*	
Plant Species[§]	^{15}N[†]	^{13}C[‡]	^{15}N-QD[†]	^{13}C-QD[‡]	QD[†]	QD control (unbound activated QDs only)	D.I. H_2O control[ψ] (nothing introduced other than 1.5 mL D.I. H_2O)
Corn	59	47	58	46	58	12	12
Sorghum Sudangrass	54	42	57	46	57	12	12
Orchardgrass	58	46	60	48	60	12	12
Red Clover	58	46	51	41	51	12	12

[§]An equal number of plants were grown on each growth environment N regime (⅓ NH_4NO_3-N only, ⅓ AA-N only, ⅓ 50:50 NH_4NO_3:AA-N)

[†]Labeled compound applied was either ammonium nitrate, arginine, asparagine, glycine or serine; with ~20% of samples receiving each labeled compound

[‡]Labeled compound applied was one of 4 AAs: either arginine, asparagine, glycine or serine; with ~25% of samples receiving each AA

[ψ] Samples used for sensitivity analyses of potential performance of each tracer, including noise and dilution-related limitations.

Table 4-4. *Settings used for all fluorescence analyses.* A Tecan Infinite M1000 analyzed all plant tissue and standards in triplicate within black, flat-bottom, 96-well plates on the same day.

Setting	*Value*	*Units*	*Notes*
Excitation Wavelength	305	nm	**Max. Signal:** 304-309 nm excitation stimulates maximum QD emissions @ 570-575 nm **Max. Noise:** Excitation above ~360 nm causes extremely high shoot tissue AF **Min. Noise:** Excitation @ 239, 245, 293, 305, and 324 nm minimizes AF emissions @ 570-575 nm **Why 305nm?** Generates highest signal: noise of all identified minimum AF noise excitation wavelengths.
Stepsize, Excitation Bandwidth	1	nm	
Emissions Wavelengths	570-575	nm	used maximum fluorescence of the 6 observations taken every 1-nm in this range as reading
Stepsize, Emissions Bandwidth	1	nm	
Fluorescence intensity mode	top		
Z-position	21374	μm	
Gain	200		
Flash number	50		
Flash frequency	400	Hz	

Table 4-5. *Shoot Autofluorescence (contributor to QD methodological noise) of Control Plants used to Interpret QD-related Fluorescence.* Values are means ± one standard error. Different lowercase letters denote means different at $p<0.05$ (Tukey post hoc tests). Bold formatting denotes value was used for interpreting QD-related fluorescence (subtracted from non-control plants of the same type).

Type of N included in Fertility Regime	Autofluorescence *(RFUs mg^{-1} shoot material)*			
	Corn	Sorghum Sudangrass	Orchardgrass	Red Clover
AAs (A.A.)	**86** ± 9a	62 ± 2	63 ± 2	**199** ± 5a
A.A. + NH$_4$NO$_3$	**57** ± 2b	65 ± 1	67 ± 2	**248** ± 12b
NH$_4$NO$_3$	**80** ± 3a	68 ± 4	65 ± 2	**268** ± 20b
all fertility regimes	N/A	**65** ± 2	**65** ± 1	N/A

Table 4-6. *Amount of tracer signal added (primary contributor to methodological potential signal) by tracer type.*

Tracer Added per Plant

Labeled Compound	^{15}N (μg ^{15}N plant^{-1})[†]		^{13}C (μg ^{13}C plant^{-1})[†]		575 nm QD (RFUs plant^{-1})[‡]	
	Corn	Other 3 Plant Spp.	Corn	Other 3 Plant Spp.	Corn	Other 3 Plant Spp.
NH$_4$NO$_3$ (^{15}N$_2$)	0.67	0.22	N/A	N/A	30,098	33,318
Arginine (^{13}C$_6$^{15}N$_4$)	1.34	0.45	1.74	0.58	63,105	65,063
Asparagine (^{13}C$_4$^{15}N$_2$)	0.66	0.22	1.15	0.38	3,885	103,753
Glycine (^{13}C$_2$^{15}N)	0.33	0.11	0.58	0.19	21,321	61,533
Serine (^{13}C$_3$^{15}N)	0.33	0.11	0.86	0.29	32,043	37,829

[†] Calculated from tracer solution molarity (15μM for corn, 5μM for the other 3 spp.) and volume (1.5 ml per plant)

[‡] Measured using settings listed in Table 4. Concentrations of QDs in corn solutions were triple those used for the other plant species (Table 1). However, despite greater QD concentrations, actual fluorescence was lower in those solutions, suggesting something went awry in the activation or compound-binding process.

Note: Differences between compounds is primarily caused by differences in chemical composition of the compounds (column 1, atm% was 98% to >99% for both ^{15}N and ^{13}C all compounds). Differences between corn and the other 3 plant species were caused by using different compound concentrations: 15μM in corn versus only 5μM in the other 3 species. We used the higher concentration for corn to compensate for its greater anticipated biomass, which we expected based on differences between plant species observed in a preliminary experiment. However, in this experiment, corn biomass was only significantly greater than red clover biomass (corn biomass was similar to orchardgrass and SSG biomass).

Theoretically QD solution fluorescence should have been the same within corn and within the other 3 species across all labeled compounds, but we observed systematic variability in solution fluorescence (although no variability within solutions).

Table 4-7. *ANOVA of Potential Signal: Noise.* Includes all shoot tissue of all plant species ("Plant Species"), rooting zone N fertility regimes ("GrowthN"), tracer types ("TracerType"), and labeled compounds ("LabeledCpd"). Analysis performed on log-transformed data to meet normality and equal variance assumptions violated by raw data.

Factor or Factor Interaction	df	Sum Sq	Mean Sq	Pr(>F)	
Plant Species[4]	3	47.00	15.66	3.93×10^{-6}	***
GrowthN[5]	2	31.80	15.89	7.55×10^{-5}	***
TracerType[6]	2	631.90	315.94	$< 2 \times 10^{-16}$	***
LabeledCpd[7]	4	76.00	19.00	5.66×10^{-9}	***
Plant Species x GrowthN	6	53.20	8.87	2.03×10^{-5}	***
Plant Species x TracerType	6	164.60	27.44	$< 2 \times 10^{-16}$	***
GrowthN x TracerType	4	27.60	6.90	0.002	**
Plant Species x LabeledCpd	12	18.40	1.53	0.51	
GrowthN x LabeledCpd	8	0.10	0.01	1	
TracerType x LabeledCpd	7	25.80	3.69	0.03	*
Plant Species x GrowthN x TracerType	12	83.60	6.97	2.17×10^{-6}	***
Plant Species x GrowthN x LabeledCpd	24	0.10	0.01	1	
Plant Species x TracerType x LabeledCpd	21	50.00	2.38	0.09	.
GrowthN x TracerType x LabeledCpd	14	0.00	0.00	1	
Plant Species x GrowthN x TracerType x LabeledCpd	42	0.10	0.00	1	
Residuals	462	757.40	1.64		

[4] Species included were corn, sorghum sudangrass, orchardgrass and red clover

[5] Rooting zone N regimes were NH_4NO_3-N only, 50:50 NH_4NO_3-N: AA-N, and AA-N only.
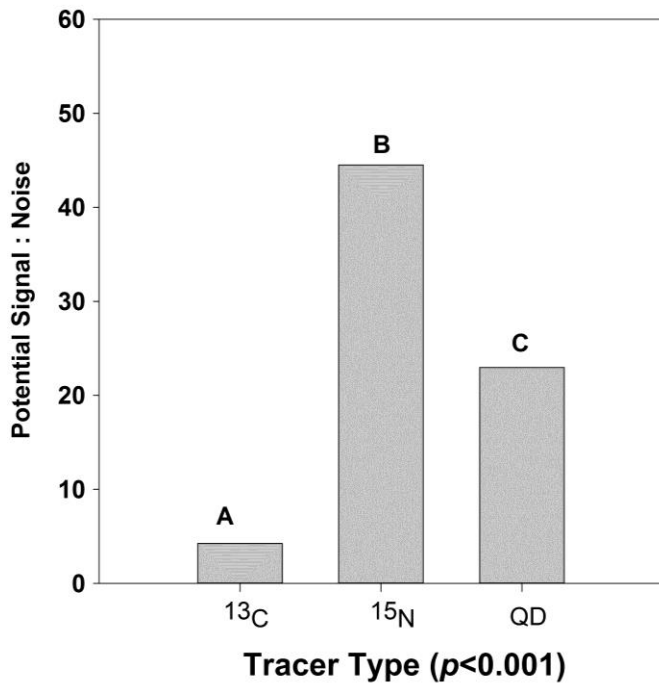
[6] Tracer Types evaluated were ^{13}C, ^{15}N and QDs

[7] Labeled Compounds evaluated were NH_4NO_3, Arginine (Arg), Asparagine (Asp), Glycine (Gly), and Serine (Ser).

FIGURES
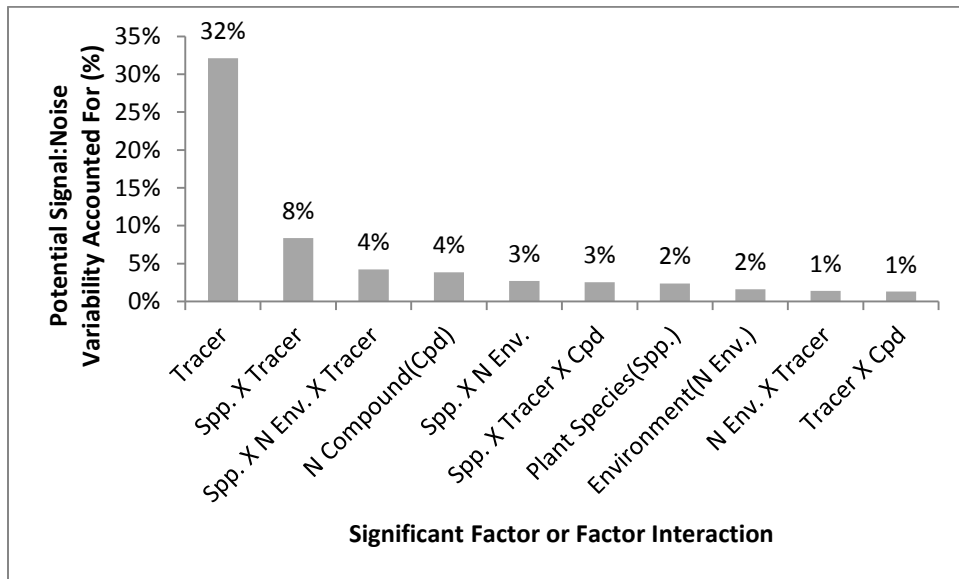
Figure 4-1. *Many Factors Significantly Affected Potential Tracer Performance, although the type off tracer (^{15}N, ^{13}C or QD) had the largest effect by far (ANOVA,* Table 4- *7).*

Figure 4-2. *Potential tracer detectability (potential signal: noise) of tracer-labeled N compounds differed mostly by tracer type.* Tracer type accounted for 32% of all variability in potential detectability (effect size and significance determined by four-way ANOVA on log-transformed data; Table 7).

a) Differences between Species within Tracers
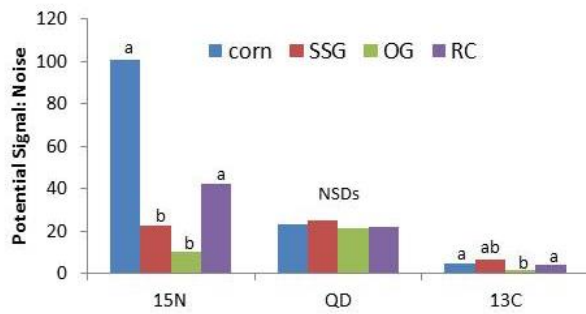
b) Differences between Tracers within Species
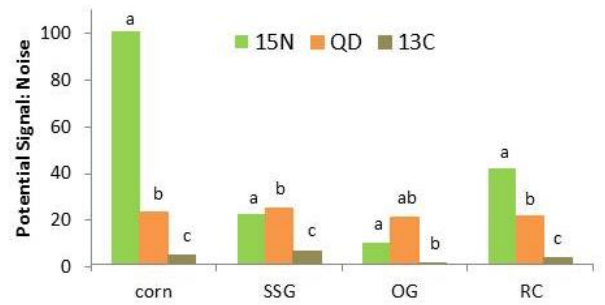
Figure 4-3. *Potential tracer signal: noise varied mostly by tracer type, but within plant species, the magnitude and significance of differences between tracer types varied.* Two-way interaction effect between Tracer and Plant Species (8% of variability in potential signal: noise, significant at $p<0.001$; ANOVA). Size and significance of differences determined by Tukey post hoc tests. Analyses performed on log-transformed data.
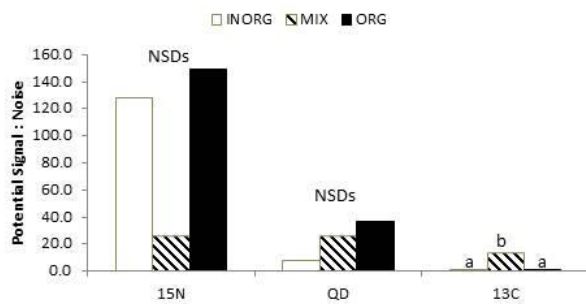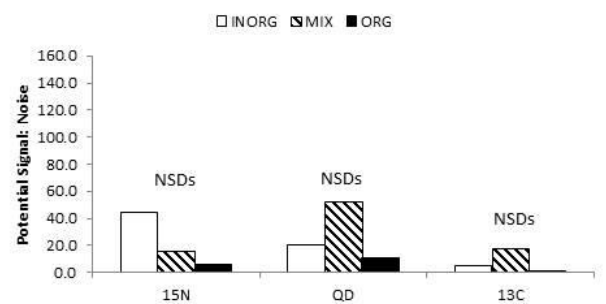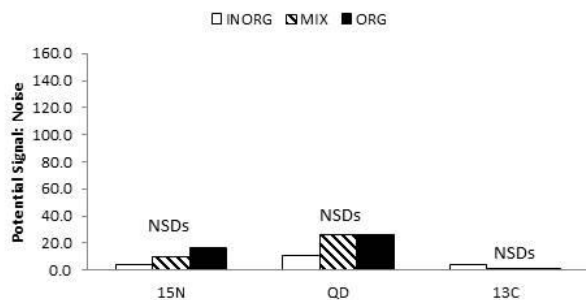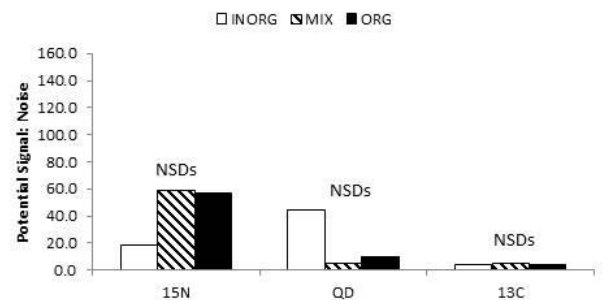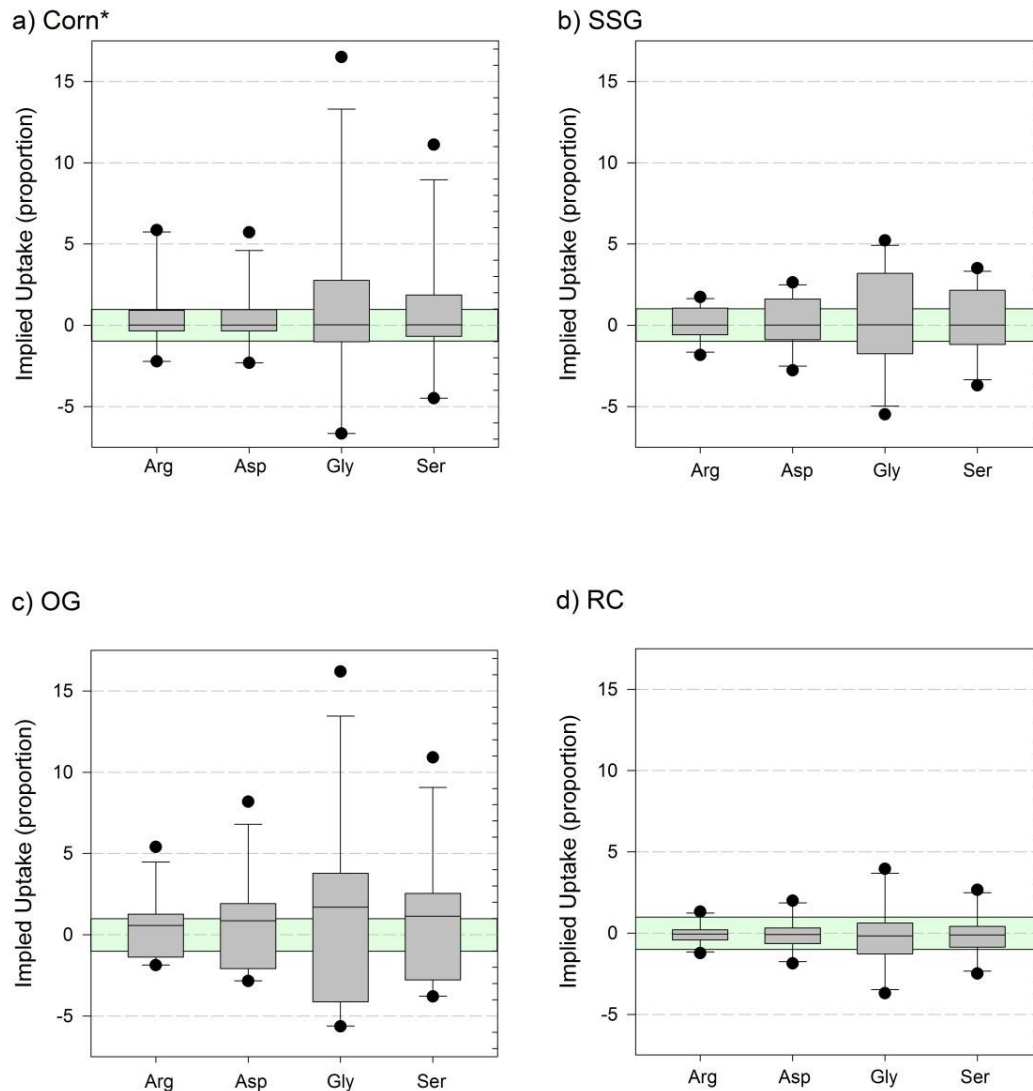
Figure 4-4. *Differences in potential signal: noise were mostly accounted for by tracer type and plant species, but in corn, potential signal: noise of ^{13}C labeled compounds also varied by N environment.* Tracer x Plant Species x N environment interaction effect accounted for 4% of variability in potential performance and was significant at *p*<0.001 (ANOVA on log-transformed data, with Tukey's HSD post hoc tests).

## ^{13}C Noise in Unlabeled Control Plants Relative to Maximum Potential ^{13}C Signal

Figure 4-5. *Variability in control plant ^{13}C signatures of all plant species exceeded the potential signal (green band) from ^{13}C of each ^{13}C-labeled AA if 100% of the applied tracer were taken up.* Differences inherent to the C content of each AA also influenced potential signal moderated by a constant (plant species-specific) biomass dilution effect. Arg is most enriched with 6 Cs, Asp has 4 Cs, Ser has 3 Cs, and Gly is least enriched with just 2 Cs. *Despite the greater concentrations of tracer applied to corn (greater potential signal), there was also greater variability in control corn plant ^{13}C signatures (noise), which resulted in control plant ^{13}C signature dispersion from the mean equivalent to more than 15 times the maximum potential signal (noise equivalent to >1500% uptake).
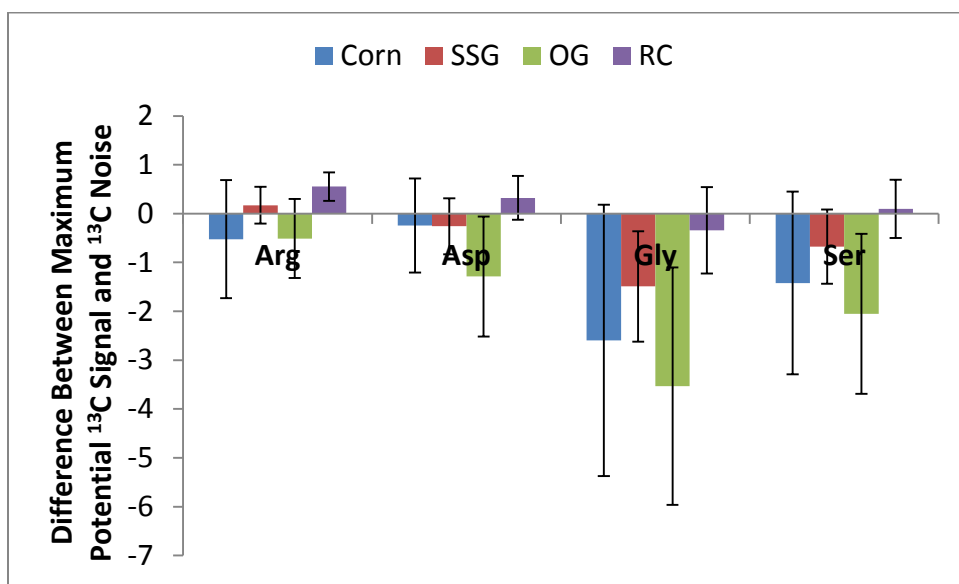
Figure 4-6. *Mean inherent ^{13}C variability in control plants (noise) exceeded or was equivalent to potential ^{13}C signal from all ^{13}C-labeled AAs in all plant species with the exception of Arg, the most ^{13}C-enriched AA, in RC.* Arg was potentially detectable in RC because RC had 40-50% lower biomass, which diluted the tracer ^{13}C less, resulting in greater potential ^{13}C signal, relative to the other 3 larger species. This suggests that tracer ^{13}C, even at 100% uptake, would not be detectable against background ^{13}C in almost all of these plants. Bars are means ± 2 SE, n=12 plants for each bar.
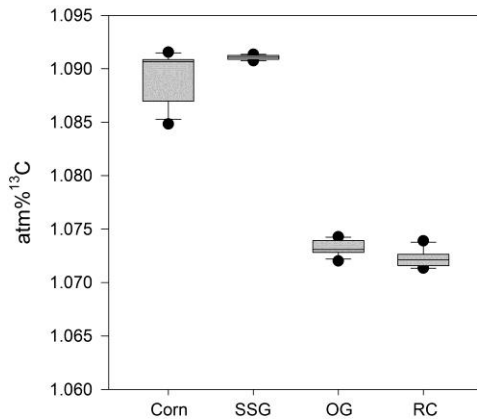
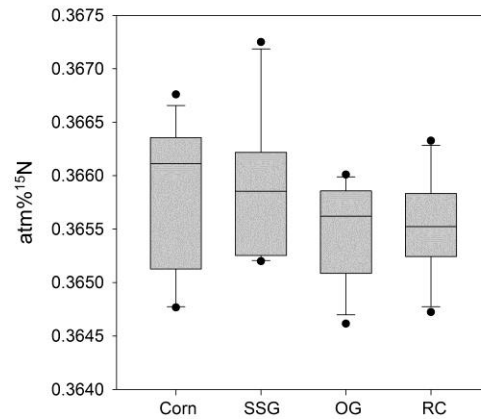a) ^{15}N-label of amino acids has 2-29x greater potential detectability than ^{13}C
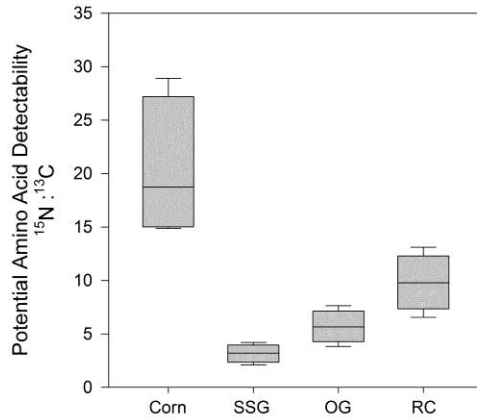
b) C dilution is 8-30x greater than N dilution in control plants
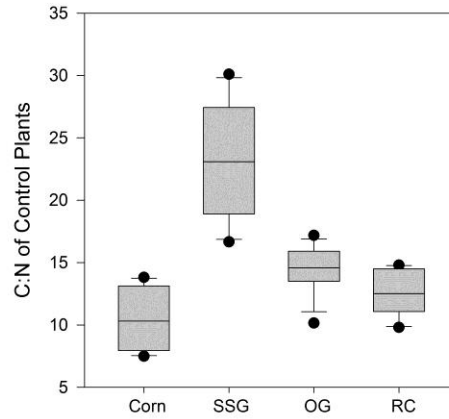
c) Variability in atm%^{13}C differed between plant species

d) Variability in atm%^{15}N was similar across plant species

Figure 4-7. ^{15}N-Labeled AAs had greater potential detectability (a) due to less dilution (b). There was also greater consistency in potential detectability across species due to less species-specific variability in background ^{15}N signatures relative to species-specific variation in ^{13}C signatures (c vs. d).

^{15}N Noise in Control Plants Relative to Maximum Potential ^{15}N Signal
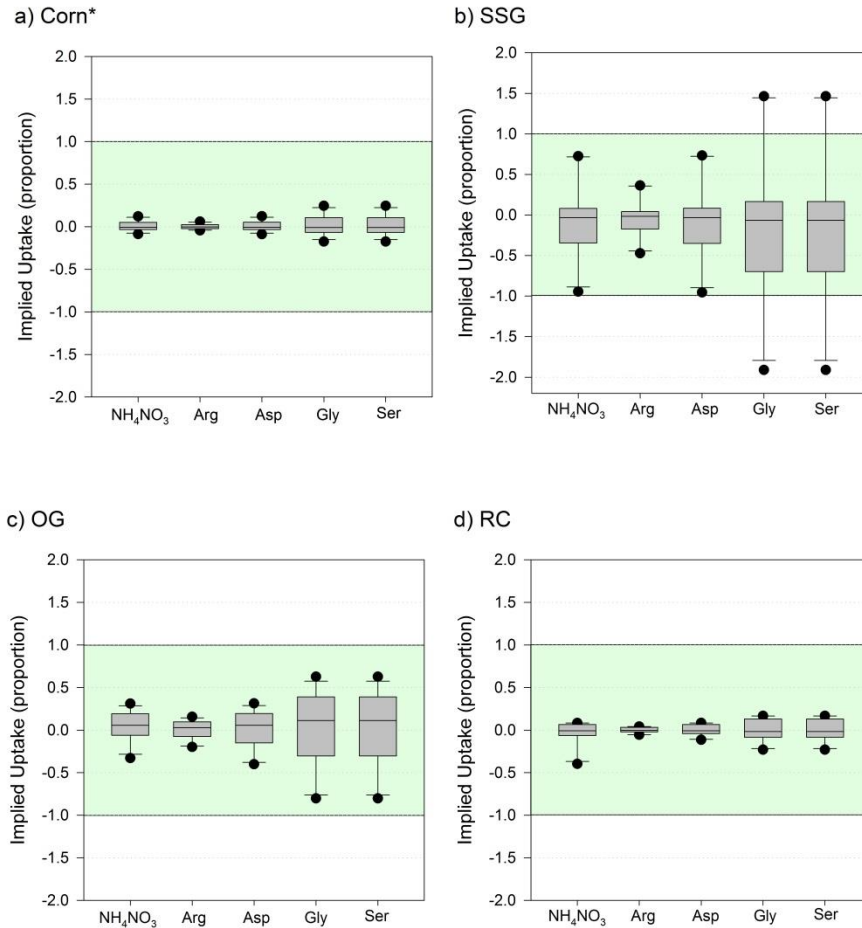
Figure 4-8. *Variability in control plant ^{15}N signatures (^{15}N tracer noise) relative to the potential signal (green band) from ^{15}N of each labeled compound if 100% of the applied tracer were taken up.* The inherent differences in chemistry between the labeled compounds – Gly and Ser each have only 1 N, Asp and NH_4NO_3 each have 2 Ns, and Arg has 3 – result in inherently different ^{15}N enrichment potentials, which translates into a significant effect labeled compound effect on potential signal: noise (Table 7; $p<0.0001$; accounting for 4% of signal: noise variability). This, in turn, either amplifies (for the less enriched compounds, i.e. Gly or Ser) or diminishes (for the more enriched compounds, i.e. Arg) the importance of background noise(variability) in ^{15}N signatures (ascertained from these patterns visible in the control plants).
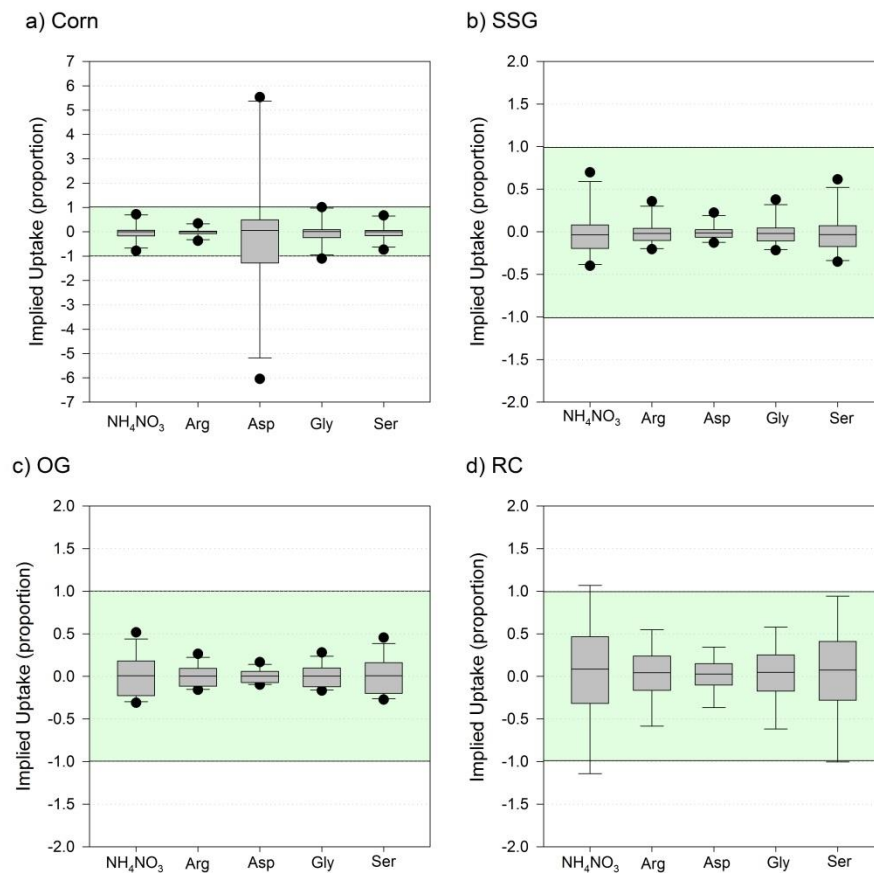
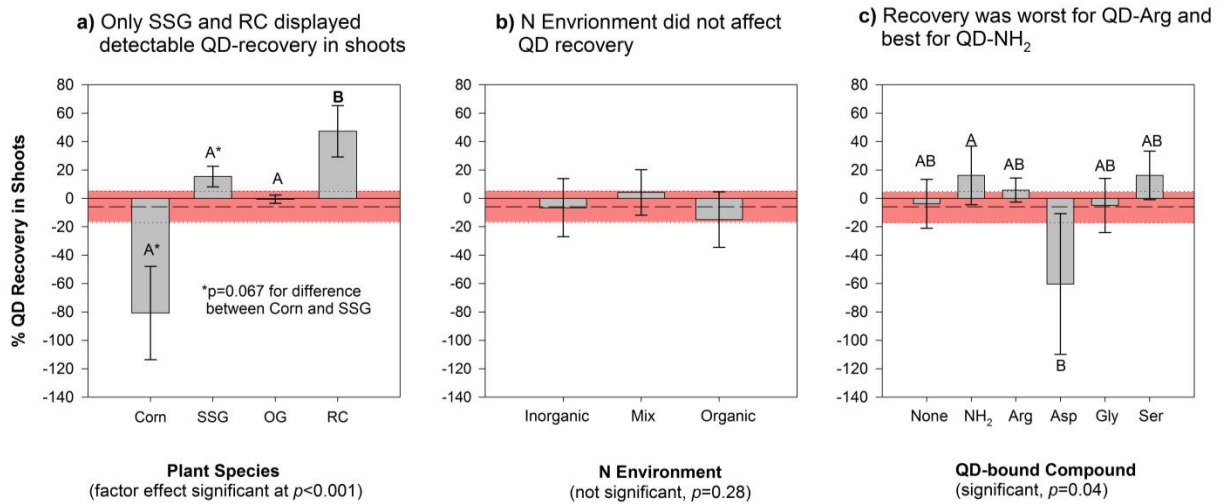**Figure 4-9.** *QD labeled compounds displayed different fluorescence signals, translating into big differences in potential detectability.* Despite using uniform concentrations of QDs across all 5 labeled N compounds (x-axis), which should have resulted in uniform potential signal regardless of the bound compound, the QD-labeled solutions varied in potential signal by target compound (Table 6). These differences are evident in the potential detectability of each labeled compound (above), with the most fluorescent solution (Asp in the 3 non-corn species), showing the greatest potential detectability relative to the background noise of control plant autofluorescence (greatest in red clover, lowest in orchardgrass) and dilution in biomass (greatest in corn). Corn solutions, despite having triple the concentration of QDs relative to the concentration of QDs used for the other 3 species, actually displayed inferior fluorescence (table 6), resulting in poorer potential performance (above). Red clover's greater variability in background fluorescence is evident, however red clover's smaller size increased the potential signal of each compound, ultimately resulting in potential detectability similar to the other plant species.
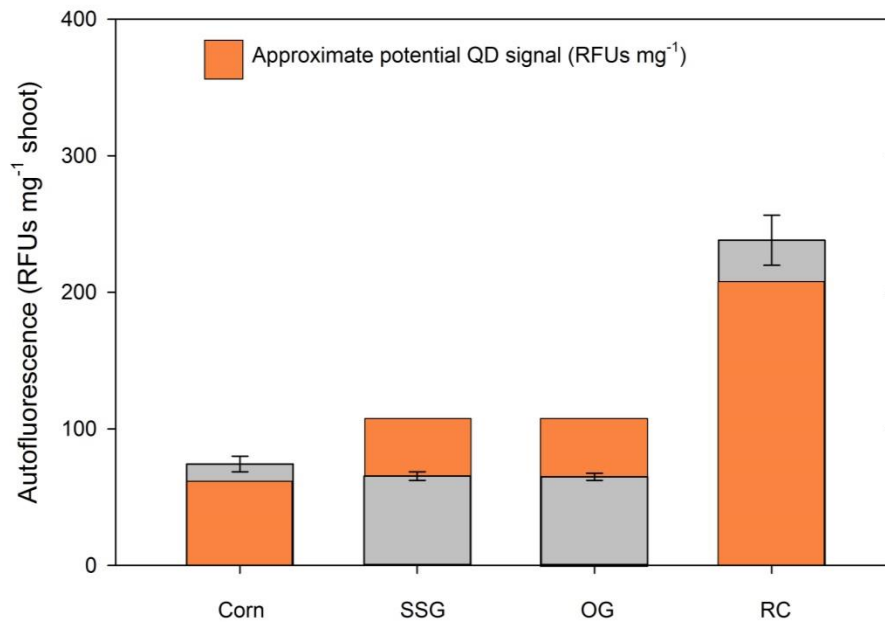
Figure 4-10. *Autofluorescence and approximate potential QD fluorescence by Plant Species.*
Shoot tissue autofluorescence (305 nm excitation, maximum emissions 570-575 nm) was
significantly greater in red clover than in each of the grass species (Kruskal-Wallis one-way
analysis of variance on ranks, Plant Species effect $p<0.001$; Tukey post hoc tests $p<0.001$ for red
clover versus each grass species). There were no significant differences between the grasses
(Tukey post hoc tests $p>0.05$). Bars are means ± 95% CIs.

a) Only SSG and RC displayed detectable QD-recovery in shoots

b) N Envrionment did not affect QD recovery

c) Recovery was worst for QD-Arg and best for QD-NH$_2$

*p=0.067 for difference between Corn and SSG

Plant Species
(factor effect significant at $p<0.001$)

N Environment
(not significant, $p=0.28$)

QD-bound Compound
(significant, $p=0.04$)

Figure 4-11. *Fluorescence-indicated QD recovery in shoots.* Actual detection of QD-fluorescence in shoots was limited (red band indicates grand mean ± 2 S.E.). Among the plant species exposed to QD-containing solutions, we were only able to detect (via fluorescence) significant QD recove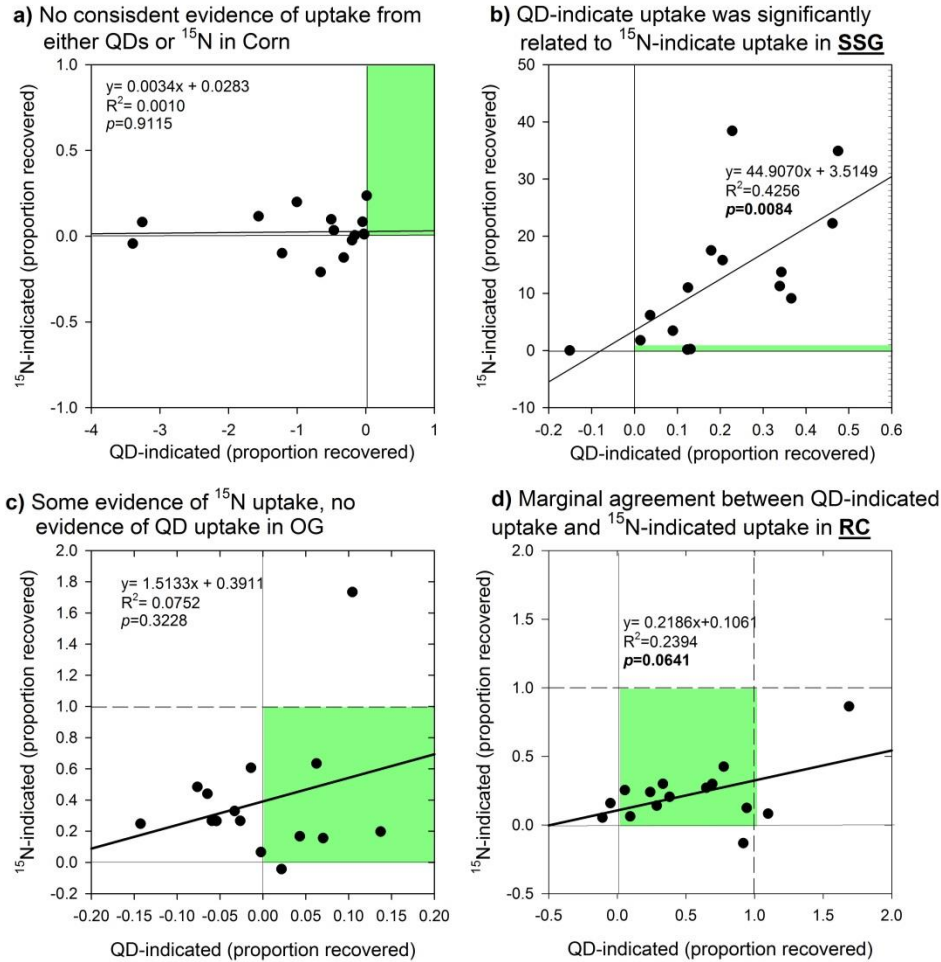ry in RC and SSG shoots (a). N environment had no effect on QD-indicated N compound uptake (b). Apparent QD-Ser uptake was greater than QD-Asp uptake, but this was likely skewed by poor potential signal of QD-Asp applied to corn and/or extremely high potential signal of QD-Asp applied to the other 3 plant species (c, Table 6). Data analyzed by ANOVA. Main factor effect significance as determined by ANOVA is indicated by p-values. Actual n=259, missing values replaced with treatment means for analytical n=288. There were 72 treatments with n=4 for each treatment. Data was cube-root transformed prior to analysis to meet normality of variance assumptions, but values shown in graphs are not transformed. Significant differences indicated by different capital letters were determined by Tukey's HSD post hoc testing.
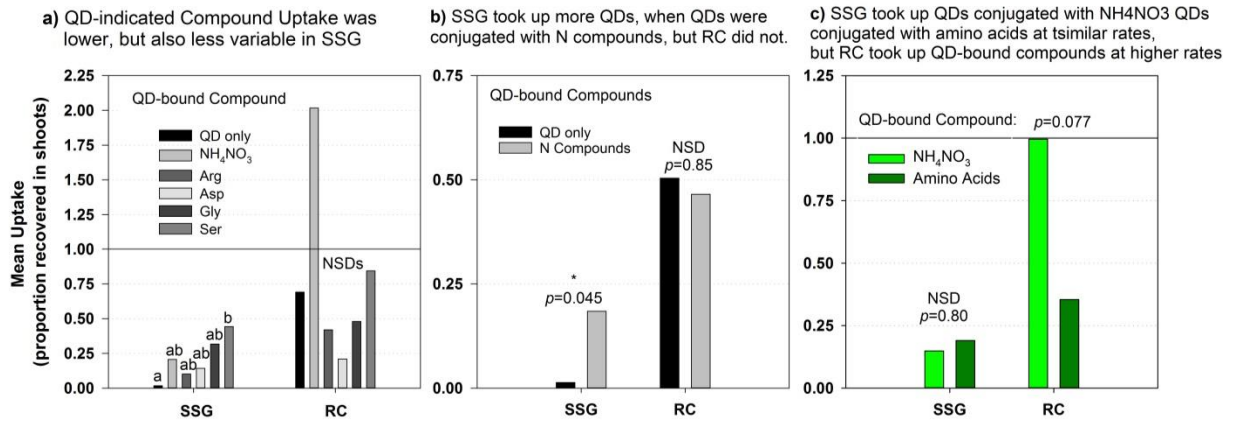
**Figure 4-12.** *QD-indicated compound uptake in SSG and RC.* Differences within species in (a) were determined via Tukey's HSD testing. Differences in (b) and (c) were determined via heteroscedastic t-tests. n=12 for both the QD-only bars in (b) and the NH_4NO_3 bars in (c). n=60 for each N Compound bar in (b) and n=14 for each "AA" bar in (c).

a) No consistent evidence of uptake from either QDs or ^{15}N in Corn

$y= 0.0034x + 0.0283$
$R^2= 0.0010$
$p=0.9115$

b) QD-indicate uptake was significantly related to ^{15}N-indicate uptake in **SSG**

$y= 44.9070x + 3.5149$
$R^2=0.4256$
$p=0.0084$

c) Some evidence of ^{15}N uptake, no evidence of QD uptake in OG

$y= 1.5133x + 0.3911$
$R^2= 0.0752$
$p=0.3228$

d) Marginal agreement between QD-indicated uptake and ^{15}N-indicated uptake in **RC**

$y= 0.2186x+0.1061$
$R^2=0.2394$
$p=0.0641$

Figure 4-13. *Linear Regressions of Mean Recovery by treatment (n=4 for each point) indicated by QDs vs. ^{15}N in plants exposed to QD-^{15}N labeled compounds.* ^{15}N-indicated recovery was significantly positively correlated with QD-indicated recovery in SSG (b) and RC (d), the 2 species which displayed fluorescence evidence of QD uptake (Figure 4-12). Corn showed no evidence of QD or ^{15}N uptake in the plants exposed to QD-^{15}N labeled compounds (a). OG showed some evidence of ^{15}N uptake, but no evidence of QD uptake (c). n=15 for each linear regression.

a) Shoot ^{15}N Reveals Over-Enrichment in SSG Exposed to QD+Isotope-labeled Compounds

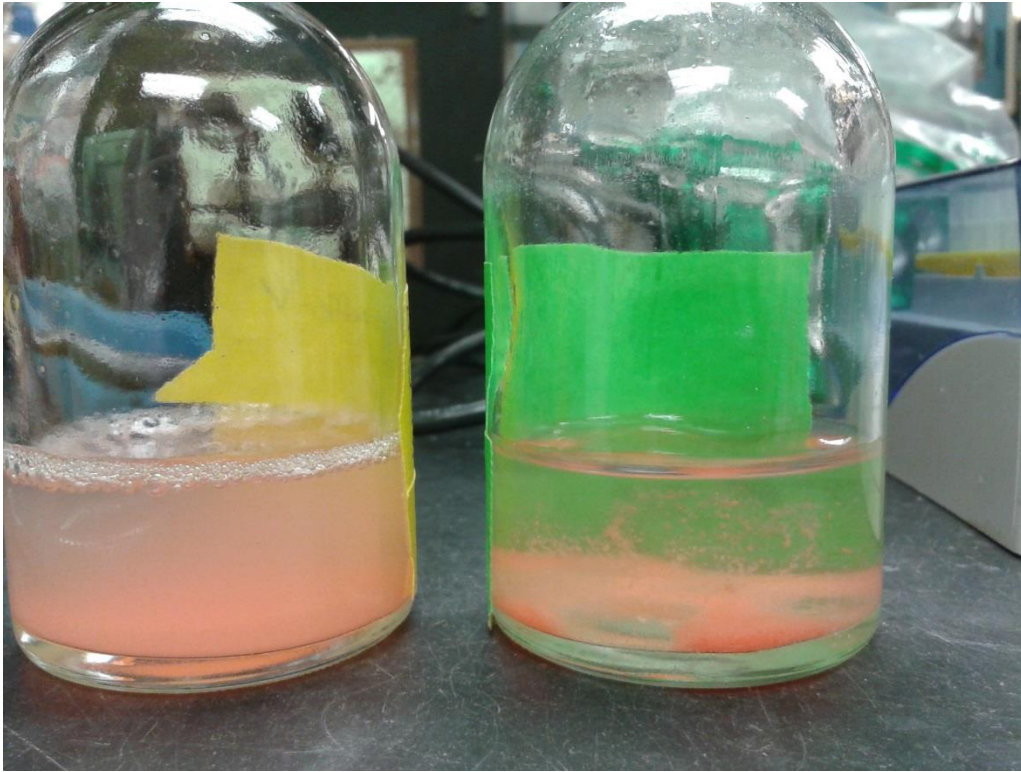**b)** Within SSG, Over-Enrichment occurred in all QD+Isotope-labeled N Compounds
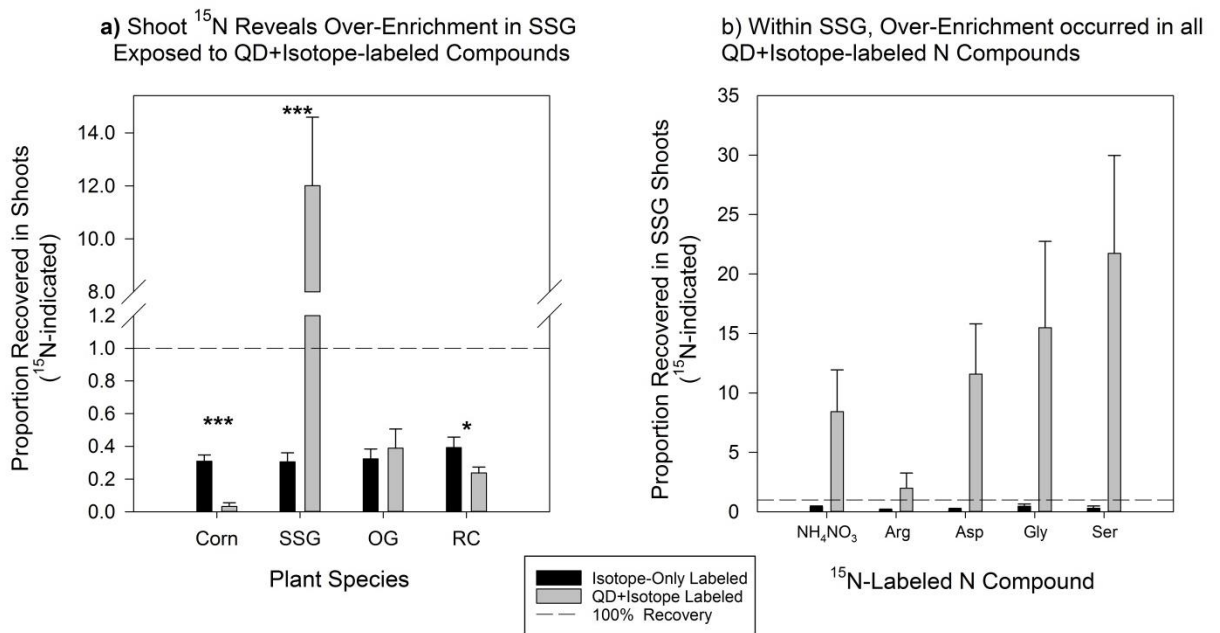
Figure 4-14. *^{15}N Recovery in Shoots by Labeling Strategy reveals over-enrichment in SSG exposed to QD+isotope labeled compounds.* Over-enrichment in plants exposed to QD+isotope labeled compounds was restricted to SSG (a). Discrimination, or reduced recovery, against QD+isotope labeled compounds relative to compounds labeled with isotopes only, was apparent in corn and red clover (a). In the SSG shoots, there was evidence of over-enrichment from each of the QD+isotope labeled compounds incubated with SSG (b; 23 SSG shoots showed over-enrichment). Bars are means + 1 S.E. Asterisks indicate significant differences between within-species isotope and QD+isotope means as determined by 2-tailed heteroscedastic t-tests ('*'p<0.05, '**' p<0.01, '***' p<0.001).
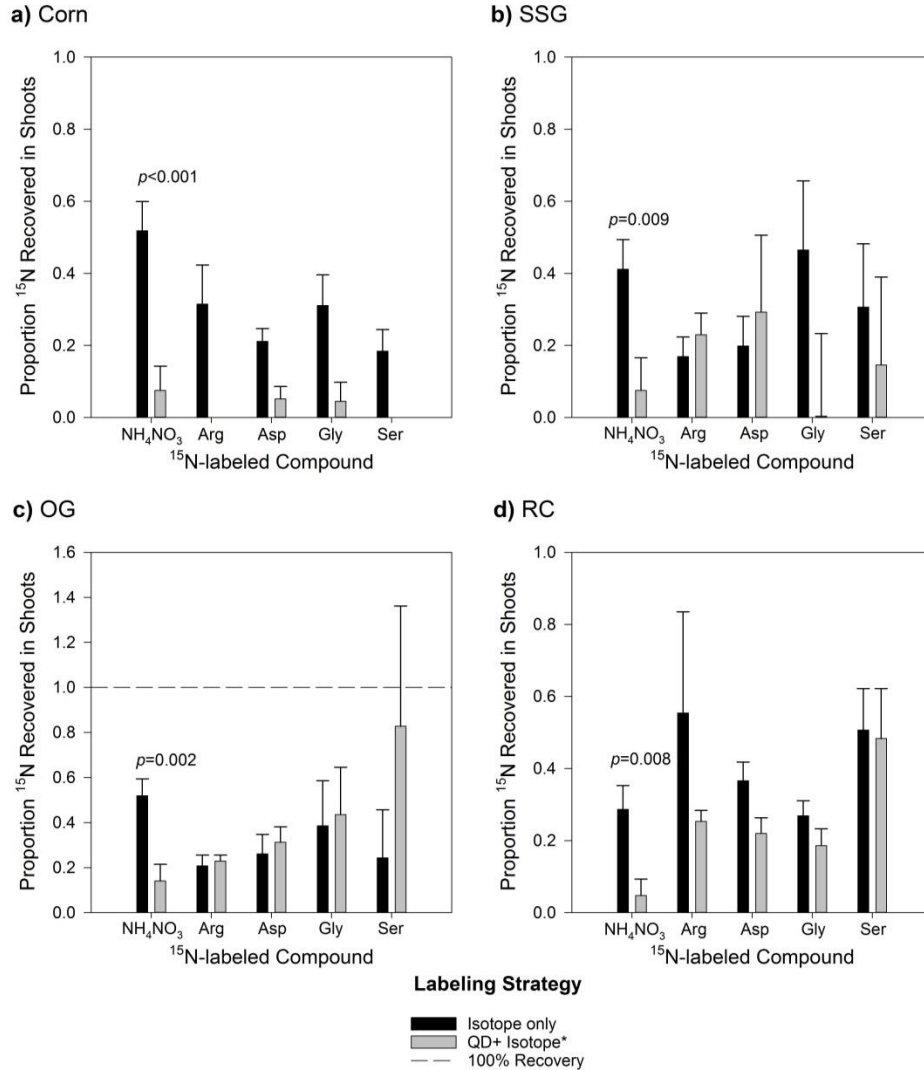
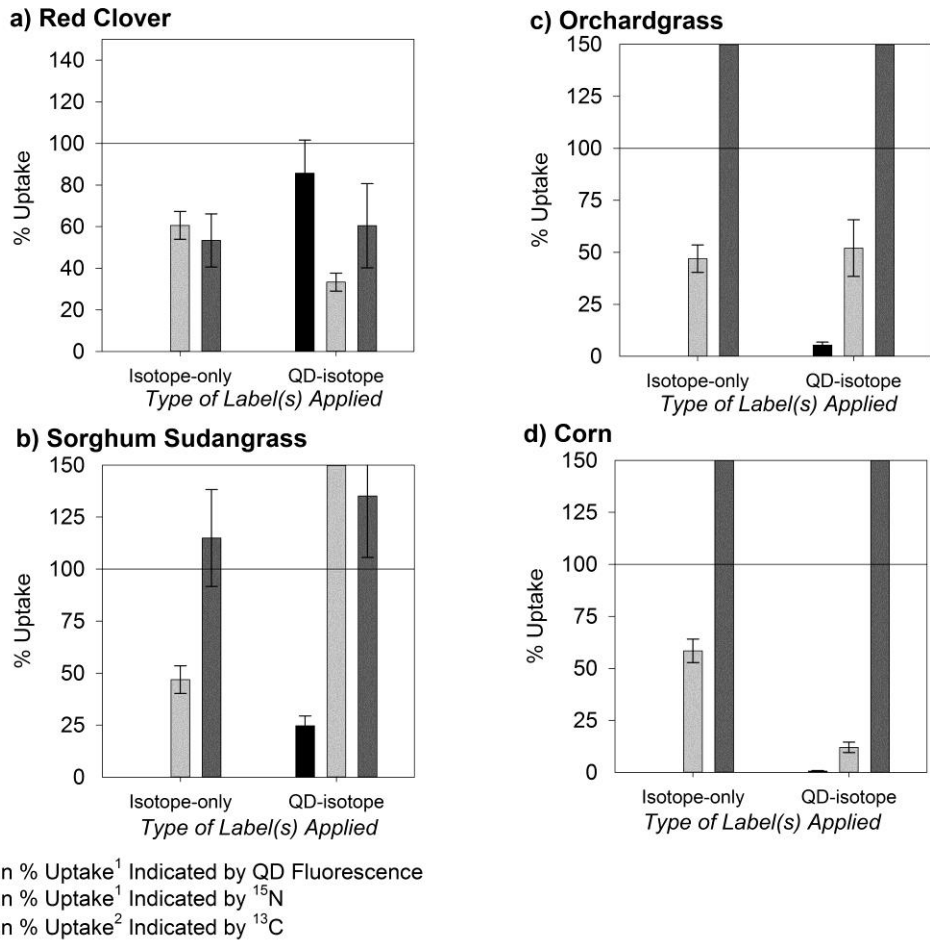**Figure 4-15.** *^{15}N Enrichment of Shoots by Species, Labeling Strategy and Labeled Compound*. ^{15}N-recovery in corn shoots confirmed there was little to no uptake of QD+isotope labeled compounds in corn (a). In all other species, plants exposed to QDs conjugated with ^{15}N$_2$-NH$_4$NO$_3$ less enriched in ^{15}N than plants exposed to un-conjugated ^{15}N$_2$-NH$_4$NO$_3$, suggesting that QD conjugation of an NH$_4$ derivative did occur, resulting in half the potential enrichment (b, c, d). This occurred because only ^{15}N-NH$_4$ was bound to the QD and the unbound ^{15}N-NO$_3$ was effectively removed from the solution prior to incubation. Bars are means + 1 SEM. Each bar has n=10-12, except for SSG QD+isotope means, which had n=7-9 due to removal of the 23 extreme over-enriched samples (*). Significant differences between isotope and QD+isotope NH$_4$NO$_3$-^{15}N recovery means within species were determined by 2-tailed heteroscedastic t-tests.

Table 4-S1. C_4 Samples Excluded Due to $^{13}\delta$ Values Suggesting C_3 Contamination

	Plant Species	Code	Growth N Regime	Inj. Trt.	$^{13}\delta$ Value
1.)	Corn	1-1-1-6	A.A. only	QD-^{15}N, 13C serine	-32
2.)	SSG	1 -2 -3 -8	NH_4NO_3	arginine(isotope only)	-25
3.)	SSG	4 -2 -2 -2	A.A.+NH_4NO_3	QD NH_4NO_3	-29
4.)	SSG	3 -2 -2 -12	A.A.+NH_4NO_3	control	-28
5.)	SSG	1 -2 -1 -3	A.A. only	glycine (isotope only)	-27
6.)	SSG	2 -2 -3 -5	NH_4NO_3	serine (isotope only)	-26
7.)	SSG	2 -2 -1 -4	A.A. only	QD-^{15}N, ^{13}C glycine	-23
8.)	SSG	4 -2 -2 -3	A.A.+NH_4NO_3	glycine (isotope only)	-22
9.)	SSG	2 -2 -2 -9	A.A.+NH_4NO_3	asparagine (isotope only)	-20

Figure 4-S1. QD Clumping and QD precipitation in the corn QD+isotope solutions

a) Red Clover

c) Orchardgrass

b) Sorghum Sudangrass

d) Corn

■ Mean % Uptake[1] Indicated by QD Fluorescence
▨ Mean % Uptake[1] Indicated by ^{15}N
▨ Mean % Uptake[2] Indicated by ^{13}C

Figure 4-S2. Uptake of label (^{15}N, ^{13}C, or QD) into the shoots relative to calculated potentials assuming equal distribution of label through entire plant biomass (roots and shoots). [1,2]Bars represent means ± 1S.E. with n of each treatment reported in Table 4.

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Appendix: Equations and Definitions

Signal (**S**): fluorescence, ^{13}C, ^{15}N in excess of the fluorescence, ^{13}C or ^{15}N of the control plants

S= fluorescence, ^{13}C, or ^{15}N of plant – mean of (control plants' fluorescence, ^{13}C, or ^{15}N)

Noise (**N**): recognized amounts of variation in fluorescence, ^{13}C or ^{15}N inherent to the plants, determined by characterization of the fluorescence, ^{13}C or ^{15}N in the control plants

$N = \frac{1}{n} \times \sum_{i=1}^{n} x_i$, where

n= the number of control plants and

x=the fluorescence or ^{13}C or ^{15}N content of each control plant

Peak signal (**PS**): maximum possible signal in terms of fluorescence, ^{13}C or ^{15}N as determined by the amount of tracer (fluorescence, ^{13}C or ^{15}N added) divided by the dilution factor determined by biomass and/or C and/or N concentration of that biomass

$$PS = \frac{Fluorescence, {}^{13}C \text{ or } {}^{15}N \text{ added}}{biomass \text{ OR } C \text{ content OR } N \text{ content of plant}}$$

Peak signal-to-noise ratio (**PSNR**): the maximum possible power of the tracer's fluorescence or isotopic signal determined by the amount of tracer added and the amount of dilution in biomass (PS, above) divided by the power of the isotopic or fluorescence noise inherent to the plant tissue (N, above)

$$PSNR = \frac{PS}{N}$$

Chapter 5
Organic Nitrogen Uptake by Plants: A Trait Primed by Rooting Environment and Relevant in Soils under Established Organic Management

ABSTRACT

Recent evidence shows both wild and domesticated plant species take up organic nitrogen (N), necessitating a critical re-evaluation of the long-held definition of plant available N as limited to ammonium (NH_4^+) and nitrate (NO_3^-). While numerous studies have documented organic N uptake abilities in diverse plants', and others have dismissed the organic N uptake pathway due to low concentrations of organic N in some field soils, no studies have evaluated the interactions between plant uptake ability and the presence of organic N sources in the rooting zone. We sought to evaluate the hypothesis that plant organic N uptake ability is not static or innate to a species, but rather can be primed by the presence of organic N in the rooting zone. We also sought to evaluate the relevance of this organic N uptake pathway in organic cropping systems where corn (*Zea mays* L.), orchardgrass ("OG", *Dactylis glomerata* L.), red clover ("RC", *Trifolium pratense* L.) and sorghum sudangrass ("SSG", *Sorghum bicolor* ssp. *drummondii*) were present by characterizing the availability of amino acids (AAs) relative to long-accepted pools of plant-available N, NH_4^+ and NO_3^-. Our results re-affirm the findings of others that AA-N is plant-available, and that it was assimilated by four of the crop species we evaluated, but our results also indicate that AA-N results in lower rates of biomass accumulation and greater biomass partitioning to roots. [15]N labelling indicated that organic N-dominated environments increase plant uptake of both AA-N and inorganic N, supporting the priming hypothesis in all of these plant species. Lastly, we found AA-N is available in forage cropping

systems under long term organic management, and makes up a critical portion of the available

N profile, especially in the early spring. These results suggest organic N is both accessible to

plants and available in the soil in organic cropping systems, and that soil availability patterns

may increase AA-N availability to plants.

INTRODUCTION

Since Liebig's 1840 assertion that only mineral forms of nutrients were plant available, NH_4^+ and

NO_3^- have been presumed to be the only forms of nitrogen accessible to plants (van der Ploeg

1999). More recently, beginning in the early 1990s, various workers have shown organic

nitrogen (N) uptake by plants to be important, primarily in inorganic N-limited environments,

such as the arctic tundra where plant N uptake exceeds the N mineralized, and many plant

species even prefer taking up organic N over inorganic N (Chapin et al. 1993, Kielland 1994).

Chapin and colleagues (1993) seminal work provided strong evidence suggesting that direct

uptake of AA-N is the primary N uptake pathway for plants in the arctic. This early work largely

attributed this novel uptake pathway to be unique to the arctic environment, where tundra soils

are frequently >80% OM, yet low temperatures prevent microbial mineralization (Kielland 1994).

Subsequent reviews have largely dismissed the potential importance of this organic N uptake

pathway outside the arctic, largely based on results with barley, which demonstrated negligible

capacity to take up inorganic N relative to tundra species (Neff 2003). They attributed these

differences to barley's mineral soil origins, concluding that organic N likely plays a negligible role

in plant nutrition outside of these extraordinarily OM-rich sites (Chapin et al. 1993). So, while

the existence of this alternative N uptake pathway in inorganic N-limited environments has been

well-documented and increasingly acknowledged, subsequent reviews of the pathway's broader

ecological importance have been largely dismissive, especially in regards to agroecosystems and crop plants (Neff et al. 2003).

Other more contemporary studies have also questioned the importance of the entire plant-organic N uptake pathway, based on experimental observations of concentration-dependent uptake rates and observations of organic N availability in agroecosystems (Christou et al. 2005, Jones et al. 2005a, Jones et al. 2005b). Jones and colleagues' (2005a) argued plant AA uptake is concentration-dependent, citing their work with corn (Zea mays L.) demonstrating that uptake was maximized only at concentrations that exceeded concentrations found in fertilized grassland soil by three orders of magnitude, and that uptake rates at concentrations more analogous to measured field soil concentrations were negligible. Therefore, they concluded that the organic N plant uptake pathway is likely unimportant in most environments, especially agricultural ones where soil AA concentrations are low (Jones et al. 2005b).

Subsequent research on plant organic N uptake work has called the environmental limits of plant organic uptake into question, as more plant species have been shown to take up intact organic nitrogen outside of OM-rich tundra environments. Some of the first work extended only as far as focusing on AA uptake capacities of various coniferous species in the Nordic region (Ohlund and Nasholm 2001). This work demonstrated many coniferous species also have not only the capacity to utilize AAs as an N source, but also that many prefer AA-forms of N over NH_4^+ and/or NO_3^- (Ohlund and Nasholm 2001, Ohlund and Nasholm 2002). Subsequent work also determined that conifer seedlings displayed divergent morphology based on the form of N they were fertilized with (Gruffman et al. 2012). Conifer seedlings grown on AA-N had larger roots and greater root to shoot ratios than those grown on inorganic nitrogen, which

improved N use efficiency in the nursery and conferred survivorship benefits when they were

transplanted from nurseries to plantations (Gruffman et al. 2012). These results suggest that

growing in an environment dominated by AA-N could increases plant N uptake abilities by

altering morphology to increase root biomass. However, Nordic pine nurseries and plantations

still have extremely high OM- and organic N-availability relative to most agricultural systems,

making the relevance of these findings beyond organic N-rich environments questionable.

Regardless, Nasholm and colleagues' (2009) review concluded that many plant species from

across high latitude or altitude ecosystems are capable of taking up and even prefer AAs relative

to mineral N, and pointed to increasing evidence that this organic N uptake pathway is

conserved in non-arctic species and perhaps more plastic than previously believed.

Plant-organic N uptake has now been documented by a wide variety of agricultural

species in agricultural settings both in the Nordic region and beyond (Reeve et al. 2008, Nasholm

et al. 2009, Puangfoo-Lonhienne et al. 2010, Vinall et al. 2012, Whiteside et al. 2012). The first

study to challenge the inorganic N paradigm in agricultural systems demonstrated that four

agricultural species (*Phleum pretense*, *Trifolium pratense*, *T. hyb*ridum, and *Ranuculus acris*),

commonly present in Swedish pasturelands, can take up 19-25% of applied dual-labeled (^{13}C, ^{15}N)

glycine intact (Nasholm et al. 2000). ^{15}N-Enrichment indicated that clover species took up

glycine-N at similar rates relative to NH_4^+- and NO_3^--N (Nasholm et al. 2000). While both the

grass and the forb exhibited the capacity to take up intact glycine, ^{15}N enrichment suggested

both species took up NO_3^--N at higher rates than glycine (Nasholm et al. 2000). Pastureland

species in the UK also demonstrated capacity to take up organic N intact (Weigelt et al. 2005).

However, in the UK study, they found an apparent inverse relationship between plant growth

potential and AA uptake ability (Wiegelt et al. 2005). The plant species with the lowest biomass

potential, took up the most AA-N, whereas the plant species with the greatest biomass potential,

took up the least AA-N (Wiegelt et al. 2005). While the work on conifers suggested AA uptake

ability may be a plastic trait that can be primed or increased by an AA-N-enriched rooting zone,

Weigelt and colleagues (2005) suggest plant AA uptake capacity may be more of an innate

characteristic, selected over a longer period of time by the relative abundance of inorganic N

versus AA-N in the rooting zone over the plant's evolutionary history (Gruffman et al. 2012).

Despite remaining questions around the mechanisms controlling the organic N uptake

pathway in plants, and whether those mechanisms are innate or plastic, others have

documented the pathway's existence in both a broader array of plant species, and in a broader

array of organic N compounds beyond AAs. Australian researchers discovered that NO_3^- is not

sugarcane's preferred source of N, suggesting AAs may be preferred (Robinson et al. 2011).

Subsequent research confirmed this, and also demonstrated that AA presence in the rooting

zone elicits the same stimulatory effect on root biomass and altered biomass partitioning effect

first observed in conifers (Vinall et al. 2012). Furthermore, additional agar plate-based research

from Australia has also documented that plants' organic N uptake pathway is not limited to AAs

(Puangfoo-Lonhienne et al. 2008, Puangfoo-Lonhienne et al. 2010). In fact, research on

Arabidopsis and tomato (*Solanum lycopersicum*) demonstrated that those species are capable of

taking up not only intact proteins to utilize as a source of N nutrition, but also entire bacteria

and even yeast to utilize as an N source (Puangfoo-Lonhienne et al. 2008, Puangfoo-Lonhienne

et al. 2010). Additional work revealed that stimulatory effect of organic N forms on root biomass

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accumulation and increased biomass allocation to roots is also conserved in Arabidopsis, further

suggesting plant uptake abilities may be more plastic than innate (Aguetoni-Cambui et al. 2011).

Despite this accumulating evidence that plants, including agricultural species, can

directly use organic forms of N as sources of N nutrition, and that rooting zone chemistry,

specifically the presence of organic N, increases plants' organic N utilization by increasing root

biomass and functioning; evaluations of the existence of both this pathway and environmental

priming in US agricultural systems is scant. One study that did evaluate AA uptake abilities in

strawberry species (*Fragaria spp*.) found AA uptake in wild strawberry species was only one-

quarter to one-fifth the rate of NH_4^+- or NO_3^--N recovery, and that the domesticated species

recovered AA-N at far lower rates still, at only one-fiftieth the rate of the wild species (Reeve et

al. 2008). Reeve and colleagues (2008) also found no evidence that organic management

increased AA-N availability relative to conventional management, suggesting the AA-N uptake

pathway may have little relevance to crop N nutrition, even in organically managed systems.

The goals of this research were to compare AA uptake ability to inorganic N uptake

ability in four commonly cultivated agricultural species, to assess whether rooting zone

influences uptake ability, and to determine what AA-N availability was and how it compared to

inorganic N availability in organic cropping systems, an environment where these species are

commonly grown. We tested the following hypotheses: (1) N uptake ability differs between

species; (2) rooting zone influences biomass partitioning of all species and organic N-containing

rooting zones increase N uptake ability; and (3) AA-N is available in quantities similar to

inorganic N in soils under long term organic management.

METHODS

Greenhouse experiment

We used a randomized factorial design to test N uptake ability indirectly through plant growth, and then directly using ^{15}N-labeled N compounds. In the growth phase of the experiment, we included two factors: plant species and rooting zone. For plant species, we included two C_4 grasses, corn (*Zea mays*) and sorghum sudangrass ("SSG", *Sorghum bicolor* ssp. *drumondii*); one C_3 grass (orchardgrass, "OG", *Dactylis glomerata*); and one legume (red clover, "RC", *Trifolum pratense*). We included 3 rooting zones that differed only in the form in which N was supplied. We supplied N in 3 ways: 1) inorganic only (NH_4NO_3), 2) a 50:50 mix of inorganic and organic N (half NH_4NO_3, half AA-N), and 3) organic N only (a mix of 4 AAs). AAs present in the organic and mix N rooting zones were arginine (Arg), asparagine (Asp), glycine (Gly), and serine (Ser). We supplied N at a constant rate of 150 mg L^{-1} (10.7 mM N). To ensure rooting zones were otherwise uniform, we supplied the balance of all plant nutrient needs except calcium with commercially-available Cornell No N fertilizer, applied at 0.5 g L^{-1} (Greencare Fertilizers, Kankakee, IL). We supplied calcium at 90 mg L^{-1} as calcium phosphate monobasic monohydrate, and adjusted the pH of all solutions to 6.8 to 7.0 using CaOH.

We took several methodological precautions to further standardize rooting zones, and exclude microbial interference to the greatest extent possible. All seeds were NaClO-sterilized prior to germination on filter paper (SSG, OG, and RC) or direct seeding into the cone-tainers (corn; SC7 Stubby Ray Leach Cone-tainers^{TM}, Stuewe & Sons, Tangent, OR). In the 107 mL cone-tainers, we cultivated plants on autoclaved sand and supplied 240 mL of fresh nutrient solutions

through a NaClO-sterilized experimental manifold/automated irrigation system every 2-4 hours,

throughout the duration of the experiment. The low water holding capacity of the sand and the

excess volume of fertigation solution facilitated complete flushing of each plant's cone-tainer in

conjunction with each irrigation event, minimizing retention of potentially mineralized AA-N in

the organic and mixed N rooting zones. From the experimental manifold, constructed of black,

12.7 mm irrigation tubing, we used 4-way pressure compensating emitters coupled with dripper

stakes via 3mm UV White PE Tubing to deliver fertilizer solution to each plant's cone-tainer

(Hummert International, Earth City, MO). To further maintain rooting zones that were consistent

with their intended N profiles, we flushed the fertilizer solution reservoirs with RO water every

24-72 hours, and replaced the solutions with freshly mixed fertilizer solutions.

We allowed the plants to grow in the greenhouse for 30 ± 5 days, or until they reached a

height approximately equivalent to the length of the cone-tainers (~14 cm). Greenhouse

temperatures were maintained between a minimum of 21°C at night and a maximum of 25°C

during the day, throughout the experiment. Throughout the experiment, we also re-randomized

the plants within the experimental manifold every 3 days to avoid any location effects on plant

growth. Plants were clustered by species within the manifold. As the plants approached the

maximum intended height, a subset of plants were removed from the experimental manifold,

and exposed to ^{15}N-labeled NH_4NO_3 or one of the four AAs for 4-5 days to assess actual uptake

rates of labeled N compounds through shifts in shoot δ^{15}N signatures.

Greenhouse Experiment - ^{15}N Tracer Phase

Prior to introduction of the ^{15}N-labeled compounds, we ceased fertilization, flushed the cone-tainers with DI H2O, and then sealed the bottoms of the cones with parafilm. We introduced one of five ^{15}N-labeled compounds (NH_4NO_3, Arg, Asp, Gly or Ser) to 4 replicate plants from each of the 12 growth phase treatments (4 plant species x 3 rooting zones). Compounds were introduced via 18-guage side port needle as 1.5 mL tracer solutions of either 15 μM (corn) or 5 μM (all other species) N compound concentrations prepared in DI H_2O (Cadence Inc., Cranston, RI). Four replicate control pants from each growth treatment were exposed to 1.5 mL DI water introduced via the same type of side port needle. Plants were incubated with the tracer solutions or water for 4-5 days. Throughout the incubation period, small amounts of DI H2O were added to each plant's cone-tainer via wash bottle to prevent desiccation. The corn incubation period occurred from days 23-28, and the incubation for the other 3 species occurred from day 30 through day 34 or 35. After the incubation period, the shoots were clipped and immediately dried at 60°C for 48 hours. Roots were washed to remove sand particles and then also dried at 60°C for 48 hours. Roots and shoots were weighed on an analytical balance, and then shoots were subsequently ground in an 8000M Mixer/Mill until all shoot tissue became fine powder (SPEX SamplePrep, Metuchen, NJ). Dried, homogenized subsamples (2 mg) of shoot tissue were analyzed for %C, %N, and $\delta^{15}N$. The Laboratory for Isotopes and Metals in the Environment (Penn State University, University Park, PA) conducted these analyses using an ECS 4010 CHNSO analyzer (Costech Analytical Technologies, Inc., Valencia, CA) connected to a ConFlo IV universal continuous flow interface (Thermo Fisher Scientific, Inc., Waltham, MA) that served as an inlet to a Delta V™ Isotope Ratio Mass Spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA). Background ^{15}N levels present in

the controls were subtracted from treatment values and uptake of ^{15}N-labeled compounds was calculated as excess ^{15}N enrichment in the shoots relative to maximum potential shoot ^{15}N enrichment. We defined maximum potential enrichment as the amount of ^{15}N added relative to (divided by) the total biomass of each plant.

Availability of AAs in Organically Managed Field Soil

We assessed the availability of AAs in field soil under organic management for 9-11 years using a fluorescent microplate assay, which measures AAs and other free primary amines in soil extracts (Darrouzet-Nardi et al. 2013). Soil samples (400-500 g sample^{-1}, 20 cm sampling depth) were collected between August 2012 and August 2014 from an organic forage systems experiment featuring seven, two-year annual forage to corn silage systems, and one perennial OG-RC system. In addition to the annual –perennial management dichotomy encompassed by this dataset, the forage systems experiment also featured a spectrum of organic N management strategies, from RC-only to RC and manure to manure-only. Lastly, systems that included manure, received either 56 kg N ha-1 broadcast manure applications and/or simulated grazing manure applications designed to mimic dung spots (Grantham et al. *in prep.*). Thus, this dataset includes a wide variety of divergent short-term N management legacies, as well as a common long-term organic soil management legacy across all samples.

After sampling, we stored soil samples at 4°C for transport back to the lab, where we weighed, homogenized and subsampled each soil sample to analyze for gravimetric water content, NH_4^+, NO_3^-, and AA-N within 24 h of sampling. We used ~10 g fresh soil for gravimetric water content determination by drying at 105°C for ~48 h. We extracted ~20 g fresh soil sub-samples in 2 M

KCl (5:1 solution: soil ratio), which facilitated analysis for NO_3^--N, NH_4^+-N and AA-N all on the same soil extracts (Darrouzet-Nardi et al. 2013, Jones and Willett 2006, Mitchell et al. 2013). We extracted soil by shaking for 1h at ~180 rpm (Mitchell et al. 2013). We then immediately filtered and froze soil extracts as described by Mitchel et al. (2013). We determined NO_3-N and NH_4-N by absorbance in microplates using the vanadium (III) chloride reaction and salicylate methods, respectively (Doane and Horwath 2003, and Hood-Nowotny et al. 2010, respectively). All extracts were analyzed in triplicate. Gly was used as the standard in the fluorescent microplate determination of soil AA content. The average N content of all AAs, 1.45 mol N mol^{-1} AA, was used to convert fluorometrically-determined AA concentrations into units of approximate AA-N. Due to potential for NH_4^+ interference in the AA assay, fluorescence values were corrected for NH_4^+ interference using the independently measured spectrophotometrically- determined NH_4+ concentration of each sample (Darrouzet-Nardi et al. 2013).

Statistical Analyses

The factorial design used in both phases of this experiment and for both potential and actual tracer performance datasets facilitated similar analytical approaches to all datasets: MANOVA on sets of related variables, followed by ANOVA on each variable, and then Tukey Post Hoc Testing conducted in SigmaPlot or R (SigmaPlot Version 13.0, Systat Software, San Jose, CA, R Core Team 2014). For multivariate datasets, we used the Wilk's λ to assess variable importance (R Core Team 2014). Two-way ANOVAs tested for plant species effects, N environment effects, and species by environment interaction effects on all measured and derived raw variables that met normality and equal variance assumptions from the growth phase of the experiment. The Shapiro-Wilk method was used to test normality, the Bonferroni Outlier Test was used to

identify outliers, and the Brown-Forsythe method was used to test for equal variance (Fox and

Weisberg 2011, R Core Team 2014). Standard log, log(x+1), square root, and cube root

transformations were used to transform raw variables characterized by violations of normality

and/or equal variance assumptions to facilitate ANOVA methods. However, if these

transformations were ineffective, we used a Kruskal-Wallis ANOVA on ranks to determine

significance of factor main and/or interaction effects (SigmaPlot Version 13.0). When ANOVA

results indicated significant effects of factors or factor interactions that included more than 2

treatments, Tukey's Honestly Significant Difference was used as a post-hoc test to determine

which treatments were significantly different from one another (at α=0.05). In R, all analyses

were performed using functions available from the core "stats" package or from the companion

to applied regression package, "car", (Fox and Weisberg 2011, R Core Team 2014). To assess

differences between unbalanced groups defined by similar management, we used

heteroscedastic t-tests (Microsoft Excel 2010). To assess relationships between pools of N in

field soil, we used linear and/or logistic regression procedures available in SigmaPlot.

RESULTS & DISCUSSION

Growth Phase: Total Plant Biomass and Biomass Partitioning

All plant species grew, and despite timing the termination of the growth phase of the

experiment to minimize species differences, plant species afftected all plant biomass related

variables: total biomass, shoot biomass, root biomass, and root : shoot ratio (Figure 5-1, Tables

1 and 2). Plant species was the main driver of variance across all 4 dependent biomass-related

variables (Tables 1 & 2, Wilks' λ MANOVA and ANOVA, Figure 5-1, 64% overall, 20-38%

depending on which biomass-related variable). Plant species effects on total biomass differed

from plant species effects on the shoot and root biomass (Figure 5-2). Grass species all had

similar total biomass, and were ~twice the size of RC (gray bars, Figure 5-2a). But, while total

biomass was similar between the three grasses, partitioning between roots and shoots differed

not only between the grass species and RC, but also between the grass species (gray bars, Figure

5-2e). SSG and OG exhibited significantly greater shoot biomass than corn, but corn had

significantly greater root biomass than either SSG or OG (gray bars Figure 5-2b, Figure 5-2d).

Among the three grass species, OG had the least root biomass, but still had significantly more

root biomass than RC (Figure 5-2d). Thus, of all the species, corn had the greatest absolute root

biomass as well as the greatest relative allocation of biomass to roots (Figures 2d and 2e). RC,

while having the least absolute root biomass (Figure 5-2d), had greater relative biomass

allocation to roots than either OG or SSG, but less than corn (Figure 5-2e). However, after

accounting for these species-specific effects on biomass, effects of rooting zone N regime were

also apparent.

While all species demonstrated a capacity to grow on all N regimes, all species tended to

accumulate more biomass when they were grown in the inorganic N environment. However, the

significance of this N environment effect difference on total biomass and shoot biomass varied

by species (species x environment interaction effect, $p<0.001$ overall, $p<0.01$ for total and shoot

biomass, Figure 5-1, Tables 5-1 and 5-2). For OG, the inorganic N environment supported

significantly greater shoot biomass and total biomass than either the mixed N environment or

the organic N environment, which did not differ significantly from one another (Figure 5-2).

However, OG was the only species to exhibit such a clear and consistent inorganic N advantage

in terms of biomass accumulation (Figure 5-2a and 5-2b). The inorganic N environment also

supported greater total biomass in corn, but shoot biomass in corn was not significantly

different between the inorganic N environment and the organic N environment (Figures 2a and

2b). There were no significant differences in total biomass between rooting zones in either SSG

or RC (Figure 5-2a). Like corn, SSG shoot biomass did not differ significantly between plants

grown in inorganic N environments and those grown in organic N environments (Figure 5-2b).

But RC, like OG, did accumulate more shoot biomass when grown on inorganic N than when

grown on organic N, with the mixed N environment supporting intermediate shoot biomass

accumulation that did not differ significantly from either the inorganic N environment or the

organic N environment (Figure 5-2b).

Effects of N environment on root biomass where less dramatic than effects on shoot

biomass, and, unlike effects on total and shoot biomass, did not vary by species, with the

inorganic N environment supporting significantly greater root biomass in all species (Figure 5-2c).

However, while inorganic N environments increased shoot biomass 30-100% relative to shoot

biomass accumulated under mixed N or organic N environments, inorganic N environments only

increased root biomass ~10%. This difference in N environment effect size between shoots and

roots, resulted in ratios of biomass allocation that differed significantly by rooting zone. Plants

grown in organic or mixed N environments tended to allocate more biomass to roots relative to

shoots (Figure 5-2e). While this difference was not significant within species (except corn), it was

relatively consistent across species, and thus across species the effect was significant (Table 2,

ANOVA, significant rooting zone effect on root:shoot).

Growth Phase: Plant Uptake of Inorganic and Organic N Suggested by N Content of Shoot Biomass

Patterns of shoot N content mirrored patterns of total shoot biomass accumulation, with differences driven by differences in shoot mass rather than differences in the concentration of N in shoots, which was constant within species regardless of rooting zone (Figure 5-3a). Similarly, the C:N ratios, while somewhat variable by species, showed no evidence of luxury consumption, or reduced C:N ratios, in the plants grown on inorganic N (Figure 5-3b). The plants' C:N ratios did not differ significantly between rooting zones for all species except SSG (Figure 5-3 b). SSG grown on inorganic N had significantly higher C:N ratios than SSG grown on either organic N or the mix of organic and inorganic N, the opposite difference in C:N ratios that would be expected if the inorganic N environments supported luxury N consumption, relative to the organic and mixed N environments.

Tracer Phase: Plant Uptake of Inorganic and Organic N Indicated by ^{15}N

While all plant species in this experiment may have an innate preference for inorganic N; or are able accumulate more biomass, especially shoots, when grown on inorganic N; results from the tracer phase of the experiment revealed that there are large feedbacks, or priming effects, of rooting zone chemistry on plants' abilities to take up ^{15}N-labeled AAs and NH_4NO_3 (Table 5-3, Figure 5-4). Specifically, plants grown in organic N environments take up significantly more ^{15}N-labeled compounds than do plants grown in inorganic N environments, with plants grown in mixed N environments taking up ^{15}N-labeled compounds at intermediate rates (Figure 5-5a).This response was robust across species, with plants grown in organic N taking up more

^{15}N than plants grown with either inorganic N or a mix of organic and inorganic N (Figure 5-5b). The concentration of the tracer seemed to have no effect on uptake rates, as corn, which was exposed to 3x as much tracer as the other 3 species, took up ^{15}N at similar rates to the other 3 species (Figure 5-5b). While the difference in uptake between plants grown in organic and inorganic N environments was consistent, the performance of the plants grown in the mixed N environments varied, with the plants grown in the mixed N environments performing more like the inorganic N-grown plants in SSG and OG, exhibiting intermediate performance in RC, and performing more like the organic N grown plants in corn (Figure 5-5b). While we had hypothesized this type of priming effect for AA uptake, we were surprised to observe that the plants grown on organic N also took up NH_4NO_3 at higher rates (as indicated by ^{15}N enrichment, Figure 5-5c). Regardless, this suggests that plants grown on organic N have superior N uptake abilities, despite their smaller roots (more N capture per unit root mass in the organic N-grown plants).

Plant preference for inorganic N was less clear in the tracer phase than in the growth phase of the experiment, with no significant differences in uptake between NH_4NO_3 and three of the four AAs (Figure 5-6a). Averaged across species, uptake of NH_4NO_3 did exceed rates of Arg uptake; a large, N-rich AA; but, within species differences were not significant (Figure 5-6). Furthermore, while not significant, within species mean uptake rates of ^{15}N-labeled compounds suggests plant species differed in their AA preferences (Figure 5-6b). Corn appeared to prefer Arg and Gly, whereas RC appeared to prefer Ser. SSG was the only species for which the highest mean uptake rate was observed in Gly, rather than NH_4NO_3, and mean Ser uptake in SSG was similarly high, suggesting SSG may take up small AAs, like Ser and Gly at similar rates as NH_4NO_3.

Thus, rather than an innate trait, N uptake ability appears to be highly responsive to rooting zone chemistry, with organic N rooting zones eliciting greater N uptake abilities for all N compounds in all four of the plant species evaluated in this experiment.

Inorganic and Organic N Availability in Field Soil under Long-term Organic Management

While results from this greenhouse experiment indicate organic N-rich rooting zones increase plant uptake of organic and inorganic N compounds 1.5 times, on average, we sought to understand to what extent these mechanisms might be playing out in the field where these species were also grown by examining availability of AA-N relative to inorganic N pools. We found AA-N (AA-N) is a less variable pool of N that had greater median availability than NH_4^+-N or NO_3^--N in field soils under long-term organic management (Figure 5-7). Mean AA-N availability was similar to mean NO_3^--N availability, but mean NH4+-N was greater than mean AA-N (Figure 5-7b). This difference in means was largely driven by the more extreme peaks in NH4+-N availability associated with manure application events meant to simulate grazing in these organic forage systems (Figure 5-8a). Still, despite the more extreme peaks in both NH_4^+-N and NO_3^--N in these systems, both pools of inorganic N were significantly positively related to AA-N availability (Figure 5-8a, 5-8b). However, in a relative sense, the AA-N pool made up the greatest proportion of total available N (sum of NO_3^--N, NH_4^+-N, and AA-N), when total N was low (Figure 5-8c). Furthermore, AA-N was the largest pool of available N in these systems overall, trailed closely by NH4+-N and with NO_3-N being the least dominant pool of N (Figure 5-9).

Perhaps most importantly, given the role of environmental priming in determining plants' ultimate N uptake abilities, AA-N is especially well-represented in the early spring,

making up more than 50% of total N availability in late April. In the perennial OG+RC system, which did not receive spring manure inputs, AA-N becomes an increasingly important portion of the available soil N pool through the early summer when it makes up more than 80% of available N in that system. During corn silage production, there is greater daily mean AA-N availability in systems containing RC than in those relying on manure alone (Figure 10c). While these mean daily differences are small (1-2 kg N ha^{-1}), their consistency over the length of the corn silage season, and especially the persistence of AA-N availability when the inorganic N pools have been largely exhausted, could give the AA-N pool even greater importance. Regardless, these data on soil AA-N availability in field soil under long-term organic management suggest AA-N is available in similar quantities to inorganic N pools averaged over the growing season, but that its relative consistency makes it an attractive complementary N resource, especially in legume-based systems. Lastly, the dominance of the AA-N pool early in the growing season suggests that the N profile inherent to field soil in organic management systems may elicit the N uptake priming effect observed in the plant species evaluated in the greenhouse experiment.

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TABLES

Table 5-1. *MANOVA of Four Biomass-Related Variables: Total Biomass, Shoot Biomass, Root Biomass, and Root:Shoot.* n=576 for each dependent variable

Factor	*Variance explained*	*df (w/in each var.)*	*Wilks' λ*	*p-value*
Plant Species	61.4%	3	0.386	$<2.2 \times 10^{-16}$
Rooting zone	14.8%	2	0.852	4.4×10^{-16}
Plant Species x Growing Env't	12.4%	6	0.876	4.1×10^{-7}
Residuals (unexplained variance)	11.4%	564		

Table 5-2. *ANOVAs of Four Biomass-Related Variables: Total Biomass, Shoot Biomass, Root Biomass, and Root:Shoot.* n=576 for each dependent variable

Factor or Factor Interaction	Dependent Variable				
	df	*Total Biomassł*	*Shoot Biomass[‡]*	*Root Biomassł*	*Root:Shoot[‡]*
		ANOVA-determined *p-value*			
Plant Species	3	< 2 x 10-16	< 2 x 10-16	< 2 x 10-16	< 2 x 10-16
Growing Environment	2	< 2 x 10-16	8.05 x 10-13	1.41 x 10-9	0.002
Plant Species x Growing Environment	6	0.002	0.002	0.184	0.018
Residuals	564				

łAnalysis performed on square root-transformed data

[‡]Analysis performed on log-transformed data

Table 5-3. *ANOVA of Percent Uptake indicated by ^{15}N-enrichment of shoots*. Data were cube-root transformed prior to analysis to meet normality assumptions. One outlier, identified with the Bonferroini outlier test was temoved and replaced with the treatment mean. Missing values (10) were replaced with treatment means to meet balance requirements of ANOVA analysis.
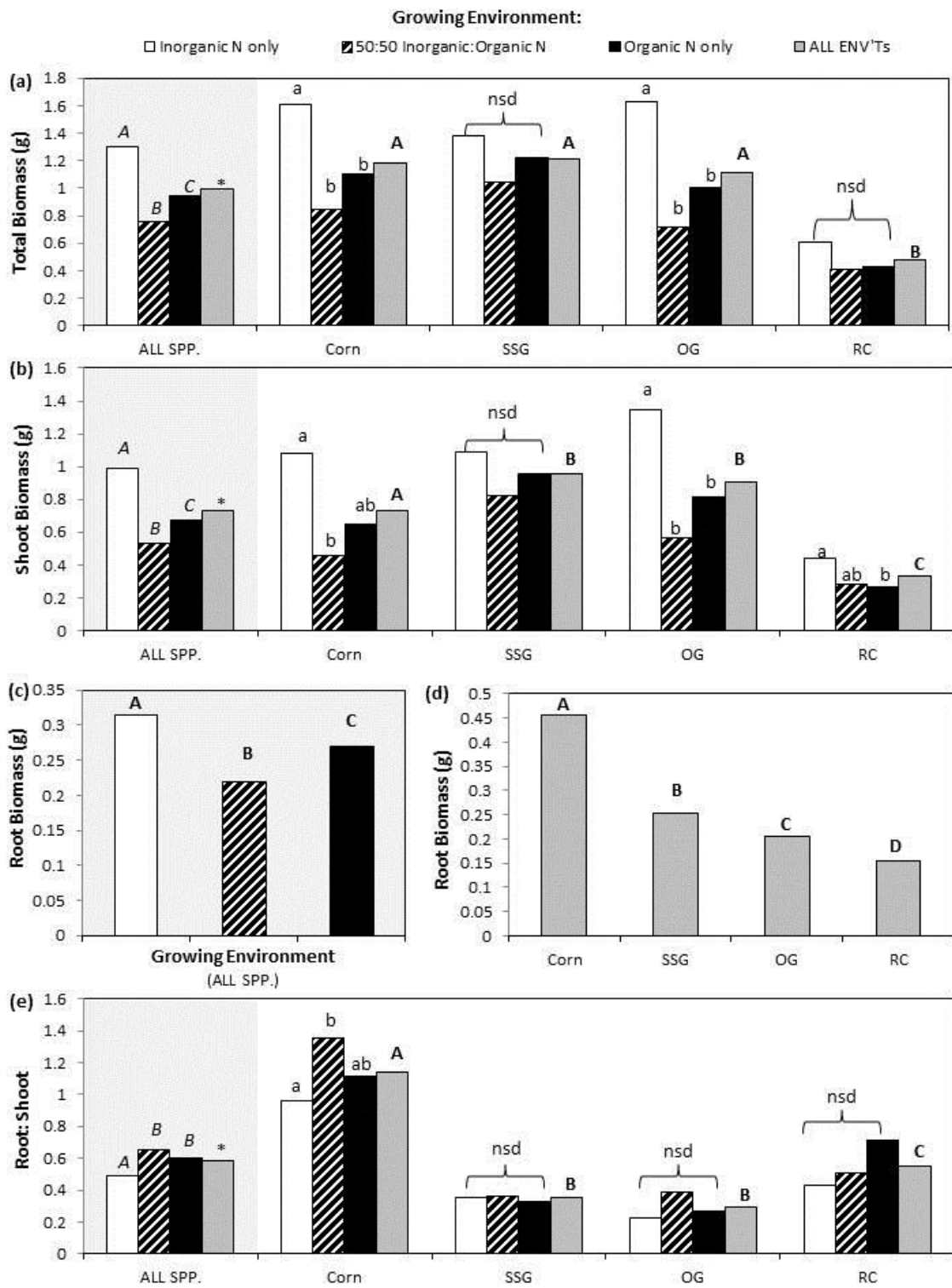
Factor or Interaction	df	p-value
Plant Species	3	0.624
Rooting zone	*2*	*3.66 x 10^{-7}*
N Compound	*4*	*3.69 x 10^{-5}*
Plant x Environment	6	0.969
Plant x Compound	*12*	*0.002*
Environment x Compound	8	0.641
3-way interaction	24	0.126
Residuals	153	

FIGURES
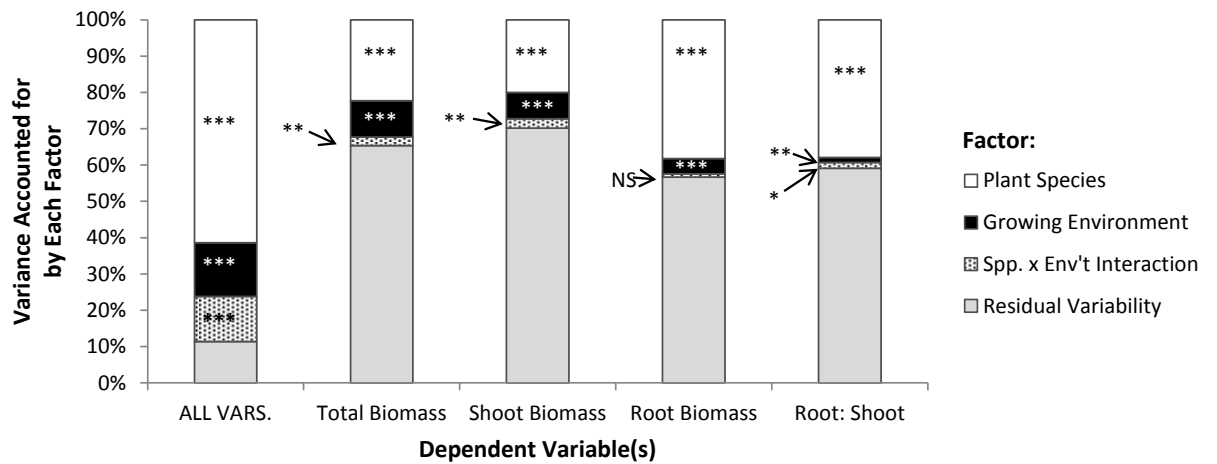

Figure 5-1. *Relative Factor Effect size and Significance on Biomass-related Response Variables as Indicated by MANOVA Wilks' λ or ANOVA sum of squares.* Planned experimental factors accounted for 88.6% of variance overall, and 30-43% of variance in each biomass-related dependent variable, with Plant Species having the largest effect on all biomass-related variables, accounting for 20-38% of explained variability. Rooting zone also had a significant effect on all four biomass-related variables, accounting for 1-10% of explained variability. The effect of Rooting zone varied by Plant Species for all response variables except root biomass (interaction effects). *** Indicates *p*<0.001, **indicates *p*<0.01, * indicates *p*<0.05, and "NS" indicates not significant (*p*>0.05).

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Growing Environment:

□ Inorganic N only   ▨ 50:50 Inorganic:Organic N   ■ Organic N only   ▩ ALL ENV'Ts
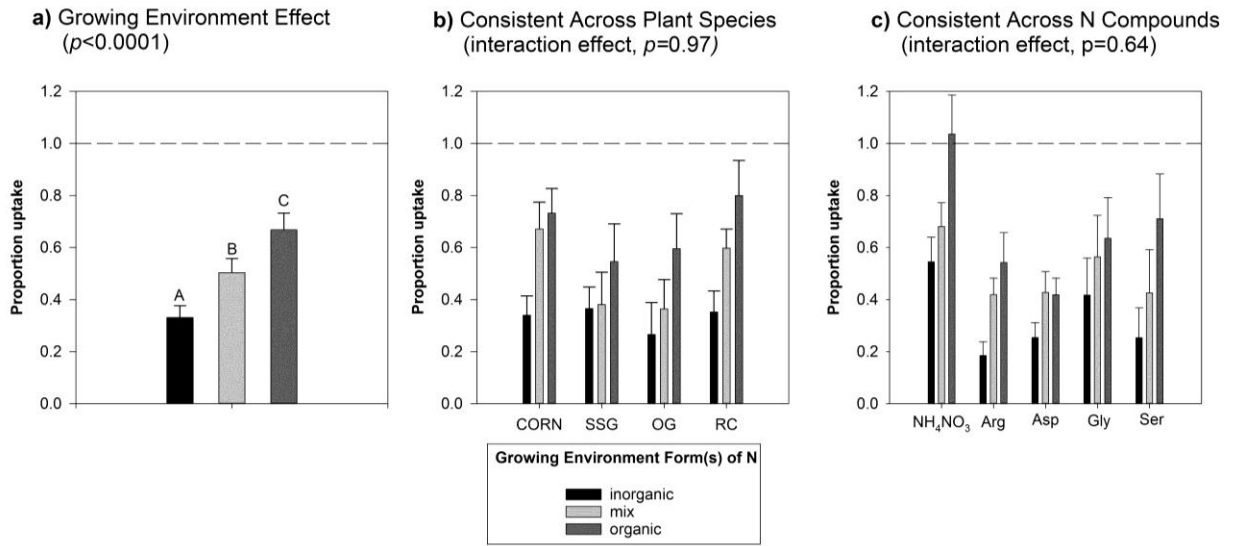
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Figure 5-2. *Effects of rooting zone chemistry and Plant Species on means of biomass-related variables.* Different capital letters indicate significant differences between means by rooting zones across all plant species (italic) or between means by plant species across all rooting zones (bold, gray bars). In biomass-related variables where ANOVA indicated a significant interaction effect, lowercase letters indicate when significant differences in biomass-related variables occurred between rooting zones, but within species. The brackets labeled 'nsd' indicated that there were no significant differences between rooting zones within that plant species. In most cases (a, b, and d), rooting zone effect size and significance varied by plant species, but overall direction was consistent with differences in total biomass, shoot biomass, and root: shoot between rooting zones observed across all species (light gray-shaded portions of graphs vs. white portions). Effects of rooting zone on root biomass (c) were consistent across all plant species (no plant species x rooting zone interaction effect, p=0.184). We used Tukey's HSD test to determine significance of differences. Asterisks (*) identify the grand mean of biomass-related variables. Analyses were performed on square root-transformed data for (a), (c), and (d) and on log-transformed data for (b) and (d).

a)

□ inorganic ▨ mix ■ organic

b)

□ inorganic ▨ mix ■ organic
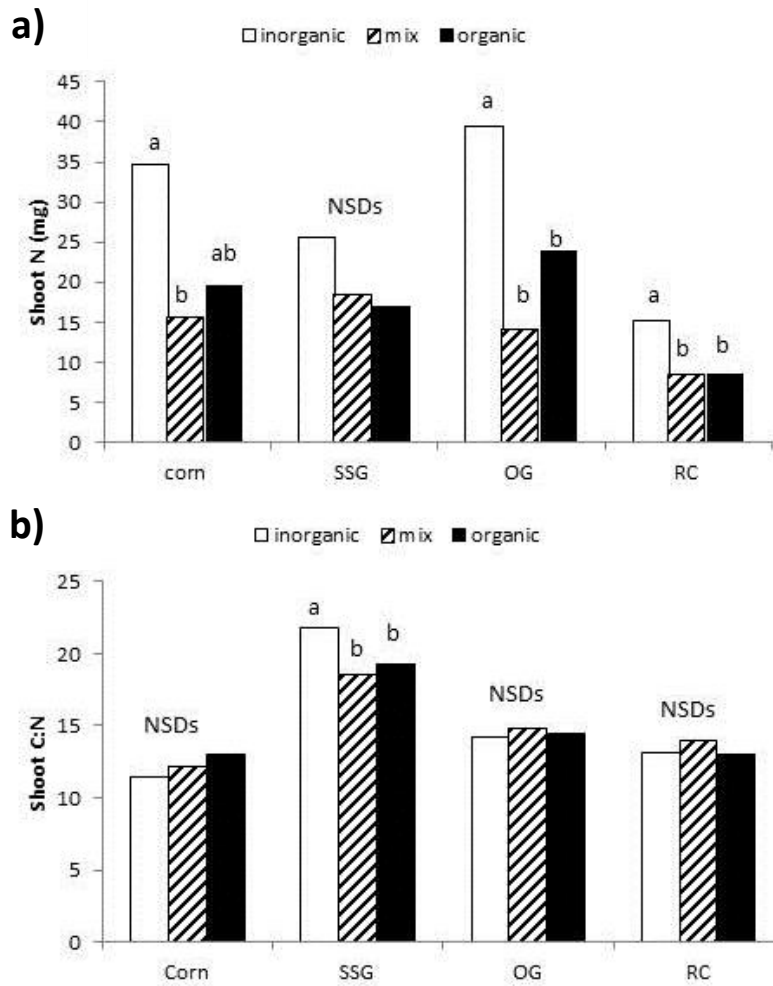
Figure 5-3. N uptake indicated by N content of shoots was higher in plants grown in inorganic N environments for corn, OG, and RC (b). However, shoot C:N ratios were consistent across rooting zones for all species except SSG, which exhibited greater C:N ratios when grown in the inorganic N environment than when grown in either a mixed N environment or in an organic N-only environment (c).
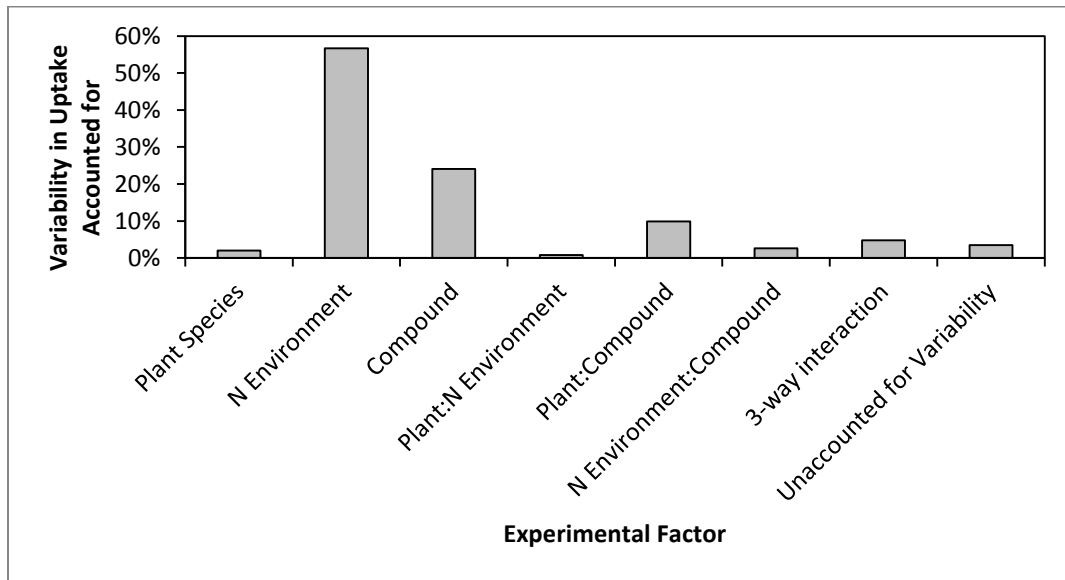
Figure 5-4. *Factor importance on N compound uptake indicated by ^{15}N enrichment of shoots (ANOVA mean square of each factor or factor interaction).*

a) Growing Environment Effect
(*p*<0.0001)

b) Consistent Across Plant Species
(interaction effect, *p*=0.97)

c) Consistent Across N Compounds
(interaction effect, p=0.64)

Growing Environment Form(s) of N
- inorganic
- mix
- organic

Figure 5-5. *Plants grown in organic N-containing rooting zones took up more ^{15}N-labeled compounds (a) regardless of plant species (b) or the type of N compound (c).*

a) Average N Compound Uptake Across All Plant Species
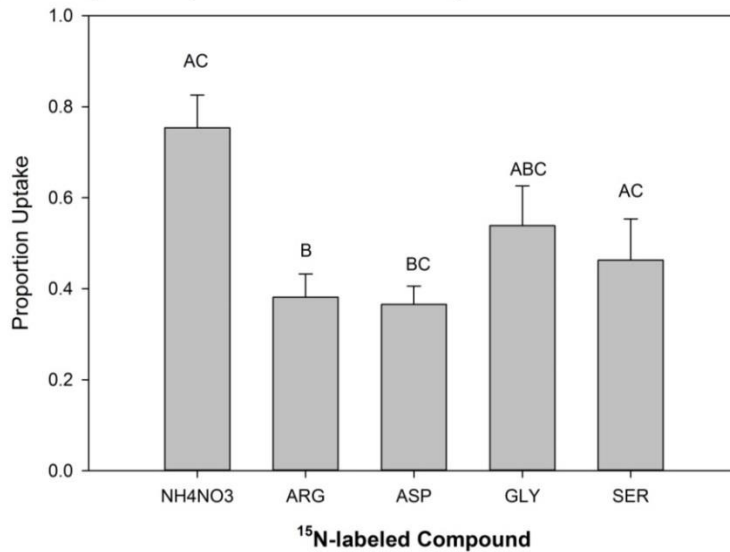
b) All Plant Species took up NH_4NO_3 at high rates, but plant species differed in which amino acids they preferred (interaction effect Plant x N Compound, $p<0.01$)
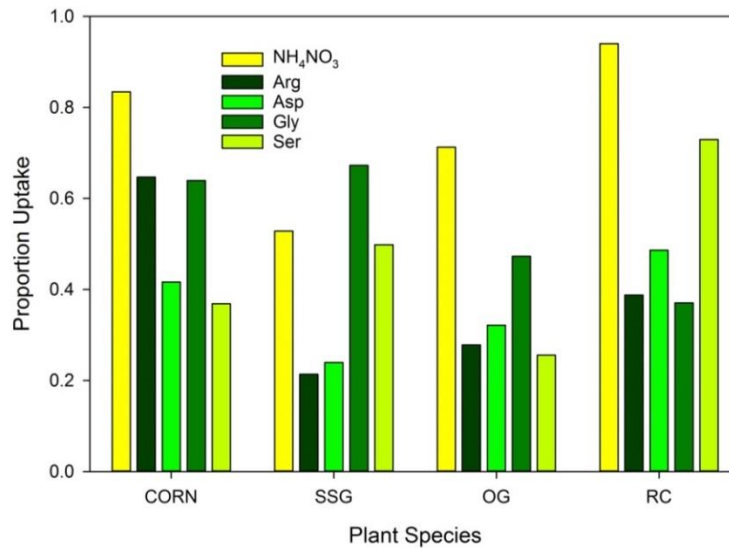
Figure 5-6. Plants did exhibit N compound preference, but preferences varied by species. High NH_4NO_3 uptake, or preference for inorganic N was consistent across species, although all species except OG took up at least one AA at mean rates within 1 SEM of their NH_4NO_3 uptake rate. Within-species differences between compound uptake did not differ significantly, except in SSG, where significant differences were consistent with differences observed across all species (a; Tukey's HSD).
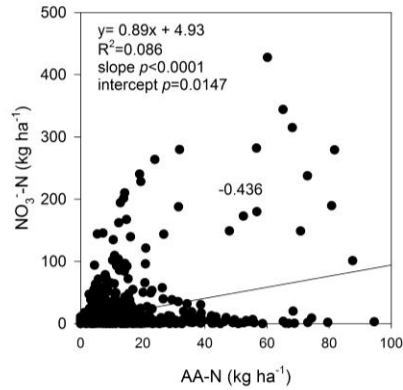
a) Inorganic N availability was more variable than AA-N availability

b) Daily median and mean ± 95% C.I. N-availability during the growing season (n=818)
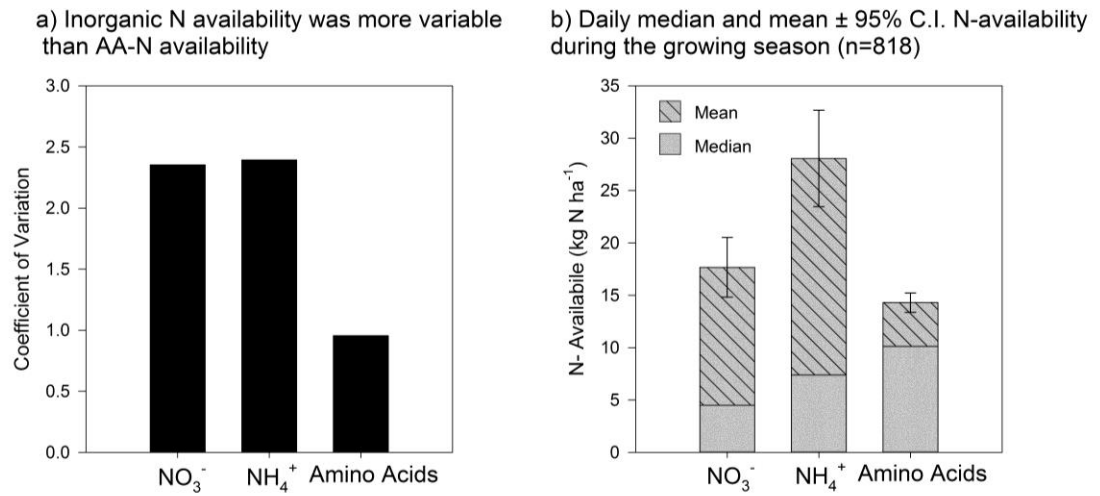
Figure 5-7. *Variation and availability of inorganic and organic N in forage cropping systems after 9-11 years of organic soil management (2012-2014).* AA-N was less variable than NO_3^--N or NH_4^+-N (a). Median daily growing season AA-N availability was greater than either daily median NH_4^+-N availability or daily median NO_3^--N availability (b). However, mean daily NO_3^--N availability was similar to mean daily AA-N availability, and mean daily NH_4^+-N availability exceeded AA-N availability (b; heteroscedastic t-tests, n=818 for each group).
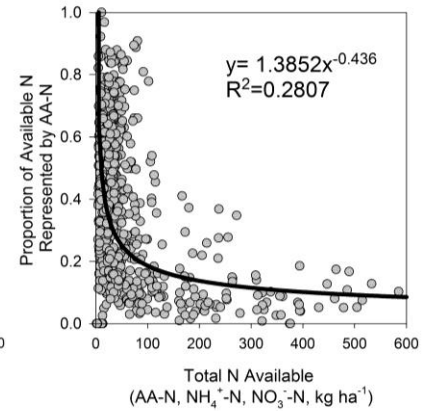
a) Ammonium-N was weakly positively correlated with AA-N

$y=1.52x + 6.36$
$R^2=0.095$
slope $p<0.0001$
intercept $p=0.0498$
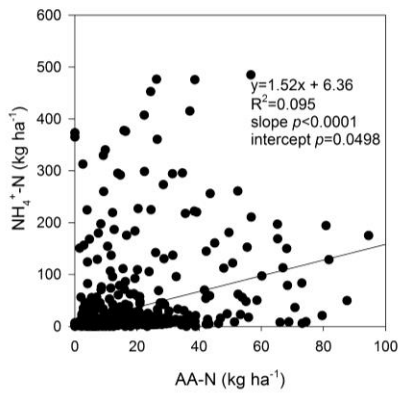
b) Nitrate-N was weakly positively correlated with AA-N

$y= 0.89x + 4.93$
$R^2=0.086$
slope $p<0.0001$
intercept $p=0.0147$

-0.436

c) AA-N made up a larger portion of available N, when total N availability was <30 kg N ha^{-1}

$y= 1.3852x^{-0.436}$
$R^2=0.2807$

Figure 5-8. *Relationships between AA-N Availability, Inorganic N Availability, and Total N Availability.* AA-N was significantly positively correlated with both NH_4^+- (a) and NO_3^--N availability (b), but the relative proportion of N represented by AA-N was greatest when total available N (AA, NH_4^+, and NO_3^-AAN) was low (c).
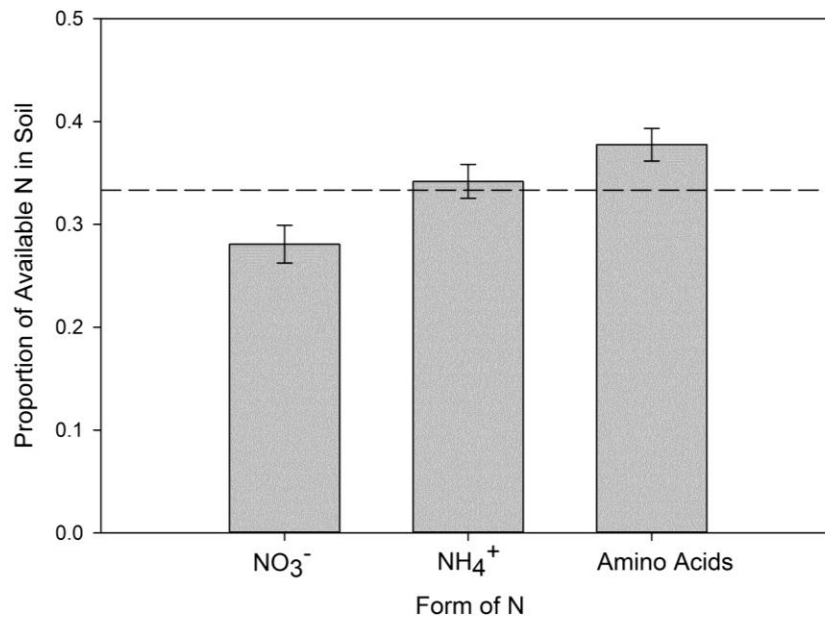
Figure 5-9. *AA-N dominated the soil available N profile in these forage systems under long-term certified organic management (means ± 95% confidence intervals, n=818 for each bar).* Data encompasses 8 forage systems over 3 years 2012-2014 in Central PA.
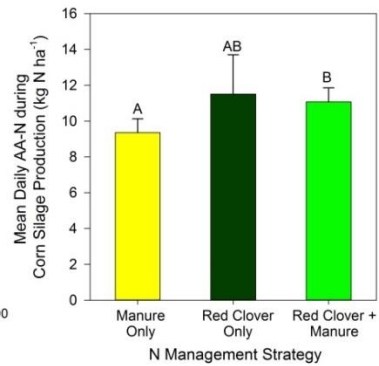
a) AA-N makes up 20-50% of available N for most of the growing season (n=818, 3 years, 8 cropping systems)
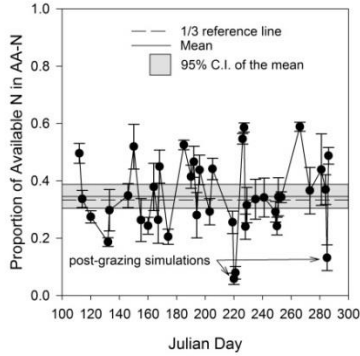
b) Available N profiles of perennial systems are especially dominated by AA-N in the late spring, early summer and fall (n=494)
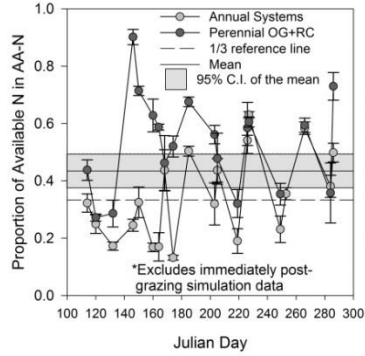
c) In annual systems, absolute mean AA-N availability was greater in systems using red clover-based managment

Figure 5-10. *Relative and absolute contributions of AA-N to available soil N in organic cropping systems.* a) Relative contributions of AA-N to soil N availability in organic cropping systems exhibited some seasonal variability, as well as effects of manure inputs associated with grazing simulation treatments (points represent daily means ± 2 SE). b) AA-N exhibited greater relative importance in the perennial system compared to the annual systems, which were more inorganic N dominated (daily means ± 2 SE). c) Within annual systems in the silage phase, absolute AA-N availability was greater in red clover-containing systems (red clover was terminated prior to silage establishment; Means + 95% CIs).

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VITA

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Education

Ph.D., Ecology and Biogeochemistry (dual-title), *expected 15 August 2015*
    The Pennsylvania State University, University Park, PA
B.A., *summa cum laude*, Biological Sciences (major), Environmental Studies (minor), 2008
    Mount Holyoke College, South Hadley, MA

Professional Experience

NSF Graduate Research Fellow & PhD Candidate in Ecology and Biogeochemistry
August 2011 – present, *Penn State University, University Park, PA*
    My research aims to improve nitrogen (N) retention in agriculture using both structural and
    chemical approaches. Motivated by the National Organic Program's Pasture Rule and the
    rapid growth in organic dairy production, I have focused on N retention benefits conferred
    by the structural conversion from confinement dairy to pasture-based organic dairy, as well
    as the role of increased organic N provisioning and direct plant uptake of organic N in
    reducing N lost as nitrous oxide, nitrate, and ammonia.

Beginning Farmer Extension Associate
November 2010 – July2011, *Penn State Extension, Nazareth, PA*
    Coordinated Penn State Extension's Start Farming Program for beginning farmers. Organized
    19 educational events in collaboration with 12 Extension Educators across PA, developed
    and wrote educational materials and curricula; taught classes; developed, conducted and
    summarized evaluations with 400+ beginning and aspiring farmers who participated in
    courses and educational events; managed websites/ facilitated media coverage.

Interim Director of Research/ Research Manager
 July 2009 – October 2010, *Rodale Institute, Kutztown, PA*
    Led development and implementation of a diverse array of organic agricultural research and
    education projects. Communicated findings to agricultural professionals, college students
    and interested community members through field days, tours, and frequent web articles.
    Supervised a staff of eight researchers and technicians. Managed 18 grant-funded projects,
    including data collection, data analysis, internal team coordination, and external
    communication of findings to collaborators and funding agencies. Wrote $1.1 million in
    successful grant and foundation proposals. Participated in Institute leadership team to
    collaboratively develop direction and policy.

Research and Policy Associate
February 2009 – June 2009, *Rodale Institute, Kutztown, PA*
    Presented scientific work to audiences ranging from local farmers and schoolchildren to
    policymakers in Washington, D.C. Prepared and delivered research briefs to state and
    national policymakers. Designed, implemented, and managed multiple organic no-till
    agricultural research projects, including field layout, data collection, presentation, and
    publication. Authored $3 M in federal, state and foundation funding applications.