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TITLE:

Microplot Design and Plant and Soil Sample Preparation for ^{15}Nitrogen Analysis

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KEYWORDS:

Fertilizer derived nitrogen, Soil derived nitrogen, ^{15}N isotope, microplot, fertilizer nitrogen use efficiency, ^{15}N enriched urea, isotope sample preparation, labeled N

SUMMARY:

A microplot design for ^{15}N tracer research is described to accommodate multiple in-season plant and soil sampling events. Soil and plant sample collection and processing procedures, including grinding and weighing protocols, for ^{15}N analysis are put forth.

ABSTRACT:

Most nitrogen fertilizer studies evaluate the overall effect of a treatment on end-of-season measurements such as grain yield or cumulative N losses. A stable isotope approach is necessary to follow and quantify the fate of fertilizer derived N (FDN) through the soil-crop system. The purpose of this paper is to describe a small research plot design utilizing non-confined ^{15}N enriched microplots for multiple soil and plant sampling events over two growing seasons and provide sample collection, handling, and processing protocols for total ^{15}N analysis. The methods were demonstrated using a replicated study from south-central Minnesota planted to corn (*Zea mays* L.). Each treatment consisted of six corn rows (76 cm row-spacing) 15.2 m long with a microplot (2.4 m by 3.8 m) embedded at one end. Fertilizer-grade urea was applied at 135 kg N·ha^{-1} at planting, while the microplot received urea enriched to 5 atom % ^{15}N. Soil and plant samples were taken several times throughout the growing season, taking care to minimize cross-contamination by using separate tools and physically separating unenriched and enriched samples during all procedures. Soil and plant samples were dried, ground to pass through a 2 mm screen, and then ground to a flour-like consistency using a roller jar mill. Tracer studies require additional planning, sample processing time and manual labor, and incur higher costs for ^{15}N enriched materials and sample analysis than traditional N studies. However, using the mass balance approach, tracer studies with multiple in-season sampling events allow the researcher to estimate FDN distribution through the soil-crop system and estimate unaccounted-for FDN from the system.

INTRODUCTION:

Fertilizer nitrogen (N) use is essential in agriculture to meet the food, fiber, feed, and fuel demands of a growing global population but N losses from agricultural fields can negatively impact environmental quality. Because N undergoes many transformations in the soil-crop system, a better understanding of N cycling, crop utilization, and the overall fate of fertilizer N are necessary to improve management practices that promote N use efficiency and minimize environmental losses. Traditional N fertilizer studies primarily focus on the effect of a treatment on end-of-season measurements such as crop yield, crop N uptake relative to the N rate applied (apparent fertilizer use efficiency), and residual soil N. While these studies quantify the overall system N inputs, outputs, and efficiencies, they cannot identify nor quantify N in the soil-crop system derived from fertilizer sources or the soil. A different approach using stable isotopes must be used to track and quantify the fate of fertilizer derived N (FDN) in the soil-crop system.

Nitrogen has two stable isotopes, ^{14}N and ^{15}N, that occur in nature at a relatively constant ratio of 272:1 for $^{14}N/^{15}N$[1] (concentration of 0.366 atom % ^{15}N or 3600 ppm ^{15}N[2, 3]). The addition of ^{15}N enriched fertilizer increases the total ^{15}N content of the soil system. As ^{15}N enriched fertilizer mixes with unenriched soil N, the measured change of $^{14}N/^{15}N$ ratio allows researchers to trace FDN in the soil profile and into the crop[3,4]. A mass balance can be calculated by measuring the total amount of ^{15}N tracer in the system and each of its parts[2]. Because ^{15}N enriched fertilizers are significantly more expensive than conventional fertilizers, ^{15}N enriched microplots are often embedded within the treatment plots. The purpose of this methods paper is to describe a small research plot design utilizing microplots for multiple in-season soil and plant sampling events for corn (*Zea mays* L.) and to present protocols for preparing plant and soil samples for total ^{15}N analysis. These results can then be used to estimate N fertilizer use efficiency and create a partial N budget accounting for FDN in the bulk soil and the crop.

PROTOCOL:

1. Field site description

NOTE: When performing ^{15}N tracer field trials, selected sites should minimize variation due to soil, topography, and physical features[5]. Cross-contamination may occur following lateral soil movement due to slope, wind or water translocation, or tillage while the vertical distribution of soil N may be impacted by subsurface water flow and tile-drainage[6].

1.1 Describe the experimental field site including past management (e.g., previous crops and tillage), latitude and longitude, soil physical and chemical properties (e.g., soil textural analysis, initial fertility conditions, pH, and soil bulk density).

1.2 Record GPS coordinates for the research site and the field corners.

1.3 Describe growing season management including pest and disease management (herbicide, insecticide, or fungicide use), soil fertility management (including rate, source, placement, and

application timing), tillage, irrigation and amounts, and residue management.

1.4 As crop growth and microbe mediated N transformations are affected by soil moisture, soil temperature, and air temperature, record climate information including daily high and low temperatures, daily precipitation, and soil moisture and temperatures at several depths that reflect the soil sampling depths.

2. Plot design

2.1 Plant six corn rows (~86,000 plants ha^{-1}) on 76 cm spacing with a final plot dimension of 15.2 m by 4.6 m.

2.1.1 Establish border areas 1.5 m from each end of the lengthwise dimension (0-1.5 m, 13.7-15.2 m) and an additional border area 1.5 m long (9.8-11.3 m) adjoining the sampling and harvest areas (**Figure 1**).

2.1.2 Designate rows 2 and 3 as the in-season plant and soil sampling area (1.5-9.8 m) and rows 4 and 5 as the harvest area (1.5-9.8 m) for corn grain yield.

2.1.3 Establish a microplot area (11.3-13.7 m) with dimensions of 2.4 m by 3.8 m centered on the width dimension. Collect all ^{15}N enriched plant and soil samples from this area, leaving 0.38 m of unsampled border on the length and width dimensions to minimize edge effects (**Figure 2**).

2.2 Delineate the treatment plot and microplot corners with different colored flags.

3. Soil and plant sample precautions

3.1 Use dedicated equipment and processing areas for unenriched and enriched materials. Contamination of unenriched materials (fertilizer, soil, or plant) by enriched materials and vice versa can drastically affect results.

3.2 Collect and process ^{15}N enriched soil and plant samples in order of lowest to highest ^{15}N expected enrichment to minimize cross-contamination. Ensure that work surfaces, gloves, utensils, and machinery are thoroughly cleaned between each sample to minimize cross-contamination from sample carryover.

3.3 Minimize foot traffic in microplots to prevent contamination of unenriched sampling areas. Wear protective shoe coverings when accessing microplots and remove them when exiting the microplot area.

4. ^{15}N enriched fertilizer preparation and application

4.1 Following guidelines put forth by Ref. 2 for fertilizer ^{15}N use efficiency ($F^{15}NUE$) studies, dilute 10 atom % ^{15}N enriched urea to 5 atom % ^{15}N enriched urea and dissolve in 2 L of deionized water

to ensure uniform enrichment of urea fertilizer.

NOTE: The required concentration of ^{15}N enriched fertilizer is dependent on the goals of the agronomic study. If the concentration of stock ^{15}N enriched fertilizer exceeds the researcher's requirements, the stock fertilizer concentration may be diluted with similar conventional fertilizer using the following formula[3].

$$X2 = [(C1/C2) - 1] * X1 \quad (1)$$

X2 is the mass of the conventional unenriched fertilizer, *X1* is the mass of the tracer fertilizer, *C1* is the isotopic concentration [expressed as atom % excess (measured atom % enrichment minus the natural background concentration assumed to be 0.3663 atom %)] of the original tracer fertilizer, and *C2* is the isotopic concentration of the final mixture. As an example, given 100 g of 10 atom % enriched urea, 92.7 g of conventional unenriched fertilizer would be required for a final isotopic concentration of 5 atom %; $X2 = \{[(10 - 0.3663)/5] - 1\} * 100$.

4.2 Analyze the solution for ^{15}N concentration to verify enrichment. The authors utilized the analytical services provided by UC Davis Stable Isotope Facility.

NOTE: Reactions of the soil-plant-microbe regime to fertilizer additions may be affected by the physical form of fertilizer. Depending on the goals of the study, the urea solution may be applied as a liquid or dehydrated to reform crystals. The crystals may be compacted into a cake using a Carver press at 10,000 psi, followed by crushing the cake and screening the particles to the desired size[3].

4.3 Evenly apply the ^{15}N enriched urea solutions to the microplots using a calibrated backpack CO_2 sprayer (**Figure 3A**). If multiple N rates or enrichment levels are used, consider using designated CO_2 sprayers for each enrichment level or use a single sprayer and apply solutions from the lowest to the highest enrichment to minimize treatment cross-contamination.

4.4 Incorporate urea-containing fertilizers with light tillage, hand rakes, or 0.64 cm of irrigation within 24 h of application to minimize volatilization loss potential.

4.5 No additional ^{15}N enriched urea fertilizer is applied to the microplot during the second growing season. Apply conventional unenriched urea to the entire treatment.

5. Field sample processing: aboveground corn biomass

5.1 At each sampling stage, collect a six-aboveground corn plant composite sample from within the sampling area (^{15}N unenriched) and a six-aboveground corn plant composite sample from the ^{15}N enriched microplot. At least two plants should separate each sampled plant to avoid significantly altering plant growth dynamics. The authors collected plant samples at the V8 and R1 corn physiological development stages[11] and at physiological maturity (**Figure 2**).

5.2 Following the principles described in steps 3.1 and 3.2, chop V8 and R1 aboveground biomass (≤5 cm by ≤5 cm); a yard waste chipper is a satisfactory option. Place chopped biomass in labeled fabric or paper bags and dry in a forced-air oven at 60 °C until constant mass. Record the biomass dry weight (**Figure 3B**).

5.3 Partition physiologically mature corn plants into stover (all vegetative tissues including leaves, husks, and stalks), grain, and cob fractions. Chop and dry in a forced-air oven at 60 °C until constant mass. Record the biomass dry weight.

5.4 Within the microplot, cut all corn stalks at the soil surface, tie into a bundle, label according to plot, and remove from the field (**Figure 3C**). Adjust microplot corner flags to be nearly flush with the soil surface to minimize the risk of removal by the combine during harvest or tillage post-harvest.

5.5 Harvest grain from the harvest area and report yield at 15.5% moisture content[12]. Harvest remaining research areas with a plot combine.

5.6 Rake unenriched biomass from off the microplot area. Chop and reapply microplot aboveground biomass to the correct plot (**Figure 3D**).

5.7 Incorporate residue into the soil surface with tillage taking care to minimize soil and corn residue transport into or out of the microplot area. Replace any microplot corner flags removed due to tillage.

5.8 Plant second-year corn on the same rows as the first-year corn.

5.9 Collect second-year aboveground corn biomass only at physiological maturity and process like first-year corn samples as described in step 5.3. Collect microplot samples from the center of the microplot area (1.52 m by 0.76 m) to avoid any potential signal dilution following tillage (**Figure 2**). Harvest grain from the harvest area and report yield at 15.5% moisture content.

5.10 Following the principles of steps 3.1 and 3.2, thoroughly mix and grind 100 to 200 g of dried plant material to pass through a 2 mm sieve. Thoroughly mix the ground material and store a subsample in a labeled coin envelope for further processing.

NOTE: A Thomas Wiley mill is a satisfactory option for plant tissue grinding while a Perten Laboratory Mill 3610 is a satisfactory option for grinding grain.

CAUTION: People grinding plant samples should wear ear protection and be protected from inhaling dust by wearing a National Institute for Occupational Safety and Health approved N95 Particulate Filtering Facepiece Respirator.

6. Field sample processing: soil

6.1 Collect first-year soil samples 8 days after ^{15}N enriched fertilizer application, V8, R1, and post-harvest before tillage. Collect second-year soil samples at pre-plant and post-harvest. Due to logistical sampling constraints, the authors collected in-season soil samples at 0- to 15-, 15- to 30-, and 30- to 60-cm depths, post-harvest soil samples at 0- to 15-, 15- to 30-, 30- to 60-, and 60- to 90-cm depths, and second-year pre-plant soil samples at 0- to 30-, 30- to 60-, 60- to 120-cm depths.

NOTE: If a soil probe is unable to collect a soil core to the deepest desired depth as a single core, collect deeper depth cores from the same boreholes as the upper depths discarding the top 1-cm of soil to avoid contamination from soil falling from upper depths.

6.1.1 Collect a four-core (1.8-cm diameter) composite soil sample from the unenriched sampling area at V8 and R1 using a hand probe. Collect one core in the corn row and three cores between the corn rows.

6.1.2 Collect a two-core (5-cm diameter) composite soil sample from the unenriched sampling area at pre-plant and post-harvest using a hydraulic probe.

6.1.3 Collect a 15-core (1.8-cm diameter) composite soil sample from the microplot area 8 days after ^{15}N enriched fertilizer application, V8, and R1 using a hand probe. Collect three to four cores in the corn row and 11 to 12 cores between the corn rows.

NOTE: Soils are extremely heterogeneous. The greater number of cores collected from within the enriched microplot provides a better estimate of the true ^{15}N enrichment of soil N[13].

6.1.4 Collect a three-core (5-cm diameter) composite soil sample from the microplot area at pre-plant and post-harvest using a hydraulic probe.

6.1.5 Homogenize each composite soil sample in a bucket and place it in a pre-labeled paper bag.

6.2.1 Dry soil samples at 35 °C in a forced-air oven until constant mass. Grind each sample to pass through a 2 mm sieve. A mechanical soil grinder is satisfactory if it can be thoroughly cleaned between each sample.

NOTE: Soil samples may be air-dried by spreading samples on trays in a thin layer. Trays should be in an area free from contamination by outside N sources. Unenriched and enriched samples should be physically separated to prevent cross-contamination.

Caution: People grinding soil samples should wear ear protection and be protected from inhaling dust by wearing a National Institute for Occupational Safety and Health approved N95 Particulate Filtering Facepiece Respirator.

7. Lab sample processing: grind soil and plant samples

7.1 Dry ground plant samples (2 mm) overnight in an oven at 60 °C.

7.2 Following the principles described in step 3, grind dried plant samples or soil material to a fine, flour-like consistency. A roller jar mill is a satisfactory option.

NOTE: The authors' jar mill is a custom-built conveyor belt system that can process 54 roller jars at a time.

7.2.1 Fill each roller jar (250 mL borosilicate glass jar with a screw-top lid) with 10 to 20 g of ground plant or soil sample and seven stainless steel rods (8.5 cm long, 0.7 cm diameter).

7.2.2 Roll roller jars at 0.4 x g for 6-24 h or until samples have a fine, flour-like consistency.

7.2.3 Transfer the finely ground material into a clean, labeled 20 mL scintillation vial.

7.2.4 Between each sample, wash roller jars, stainless steel rods, and lids with soap and water to remove any residue.

7.2.4.1 Immerse roller jars and lids in a 5% HCl acid bath (prepared from 36-38% concentrated stock) overnight[14].

CAUTION: Hydrochloric acid is corrosive. It can cause severe skin burns, eye damage, and is harmful if inhaled. Always wear protective clothing, gloves, and eye and face protection. Flush contacted tissue thoroughly with water. Always use a secondary container when transporting acids. Always add acid to water as this reaction is exothermic. Immediately neutralize acid spills with baking soda.

NOTE: A large acid bath may be prepared as 100 L of 5% HCl in a 208 L plastic container. Prepare several smaller volumes in a fume hood and then transfer the solutions to the plastic container. Replace the solution quarterly.

7.2.4.2 Triple rinse roller jars and lids with deionized water and air dry.

7.2.4.3 Immerse stainless steel rods in a 0.05 *M* NaOH bath (prepared by dissolving 2 g of NaOH in 1 L of deionized water) overnight[14]. Prepare a new 0.05 *M* NaOH bath each day.

CAUTION: Sodium hydroxide can cause severe skin burns and eye damage. Always wear protective clothing and eye protection. Immediately remove contaminated clothing and rinse skin or eyes with water for several minutes.

7.2.4.4 Rinse the rods under running hot tap water for 5 minutes. Decant and triple rinse the rods with deionized water. Allow the rods to air dry on a paper towel-lined tray.

8. Weigh ground plant and soil samples for total N and ^{15}N analysis

8.1 Analyze a few representative plant and soil samples for total N content (e.g., combustion analysis[15]). Calculate the sample mass that provides adequate N content for ^{15}N analysis according to the analyzer specifications.

NOTE: The authors utilized the analytical services provided by UC Davis Stable Isotope Facility. Enriched sample weights were optimized for 20 µg of N with a maximum of 100 µg of N.

8.2 Organize like-samples from lowest to highest expected ^{15}N enrichment. Duplicate every eighth to twelfth sample in each run to check sample precision. Include at least one check sample per run[16].

8.3 Label a clean 96-well plate and fitted lid with individual well evaporation rings. Cut a clean index card to fit just inside the lid to prevent sample movement between wells during transport.

8.4 Wearing nitrile gloves, clean the microscale, work surfaces, spatula, and forceps with laboratory wipes and ethanol. Place cleaned utensils on a Kimwipe on the lab bench.

NOTE: Unenriched and enriched samples should be processed using separate scales and utensils to prevent cross-contamination.

8.5 Use forceps to place a pre-formed 5 mm x 9 mm tin capsule on a clean work surface, such as a stainless steel block with 5 mm x 8 mm well. Gently tap the capsule into the well to reform the cylindrical shape and flatten the bottom of the capsule if needed.

NOTE: Because sample masses will be very small, the risk of sample contamination is high. Never touch the capsules with gloves. Discard the capsule if it touches any surface other than the forceps, clean work surface, scale weigh pan, or 96-well plate.

8.6 Use forceps to gently flare out the top 1 mm of the capsule to facilitate manipulation. To avoid scale damage when taring the weight of the capsule, hover and release the capsule 1 to 2 mm above the microscale weigh pan. Tare the capsule. Use forceps to return the capsule to the clean work surface.

8.7 Use a spatula to carefully add the required mass of finely ground sample material to the capsule. Avoid spilling sample material on the outside surface of the capsule or the work surfaces.

8.8 Using forceps, slowly crimp the top third of the capsule and fold over to seal. Using forceps, continue to fold and compress the capsule into a spherical shape taking care not to puncture or tear the tin.

NOTE: Samples with low N content may require sample volumes that exceed the capacity of the 5x9 mm capsule. Larger capsules (e.g., 9 mm x 10 mm) may be used in these instances.

8.9 Use forceps to drop the wrapped capsule several times from a height of 1 cm onto a clean, dark surface or mirror to check for leaks. If no dust appears, weigh the sample using the same technique as described in step 8.6. Record the sample weight. Place the capsule in a 96-well plate and record the well placement.

8.9.1 If dust appears on the dark surface, record the sample weight. Wrap the sample in a second tin capsule, recheck for leaks, and place it in a clean 96-well plate.

NOTE: If the wrapped capsule is too large to fit in a 96-well plate, use a 24- or 48-well plate.

8.10 Between samples, clean each of the utensils and surfaces with ethanol and laboratory wipes paying especial attention to the spatula and forceps edges.

8.11 Secure the lid to the 96-well plate using tape and store in a desiccator.

9. **Calculations**

9.1 Calculate the mass of N (kg·ha^{-1}) contained in the plant or soil samples using the following equations.

$$Total\ N_{Plant} = \frac{mass\ of\ N\ in\ sample\ (ug)}{mass\ of\ sample\ (mg)} \times \frac{dry\ matter\ mass\ (g)}{\#\ of\ plants} \times plant\ population\ (\#\ ha^{-1}) \div 1{,}000{,}000 \quad (2)$$

$$Total\ N_{Soil} = \frac{mass\ of\ N\ in\ sample\ (ug)}{mass\ of\ sample\ (mg)} \times soil\ sampling\ depth\ (m) \times bulk\ density\ (Mg\ m^{-3}) \times 10 \quad (3)$$

9.2 Calculate the fertilizer N fraction (N_f), fertilizer derived N (*FDN*), and soil derived N (*SDN*) for plant and soil samples[17].

$$N_f = \frac{(A_{microplot} - A_{unenriched\ sampling\ area})}{(A_{fertilizer} - A_{unenriched\ sampling\ area})} \quad (4)$$

Where *A* is the atom % ^{15}N enrichment.

$$FDN_{Plant\ or\ Soil}(kg\ ha^{-1}) = N_f \times Total\ N_{Plant\ or\ Soil}\ (kg\ ha^{-1}) \quad (5)$$

$$SDN_{Plant\ or\ Soil}(kg\ ha^{-1}) = Total\ N_{Plant\ or\ Soil}\ (kg\ ha^{-1}) - FDN_{Plant\ or\ Soil} \quad (6)$$

9.3 Calculate fertilizer ^{15}N use efficiency[17].

$$F^{15}NUE\ (\%) = \frac{FDN_{Plant}\ (kg\ ha^{-1})}{Applied\ fertilizer\ N\ rate\ (kg\ ha^{-1})} \times 100 \quad (7)$$

REPRESENTATIVE RESULTS:

The results presented in this paper come from a field site established in 2015 at the University of Minnesota Southern Outreach and Research Center located near Waseca, MN. The site was managed as a corn-soybean [*Glycine max* (L.) Merr] rotation prior to 2015 but was managed as a corn-corn rotation during the 2015 and 2016 growing seasons. The soil was a Nicollet clay loam (fine-loamy, mixed, superactive, mesic Aquic Hapludolls)–Webster clay loam (fine-loamy, mixed, superactive, mesic Typic Endoaquolls) complex. Soil fertility was managed according to university guidelines except for N[18]. Several N fertilizer treatments were arranged in a randomized complete block design with four replications but only the 135 kg N·ha^{-1} rate applied as urea at planting is presented in this paper. Soil bulk density was measured at the center of 0- to 15-, 15- to 30-, 30- to 60-, 60- to 90-, and 60- to 120-cm depth layers from two 5-cm deep samples per replication using the intact core method[19]. Bulk density was averaged within depth across replications and assumed to be constant across the field. Plot setup and plant and soil samples were collected and processed as described in the protocol section.

Total (FDN + SDN) aboveground biomass N increased with each successive sampling event over the first growing season (**Figure 4**). Fertilizer derived N concentration was greatest earlier in the growing season accounting for 44 ± 4% (mean ± standard error) of the total aboveground biomass N at V8 and decreased with each successive sampling period (**Figure 4A**). However, SDN consistently was the greatest fraction of aboveground biomass N illustrating the importance of soil N supply for optimal corn growth. At physiological maturity in the first year, 27 ± 1% of aboveground biomass N was from FDN with similar proportions in grain, stover, and cob fractions (**Figure 4B**). At physiological maturity in the second year, only 2 ± 0.1% of first-year FDN was recovered in the aboveground biomass with 1.6 ± 0.2 kg of first-year FDN ha^{-1} exported in the grain (**Figure 4A**).

The soil-crop FDN budget is useful for quantifying FDN cycling within the system over time. Within 8 d of fertilizer application, the majority of FDN was in the top 15 cm of the soil profile, as expected (**Figure 5**). However, 22.2 ± 4.4 kg N ha^{-1} had already moved into the deeper depths while 4 ± 10% of the FDN was unaccounted for. Unaccounted-for FDN is likely primarily driven by N loss mechanisms including leaching, denitrification, and volatilization that either move FDN below the soil sampling depths or remove the FDN from the system entirely. At V8 and R1, unaccounted-for FDN increased to 60.4 ± 4.7 kg N ha^{-1} on average while soil N (0–15 cm) was 31.6 ± 6.8 kg N ha^{-1} on average. Corn's rapid growth and high N demand from V8 to R1 resulted in an increase of 19.0 ± 4.4 kg FDN ha^{-1} in aboveground plant biomass mirroring the 17.7 ± 5.2 kg FDN ha^{-1} reduction from the 15- to 60-cm soil depths. Soil temperature and moisture conditions between these corn development stages tend to favor microbial growth resulting in rapid turnover of organic residues and re-utilization of mineralized N. These results suggest that corn roots mined inorganic FDN from the 15- to 60-cm depths while FDN in the 0- to 15-cm depth was primarily cycled between soil organic matter and microbial fractions. Additional isotopic analysis of soil inorganic and organic N pools is necessary to validate this hypothesis and provide greater detail and insight into FDN cycling dynamics[10]. By post-harvest year 1, 59 ± 2% of the original FDN was unaccounted for while 18.1 ± 3.9 kg FDN ha^{-1} was in the top 30 cm of the soil (**Figure 5**) and 22.1 ± 2.3 kg FDN ha^{-1} was exported in the grain (**Figure 4B**). Fertilizer ^{15}N use efficiency was 24% (**Equation 7**) and is at the low end of commonly reported F^{15}NUE measures (25-45%) reported by

other studies[20]. Although equipment was thoroughly cleaned between each sample, the lower $F^{15}NUE$ measures of the study could be an artifact of enriched sample dilution by processing enriched samples in order of lowest to highest expected enrichment. The amount of FDN in the top 30 cm doubled (36.0 ± 5.2 kg FDN ha^{-1}) from post-harvest year 1 to pre-plant year 2 due to partial residue breakdown since the previous fall but by post-harvest year 2 only 17.3 ± 3.3 kg FDN ha^{-1} was still found within the soil-corn system (**Figure 5**). This study indicates that by the end of the first and second years, only 41 and 29%, respectively of first-year FDN was accounted for within the soil-corn system (including FDN exported in the grain) while the remainder was either lost to the environment or leached below the 90 cm soil sampling depth.

Spurious results may be obtained when samples are cross-contaminated affecting calculations of N_f, FDN, and SDN. For example, suppose a ^{15}N enriched plant sample with an actual enrichment of 3.000 atom % ^{15}N is contaminated with unenriched material diluting the ^{15}N concentration to 2.500 atom % ^{15}N. Further, assume Total N_{Plant} is 100 kg N ha^{-1}, the atom % ^{15}N enrichment of the fertilizer was 5.000, and the atom % ^{15}N enrichment of the unenriched plant sample was 0.366. The ^{15}N enriched plant sample N_f would be reduced from 0.568 (actual) to 0.461 (contaminated sample) underestimating the true FDN by 10.7 kg N ha^{-1}. Overestimations of FDN may occur when samples with low ^{15}N enrichment are contaminated with additional ^{15}N. Thus, extreme care should be taken in all steps of sample collection and processing to minimize sample contamination, but most especially when sample masses are reduced (e.g., grinding and weighing procedures).

FIGURE AND TABLE LEGENDS:

Figure 1: Plot design for the treatment plot and microplot. The figure illustrates the dimensions and relative placements of the border areas, unenriched sampling area, harvest area, and microplot area within the treatment plot.

Figure 2: Microplot plant and soil sampling diagram. The figure illustrates the relative plant and soil sampling positions at each sampling stage that avoids altering corn N uptake patterns of later sampled corn plants. Sampling events occurred 8 days following the ^{15}N enriched fertilizer application, at the **V8** and **R1** corn physiological development stages, at physiological maturity in the year of ^{15}N enriched fertilizer application (**PMY1**) and the following year (**PMY2**), and prior to planting the second year (**PPY2**).

Figure 3. **Chronological depiction of microplot management**. (**A**) Dissolve ^{15}N enriched urea into 2 L of deionized water and spray onto the microplot at planting. (**B**) Collect and chop a six-aboveground corn plant composite sample from within the sampling area (^{15}N unenriched) and a six-aboveground corn plant composite sample from the ^{15}N enriched microplot at the pre-determined sampling times. (**C**) Following sample collection at physiological maturity, remove all remaining aboveground biomass from within the microplot. (**D**) Post-harvest, rake unenriched aboveground corn biomass from the microplot area. Chip and reapply the microplot corn aboveground biomass to the microplot area.

Figure 4: Example of aboveground biomass N partitioned into fertilizer derived N (FDN) and soil derived N (SDN) fractions. Total aboveground biomass N was separated into its individual sources of FDN (solid color) and SDN (hashed color) in (**A**) and (**B**). Error bars represent the standard error of the mean. (**A**) Aboveground biomass N was measured at the V8 and R1 corn physiological development stages and at physiological maturity in the year of ^{15}N fertilizer application (**PMY1**) and the year following ^{15}N fertilizer application (**PMY2**). The value above each column represents the percentage of the total N that was FDN. (**B**) Aboveground biomass N measured at PMY1 and PMY2 is shown in its individual parts of cob (only Year 1), stover (stalk and leaves; includes cob for PMY2), and grain for FDN and SDN.

Figure 5: Example of the soil-corn fertilizer derived N (FDN) budget. The mass of FDN recovered in aboveground (**Abvgd**) corn biomass and at various soil sampling depths is reported for six sampling events over two growing seasons. Sampling events occurred 8 days following the ^{15}N enriched fertilizer application (**PA**), at the **V8** and **R1** corn physiological development stages, at physiological maturity in the year of ^{15}N enriched fertilizer application (**PMY1**) and the following year (**PMY2**), and prior to planting the second year (**PPY2**). The difference between the applied fertilizer rate (135 kg N ha^{-1}) and the mass of FDN recovered in the soil-corn portions is the unaccounted for FDN fraction. The total mass of FDN for PPY2 and PMY2 was 113 kg FDN ha^{-1} because 22 kg FDN ha^{-1} was exported out of the soil-corn system as first-year grain. Error bars represent the standard error of the mean.

DISCUSSION:

Stable isotope research is a useful tool for tracking and quantifying FDN through the soil-crop system. However, there are three main assumptions associated with N tracer studies that if violated may invalidate conclusions drawn from using this methodology. They are 1) the tracer is uniformly distributed throughout the system, 2) processes under the study occur at the same rates, and 3) N leaving the ^{15}N enriched pool does not return[3]. Because this study is interested in the distribution of total FDN throughout the soil-crop system, assumptions 2 and 3 are of minimal concern[21].

The high cost of ^{15}N enriched material generally limits the size of ^{15}N tracer studies. Therefore, prior to initiating a N tracer study, the researcher should carefully plan the research project's objectives considering: the number of sampling events, the length of the study (days to years), the N fertilizer application rate, and the ^{15}N enrichment concentration required to measure differences from natural abundance (0.366 atom %) following ^{15}N enriched fertilizer dilution by bulk soil[2]. Commonly used ^{15}N enrichment levels and application rates are reported for different types of agronomic research in Ref. 2. After determining the study objectives, the microplot must be sufficiently large to accommodate soil and plant sampling and avoid edge effects. The plot design described in this protocol uses a non-confined plot requiring that non-sampled border areas be employed[6]. The ^{15}N concentration in border areas is diluted by mass flow across the microplot boundary and N uptake from outside the microplot by lateral corn roots growing in rows 1 and 6. Confined plots, where physical barriers are driven into the soil, do not require border areas but do require additional work during microplot establishment and may limit routine field operations[6]. References 3, 6, 22–25 provide additional guidance on selecting

526 microplot sizes, border widths, and when confined or non-confined plots may be most
527 appropriate.
528
529 The plant and soil sampling scheme of this study is designed to allow for multiple sampling events
530 over two consecutive growing seasons. Early season plant and soil samples are taken near the
531 outside edges of the microplot. Each successive sampling event moves closer to the center of the
532 microplot to avoid sampling previously sampled areas. At least two corn plants separate each
533 sampled plant to minimize changes in corn physiological development. One challenge with this
534 study's soil sampling technique is that the soil core sampling method may not accurately
535 intercept the heterogeneous distribution of ^{15}N in the soil profile[3]. Spatial variability of soil total
536 N is high with an estimated coefficient of variation of 15%[3]. Complete microplot excavation would
537 improve ^{15}N quantification accuracy but requires processing significant volumes of soil and limits
538 sampling to a single event[3] that is not in line with the objectives of this study. Subdividing the
539 microplot into smaller sampling units allows for multiple excavation events but may increase the
540 required microplot size to ensure non-sampled units are unaffected by modifications to the crop
541 canopy and soil water dynamics. Despite the potential reduction in accuracy, many studies use
542 the soil core technique for microplots ≥1 m^2 [9,22,26–28]. Sample precision may be increased by
543 increasing the number of soil cores collected and composited per microplot using the following
544 formula[13]:
545
546 $$n = (Z^2)(CV^2)/(d^2) \quad (8)$$
547
548 where *n* is the number of soil cores, *Z* is the standardized normal variate for the corresponding
549 alpha level (1.96 for 0.05 and 1.65 for 0.10), *CV* is the coefficient of variation, and *d* is the margin
550 of error in the plot mean (as a decimal). Based on this formula, the authors expect that 15 cores
551 per microplot would estimate total N to ±7.6% on 95% of the plots (*n* = 15; *Z* = 1.96; *CV* = 15%; *d*
552 = 0.076). Reference 25 used a similar number of cores but subdivided the microplot into 32
553 sampling units collecting plant and soil samples from four units at each sampling event.
554
555 Others have shown that the microplot data can be extrapolated to the entire plot[29]. However,
556 for this assumption to be valid, the treatment plot and microplot must be similarly managed. If
557 possible, fertilizer N should be applied in the same chemical and physical forms (e.g., urea
558 dissolved in water) as these properties impact fertilizer-soil dynamics including N loss
559 mechanisms, immobilization, and availability to soil microbes and plants[3].
560
561 The roller jar grinding method described in this protocol is capable of pulverizing large volumes
562 of plant and soil samples, ideal for ensuring a representative, homogenized sample. However,
563 the technique requires significant manual labor and time to load, unload, roll, and clean the roller
564 jars. Sample processing is limited by the available number of roller jars, the capacity of the
565 conveyor belt unit, and the size of the acid bath. Commercial grinding vials may be an alternative
566 to roller jars but may limit the volume of plant and soil samples processed. Lab-made, single-use
567 grinding vials may be constructed that potentially serve as both the grinding and sample storage
568 vessel. The main consideration of any of these grinding methods is to minimize cross-
569 contamination between samples.

Finally, because ^{15}N enriched fertilizer material is expensive, ^{15}N enriched aboveground biomass and soil samples may be retained and homogenized for use in future studies. These products may be especially useful when investigating residue decomposition, mineralization potential, or other nutrient cycling processes[21].

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DISCLOSURES:
The authors have nothing to disclose.

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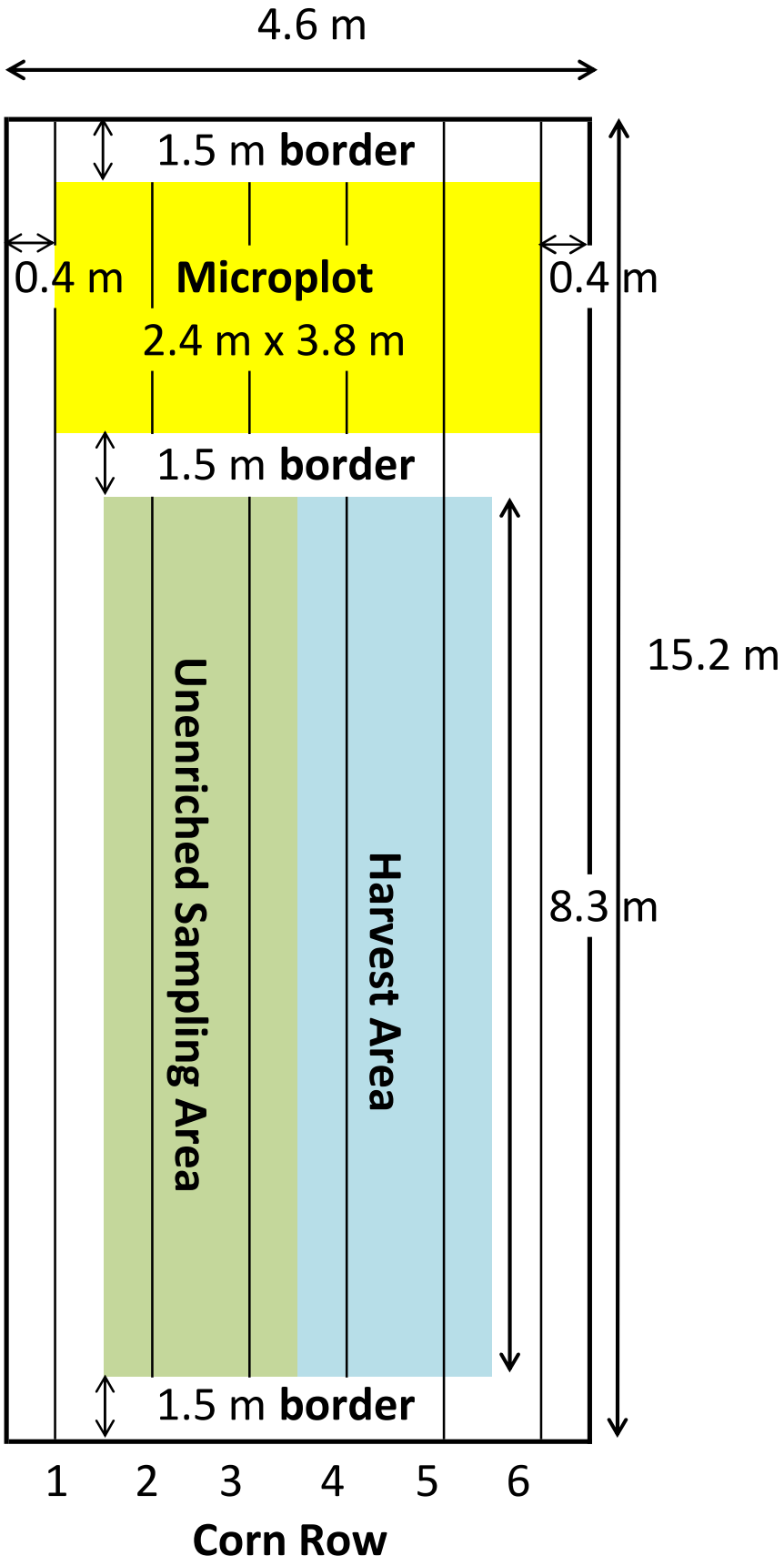

Figure 2 pdf

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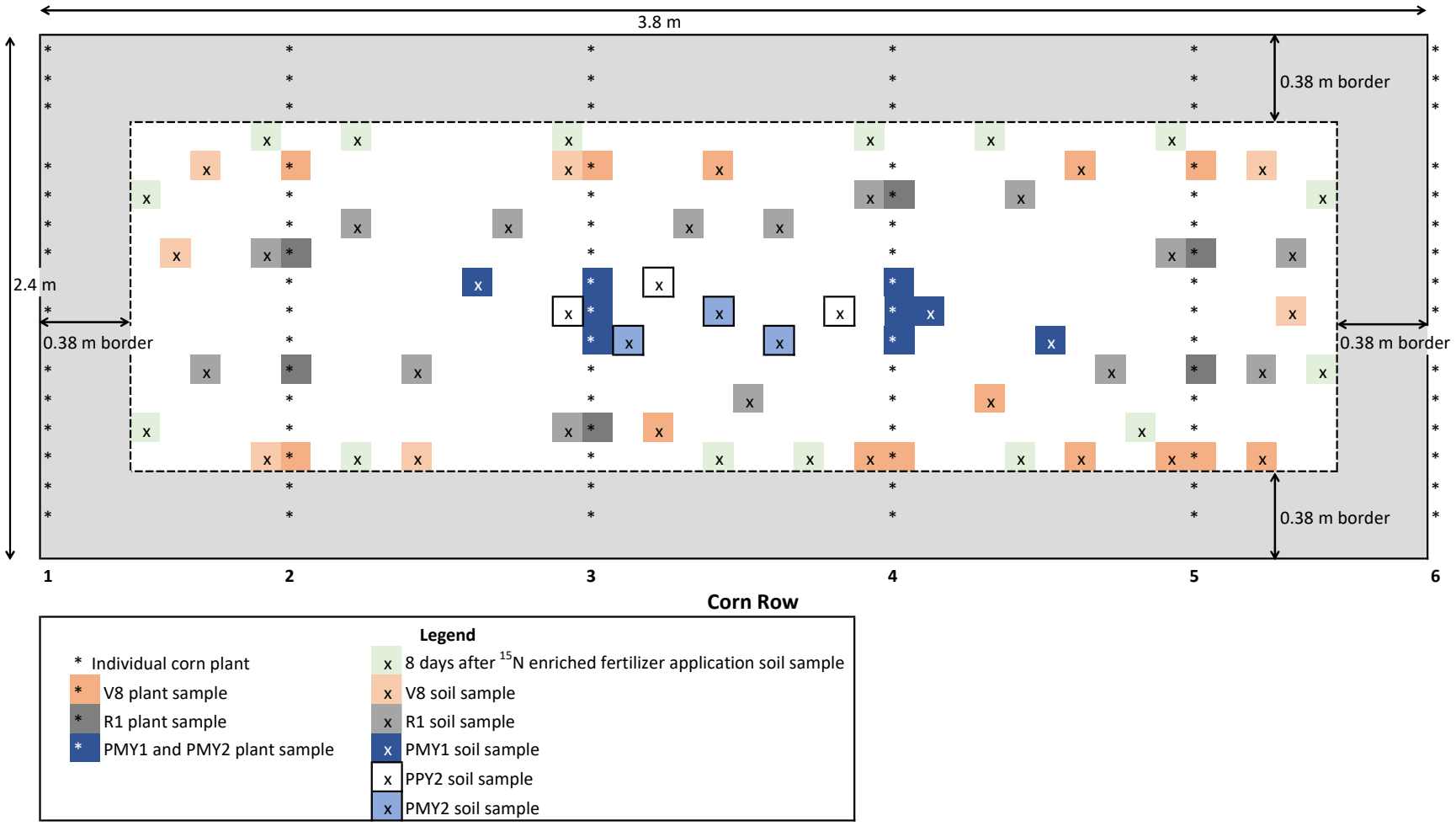

Figure 3 pdf

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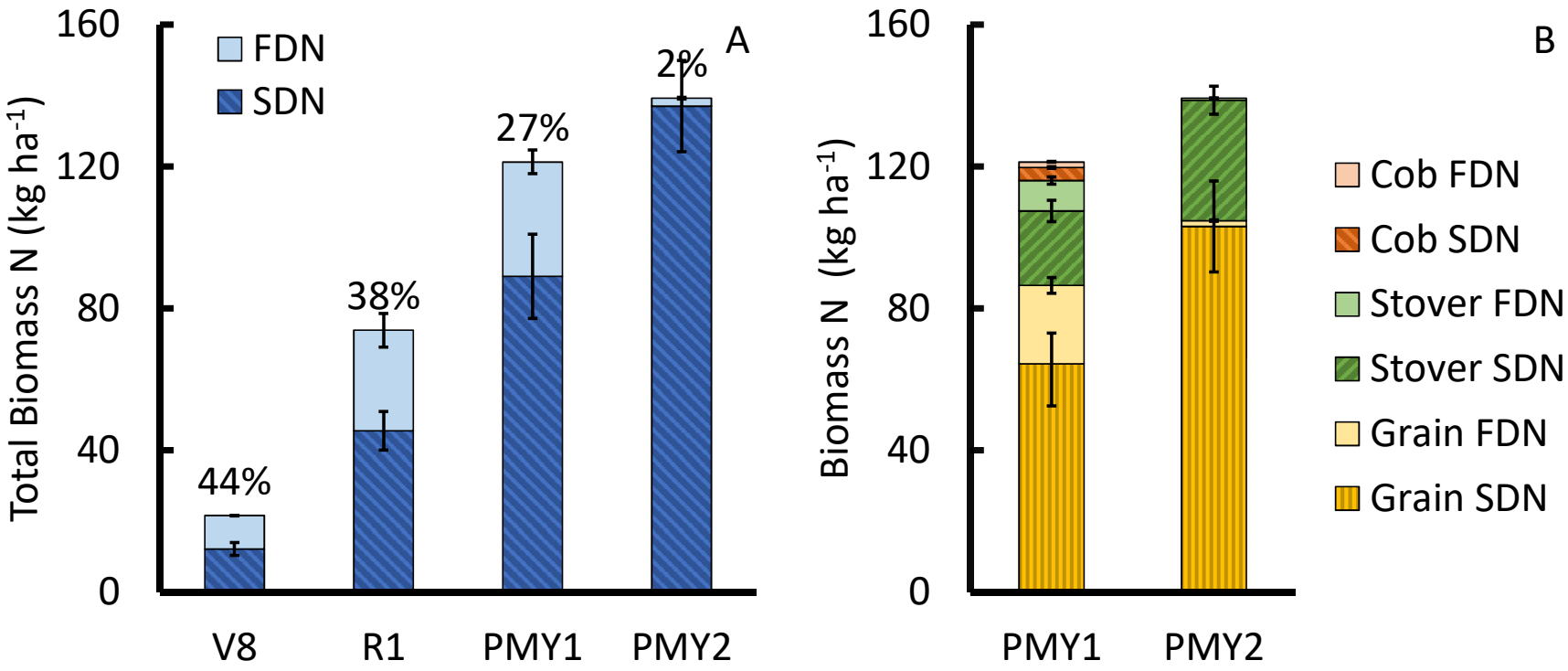

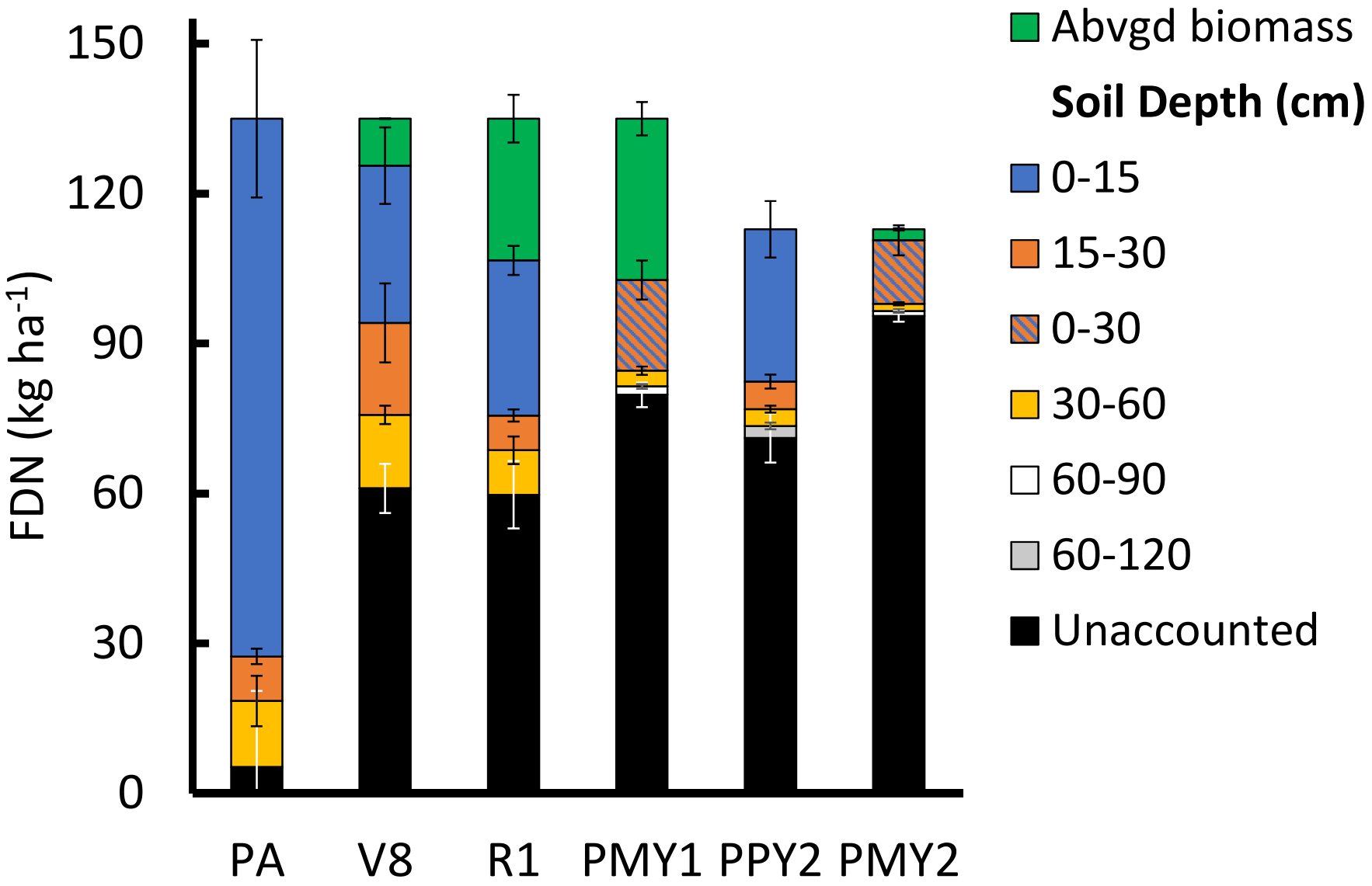

Table of Materials

Click here to access/download;Table of Materials;Copy of JoVE_Materials - Revised.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
20 mL scintillation vial	ANY; Fisher Scientific is one example	0334172C	
250 mL borosilicate glass bottle	QORPAK	264047	
48-well plate	EA Consumables	E2063	
96-well plate	EA Consumables	E2079	
Cloth parts bag (30x50 cm)	ANY	NA	For corn ears
CO2 Backpack Sprayer	ANY; Bellspray Inc is one example	Model T	
Coin envelop (6.4x10.8 cm)	ANY; ULINE is one example	S-6285 SKU:CS23030BM	For 2-mm ground plant samples
Corn chipper	ANY; DR Chipper Shredder is one example	N0	For chipping corn biomass
Corn seed	ANY	NA	Hybrid appropriate to the region
Disposable shoe cover	ANY; Boardwalk is one example	BWK00031L	
Ethanol 200 Proof	ANY; Decon Laboratories Inc. is one example	2701TP	
Fabric bags with drawstring (90x60 cm)	ANY	NA	For plant sample collection
Fertilizer Urea (46-0-0)	ANY	NA	~0.366 atom % 15N
Hand rake	ANY; Fastenal Company is one example	5098-63-107	
Hand sickle	ANY; Home Depot is one example	NJP150	For plant sample collection
Hand-held soil probe	ANY; AMS is one example	401.01	
Hydraulic soil probe	ANY; Giddings is one example	GSPS	
Hydrochloric acid, 12N	Ricca Chemical	R37800001A	
Jar mill	ANY; Cole-Parmer is one example	SI-04172-50	
Laboratory Mill	Perten	3610	For grinding grain
Microbalance accurate to four decimal places	ANY; Mettler Toledo is one example	XPR2	
N95 Particulate Filtering Facepiece Respirator	ANY, ULINE is one example	S-9632	
Neoprene or butyl rubber gloves	ANY	NA	For working in HCl acid bath

Paper hardware bags (13.3x8.7x27.8 cm)	ANY; ULINE is one example	S-8530	For soil samples and corn grain
Plant grinder	ANY; Thomas Wiley Model 4 Mill is one example	1188Y47-TS	For grinding chipped corn biomass to 2-mm particles
Plastic tags	ULINE	S-5544Y-PW	For labeling fabric bags and microplot stalk bundles
Sodium hydroxide pellets, ACS	Spectrum Chemical	SPCM-S1295-07	
Soil grinder	ANY; AGVISE stainless steel grinder with motor is one example	NA	For grinding soil to pass through a 2-mm sieve
Tin capsule 5x9 mm	Costech Analytical Technologies Inc.	041061	
Tin capsule 9x10 mm	Costech Analytical Technologies Inc.	041073	
Urea (46-0-0)	MilliporeSigma	490970	10 atom % 15N

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Done
2. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

3. Please ensure that all the panels of a figure appear in the same image: Figure 3, etc.

Done

Reviewers' comments:
Reviewer #1:

This manuscript describes a protocol for executing a field study involving labeled urea in a conventional agricultural system, the authors should be commended for the effort involved in working with 15N. The topic is appropriate for JoVE, but major revisions are necessary before publication. There are some areas of the protocol where extreme detail is given and not in others. Additionally, see the following list for some of the writing/grammar issues as well as the gaps in information that should be included.

Line numbers listed below correspond to the tracked changes document under the setting “Simple Markup”.

Major:

Title: The use of the word "solid" seems unnecessary, if so, it should be removed. "Isotope" and "15nitrogen" are redundant.

We agree that the words “solid” and “isotope” is redundant. They have been removed from the manuscript. (Line 4)

Abstract:
1) Line 42 - 15N should appear with the unit atom %, i.e. "urea enriched to 5 atom % 15N."a

We agree (line 41-42)

2) Lines 50 - 51 - While 15N does provide a powerful tool for tracing N through the plant-soil system, we can never quantify the amount lost, especially in a field setting. At best we can use the mass balance approach to estimate. Rewording to reflect this should be incorporated.

Thank you for this comment. We have edited the sentence to read:

"However, using the mass balance approach, tracer studies with multiple in-season sampling events allow the researcher to estimate FDN distribution through the soil-crop system and estimate unaccounted-for FDN from the system." Lines 47-50

Protocol:
2. Plot design
1) Lines 118 - 119 - Sampling among center rows is noted to minimize edge effects, but there is no mention of sampling center of the row for this same reason.

These lines (115-118) were clarified by rewording section 2.1.3 as follows:

2.1.3 Establish a microplot area (11.3-13.7 m) with dimensions of 2.4 m by 3.8 m centered on the width dimension. All 15N enriched plant and soil samples are collected from this area leaving 0.38 m of unsampled border on the length and width dimensions to minimize edge effects (Figure 2) [Place Figure 2 here].

4. 15N Enriched Fertilizer Preparation and Application
1) Line 148 - Description of how background 15N enrichment was obtained is missing from the manuscript.

Lines 151-152 Additional clarification has been added as follows:

"… *C1* is the isotopic concentration [expressed as atom % excess (measured atom % enrichment minus the natural background concentration **assumed to be 0.3663 atom %)]** of the original tracer fertilizer…"

5. Field Sample Processing: Corn Biomass
1) Line 203 - How was this grinding done? With what instrument? Was the same care taken during this initial grinding as during that described in 7.4?

Section 3 describes precautions that apply to every stage of sample processing. This line was modified to read as follows:

5.10 Following the principles of steps 3.1 and 3.2, thoroughly mix and grind 100 to 200 g of dried plant material to pass through a 2-mm sieve. Thoroughly mix the ground

material and store a subsample in a labeled coin envelope for further processing. A Thomas Wiley mill is a satisfactory option for plant tissue grinding while a Perten Laboratory Mill 3610 is a satisfactory option for grinding grain. (Lines 216-220)

6. Field Sample Processing: Soil
1) Lines 221 - 222 - The paper cited does not include 15N, and is inappropriate. Additionally, Ref 2 in your reference list identifies plot excavation as the only way to successfully quantify 15N applied in field conditions to the soil. Unless you include data supporting successful quantification of 15N with banded applications, this suggestion should be removed.

Thank you for your comment. We have removed the reference and discussion about banded applications. (Line 241)

7. Roller Jar Milling of Soil and Plant Samples
1) There's mention of cleaning utensils, but not of the sample jar. This would be a critical step in eliminating sample cross-contamination. Was this cleaning done between samples? If so, it should be noted here in the manuscript.

The cleaning protocol has been modified for inclusion in step 7. Roller jars are washed between each sample. The sentence has been edited as follows.

7.2.4 Between each sample, wash roller jars, stainless steel rods, and lids with soap and water to remove any residue. (Line 287-288)

8. Weigh Ground Plant and Soil Samples for Total N and 15N Analysis
1) Lines 334 - 335 - This note could be included in the above section (8.6) with a simple phrase i.e. "To avoid scale damage when taring the weight of the capsule,…", which would improve readability. Additionally, the action of taring is not completed by simply releasing it onto the weigh pan; this sentence (lines 329-331) needs major revision.

Section 8.6 was edited to read:

8.6 Use forceps to gently flare out the top 1 mm of the capsule to facilitate manipulation. To avoid scale damage when taring the weight of the capsule, hover and release the capsule 1 to 2 mm above the microscale weigh pan. Tare the capsule. Use forceps to return the capsule to the clean work surface. Line 346-349

Representative results:
1) Line 421 - This is the first mention of soybean and should thus include the scientific name as was done with corn.

Done line 407

2) Lines 465 - 469 - The purpose of this text is unclear to me, is this to explain lines 456 - 457? If so, that should be made more clear. This portion of the text illustrates the cross-contamination issues seen in 15N tracer studies. Were checks used throughout the process (grinding through 15N determination), if so, it seems relevant to present those results here as evidence of your precautions being successful.

This paragraph was included to illustrate the importance of the researcher being extremely conscientious in sample handling. While contamination can occur in all steps of the experiment (fertilizer application, sample collection, and processing), the risk of ^{15}N sample enrichment or dilution is less when the material masses are large. As the sample is reduced from several kilograms to milligrams, a small amount of contamination can have much larger effects on diluting or enriching the ^{15}N concentration in a sample.

Figure and Table Legends:
Figure 5: Example of the soil-corn fertilizer derived N (FDN) budget.
1) Lines 503 - 507 - In line 504, "Fertilizer derived N was accounted for…" this needs to be changed as in figure 5, the black (unaccounted) shows you did not account for the FDN. This statement is misleading and in direct opposition to the data you are presenting.

This caption has been edited as follows (Lines 503-512):

Figure 5: Example of the soil-corn fertilizer derived N (FDN) budget. The mass of FDN recovered in aboveground (Abvgd) corn biomass and at various soil sampling depths is reported for six sampling events over two growing seasons. Sampling events occurred 8 days following the 15N enriched fertilizer application (PA), at the V8 and R1 corn physiological development stages, at physiological maturity in the year of 15N enriched fertilizer application (PMY1) and the following year (PMY2), and prior to planting the second year (PPY2). The difference between the applied fertilizer rate (135 kg N ha-1) and the mass of FDN recovered in the soil-corn portions is the unaccounted for FDN fraction. The total mass of FDN for PPY2 and PMY2 was 113 kg FDN ha-1 because 22 kg FDN ha-1 was exported out of the soil-corn system as first-year grain. Error bars represent the standard error of the mean.

Minor:

Summary:
1) Line 25 - "Isotope" is redundant and could be deleted, or consider replacing with "tracer".

We agree. Line 26

Abstract:
3) Line 36 - "Of adequate size" is unnecessary and could be deleted.

We agree. Line 35-37

4) Line 38 - "Isotopic" is unnecessary and could be deleted.

We agree. Line 37

5) Line 41 - Consider replacing "Conventional" with "fertilizer grade" to improve clarity.

Thank you for this suggestion. We changed the wording to improve clarity. Line 40

6) Line 41 - "To the plot" is unnecessary and could be deleted.

We agree. Line 40

7) Line 41 - "but" should be replaced with "while".

We agree. Line 41

8) Line 43 - "sample" is unnecessary and could be deleted.

We agree. Line 43

Introduction:
1) Line 71 - The potential for measuring different N fractions in the soil is possible with current methodology, but considering this manuscript only describes total 15N analysis, I'm wondering if mentioning so is appropriate.

This sentence has been modified as follows: As 15N enriched fertilizer mixes with unenriched soil N, the measured change of 14N/15N ratio allows researchers to trace FDN in the soil profile and into the crop 3, 4. Line 68-70

2) Line 76 - "Of adequate size" is unnecessary and could be deleted.

We agree. Line 74

Protocol:
1. Field Site Description

1) When doing 15N field trials, there are many considerations when choosing site locations. Here there is no mention of slope, which can lead artifacts in neighboring plots via lateral movement. Other important considerations would be those that affect uniformity of movement down the profile such as tiling. Consider adding a section for locating appropriate sites.

A note was added just after the Field Site Description heading. It reads: NOTE: When performing 15N tracer field trials, selected sites should minimize variation due to soil, topography, and physical features5. Cross-contamination may occur following lateral soil movement due to slope, wind or water translocation, or tillage while the vertical distribution of soil N may be impacted by subsurface water flow and tile-drainage6. Line 83-86

2. Plot Design

2) Line 102 - 103 - "Large enough" and "large gap" should be more quantitative.

This note was removed, but a new paragraph was added to the discussion section. See line 544-545

3) Line 107 - "five sections" is a little unclear. I'm assuming this is in reference to the three 1.5m boarders along with the unenriched plot and microplot. Consider rewording.

To improve clarity, we removed the sentence “Divide the treatment plot into five sections as described below” from Section 2.1. The text in sections 2.1.1- 2.1.3 is sufficiently clear to describe the layout of the treatment plot. Line 105-118

3. Soil and Plant Sample Precautions

1) Lines 125 - 126 - Depending on the end goal for the researcher, contamination of the labeled material by the unlabeled material can be just as detrimental. You're representative data show lower than average uptake efficiencies, attention to contamination by labeled and not unlabeled material may be a key factor in this discrepancy.

Cross-contamination is a serious concern with ^{15}N tracer studies. As you point out, contamination of labeled material with unlabeled material and vice versa can lead to spurious results. An example of the effect of contamination is given in the Representative Results section. Line 459-469

We also modified Section 3.1 to read: “Contamination of unenriched materials (fertilizer, soil, or plant) by enriched materials and vice versa can drastically affect results…” line 124-125

To further acknowledge your point, we have added the following sentence following our report of $F^{15}NUE$ measures in line 448-451.

“Although we thoroughly cleaned our equipment between each sample, the lower F15NUE measures of our study could be an artifact of enriched sample dilution by processing enriched samples in order of lowest to highest expected enrichment.”

4. 15N Enriched Fertilizer Preparation and Application
5. Line 141 - It's unclear to me what is meant by "the requirements for agronomic research", justification is needed for how or why the enrichment was chosen.

4.1 Following guidelines put forth by Ref. 2 for fertilizer 15N use efficiency (F15NUE) studies, dilute 10 atom % 15N enriched urea to 5 atom % 15N enriched urea and dissolve in 2 L of deionized water to ensure uniform enrichment of urea fertilizer.

“NOTE: The required concentration of 15N enriched fertilizer is dependent on the goals of the agronomic study. If the concentration of stock 15N enriched fertilizer exceeds the researcher’s requirements, the stock fertilizer concentration may be diluted with similar conventional fertilizer using the following formula 3.” Line 139-146

6. Line 155 - 156 - Fertilizer grade urea pellets (prills) are formed by first creating molten urea. Due to the formation of biuret when urea is brought to high temperatures, this is a very difficult task. If the author has a protocol for this it should be cited.

Rather than creating molten urea, the crystals may be compressed into a cake using a Carver press. The cake can then be crushed and screened according to the desired particle size. While the prill is not exactly the same as conventional urea pellets, this method is more similar than applying dissolved urea. Line 160-164

5. Field Sample Processing: Corn Biomass
1) Line 177-178 - This portion of the text could be rewritten for clarity. Consider "Biomass samplings taken prior to stage X were chopped and placed into labeled fabric bags to dry...". The version you have now has a subject issue (you chop biomass into bags?).

We modified the text to read as follows:

5.2 Following the principles described in steps 3.1 and 3.2, chop V8 and R1 aboveground biomass (≤5 cm by ≤5 cm); a yard waste chipper is a satisfactory option. Place chopped biomass in labeled fabric or paper bags and dry in a forced-air oven at 60 ºC until constant mass. Record the biomass dry weight (Figure 3B). Line 185-188

2) Line 185 - Figure 3D provides no further clarification for the sentence in question, consider moving "(Figure 3D)" behind the first sentence in 5.4 if this is the case.

Two images were removed so 3D is now 3C. Line 195

6. Field Sample Processing: Soil
2) Lines 231-232 - While more cores equate to a better representation, removing 15N from the plots degrade the data obtained from plant samples obtained later. You are already taking 15 cores, at what point does the number of cores result in poor plant uptake conclusions?

This is a valid concern. The sampling scheme is such that the early season plant and soil samples are collected near the outside edges of the microplot. As the season progresses, soil and plant sample collection is taken closer towards the center of the microplot to minimize the risk of poor plant uptake. We have added an additional paragraph addressing how the number of cores may affect sample precision. Lines 541-565

3) Lines 239 - 240 - The attention to detail in soil grinding is lacking in comparison to the detail described in section 7.4.

As was previously noted by the reviewer, some sections were far more detailed than others. Upon review of the protocol, we deemed that less detail was needed when describing our roller jar methodology as this step is not crucial to obtain a fine, flour-like powder. However, we retained cleaning protocols for this methodology that may be useful for other researchers using similar equipment.

The reader is directed to section 3 for cautions related to sample processing.

7. Roller Jar Milling of Soil and Plant Samples
2) Line 254 - Writing could be improved, consider: "…clean prior to placing damp paper towels on the work area to catch…"

After editing the paper, this line was removed.

3) Lines 267 - 268 - This could be written as one sentence, which would improve readability.
After editing the paper, this line was removed.

8. Weigh Ground Plant and Soil Samples for Total N and 15N Analysis
2) Line 348 To aid the reader, "described in section 8.6." could be added to the end of this sentence.

Done line 364

9. Clean-up of roller jars and stainless steel rods.
1) Title - This is the only title that is not in title case and ends in a period, change for consistency.

2) There is no mention of how often this cleaning is completed. Between each sample? Between X amount of samples?

Due to editing, section 9 was removed. Portions of section 9 were incorporated into section 7. Cleaning is performed between each sample. This information was added to Section 7.2.4 Line 287-313

10. Calculations
1) Lines 400 - Was bulk density measured for each sample, or was constant bulk density assumed across the field and down the profile?

. Soil bulk density was measured at the center of 0- to 15-, 15- to 30-, 30- to 60-, 60- to 90-, and 60- to 120-cm depth layers from two 5-cm deep samples per replication using the intact core method19. Bulk density was averaged within depth across replications and assumed to be constant across the field.

This information has been added to the discussion section. Line 413-416

Representative results:
3) Line 419 - Manuscript should be changed to paper.

Done Line 413

4) Line 420 - Edit to "…Research Center located near…"

Done line 406

5) Line 428 - Change "methods section" to "protocol" for consistency.

Done Line 417

6) Line 430 - 437 - "Aboveground biomass" and "biomass" are both used, because you did not take root material, consistency on including this adjective is important.

Thank you for this comment. We have gone through the rest of the manuscript and changed biomass to aboveground biomass where appropriate.

Line 419-428

7) Line 447 - Consider replacing "that was like" with "mirroring" or "reflecting".

Done line 438

8) Line 450 - Remove "may", results either suggest or don't suggest.

Done line 441

Figure and Table Legends:
Figure 5: Example of the soil-corn fertilizer derived N (FDN) budget.
2) Lines 503 - 507 - This is a very long sentence, if possible, break up to improve readability.

Done Line 503-512

Discussion:
1) Line 517 - Nonsensical line break.

Done
2) Lines 518 - 521 - This sentence is irrelevant because N fractionation of 15N was not done in this study, only total N.

This was modified to read as:

They are 1) the tracer is uniformly distributed throughout the system, 2) processes under the study occur at the same rates, and 3) N leaving the 15N enriched pool does not return3. Because this study is interested in the distribution of total FDN throughout the soil-crop system, assumptions 2 and 3 are of minimal concern21. Line 518-522

Table of materials:
JoVE states that basic lab materials/equipment do not need to be included in this list, for this reason, remove:
Earplugs Done
Kimwipes Done
Nitrile gloves Done
Scoopula Done
Masking tape Done
Safety goggles Done
Lab coat Done

Consider removing:
Stake flags Done
3.78 L plastic bucket Done
SS spatula Done
SS forceps Done
Wash basin Done
55 gallon trash can Done

Reviewer #2:

Manuscript Summary:
A 15-nitrogen microplot design and sampling scheme is described for a field study to determine the N budget and fertilizer nitrogen use efficiency in a corn system. The protocol is described and results from a field study presented. Data show the contribution of fertilizer-derived N and soil-derived N to above ground plant biomass and total soil N. Considerations for using labeled N and the proposed protocol are discussed. A well-written manuscript describing a reasonable protocol for a powerful research tool. Some critical considerations such as avoiding cross contamination of samples are addressed.

Major Concerns:
None

Minor Concerns:

The protocol seems very specific to the study the authors performed and to the equipment they had available. Some of the very experiment-specific details could possibly be eliminated. Others could be written in more general terms with specific details described as 'a satisfactory option' for the given circumstances of the authors' study.

Thank you for this comment. We have edited the manuscript as you recommended. The yard waste chipper (line 186), Thomas Wiley mill (line 218), Perten Laboratory mill (line 218), mechanical soil grinder (line 260), and roller jar mill (line 276) have been described as satisfactory options for plant and soil sample processing as these pieces of equipment are not required to successfully complete the ^{15}N analysis.

The in-depth description of the roller jar mill has been reduced. Less emphasis is placed on this portion of the protocol, but we retained information regarding our jar mill protocol (including the cleaning procedures) as one example.

Section 8, that describes the sample weighing procedures, has been modified only slightly as this step will likely be ubiquitous across labs.

There was brief mention in the introduction of why the 15nitrogen method might be preferable to a typical N fertilizer study with unlabled N. There was no discussion of advantages or drawbacks of this particular 15nitrogen protocol compared to others in the literature. Several papers are cited where similar protocols were used. Some

discussion comparing these approaches to the proposed method would be beneficial. What makes the proposed method better than the others?

A new paragraph was added to the discussion section that discusses different soil sampling techniques and types of microplots (line 541-565). Each methodology has pros and cons. While we do not think our method is better than others (after all other techniques are equally viable and scientific papers have been published with those techniques as well as the one we used), we feel that the technique we used was the best choice for our objectives. The technique we used allowed us to conduct several measurements during the first growing season and to continue the study during a second year.

Line 68: These three values should be equivalent since they are just different ways of reporting the same thing. The natural abundance ratio is 272 not 242. As a concentration, that would be 0.366 atom% or 3660 ppm. Sometimes the concentrations are rounded to 0.37 atom% or 3700 ppm. Cited references

Thank you for catching that error. 242 has been changed to 272. The entry has been edited to denote equivalency between the values and show the reader three ways that they may see the natural abundance ratio of $^{14}N/^{15}N$ presented in the literature. Line 67

Line 107: I can ID only four areas and only four are named in the Fig 1 caption. I would consider all borders a single 'area' since they are all treated the same.

To improve clarity, we removed the sentence “Divide the treatment plot into five sections as described below” from Section 2.1. The text in sections 2.1.1- 2.1.3 is sufficiently clear to describe the layout of the treatment plot. Line 108-118

Line 151: It might be helpful to plug these values into equation 1 as an illustration.

Done. Line 155

Line 171: It would be helpful to clarify details here. Are there two sets of six samples collected, i.e., one from the unenriched plot area and another from the enriched area? Is each of the six 'plant samples' one individual plant? (if just one plant I suggest saying six plants instead of six plant samples). Are the six samples processed and analyzed separately or composited?

Thank you for pointing out the ambiguity of this section. We have modified section 5.1 to read as follows:

5.1 At each sampling stage, collect a six-aboveground corn plant composite sample from within the sampling area (15N unenriched) and a six-aboveground corn plant composite sample from the 15N enriched microplot. At least two plants should separate

each sampled plant to avoid significantly altering plant growth dynamics. The authors collected plant samples at the V8 and R1 corn physiological development stages11 and at physiological maturity (Figure 2). Line 179-183

177: Any suggestions for how to chop samples? It looks like a yard waste chipper was used in this study. Did that work well? Are there other satisfactory alternatives? Do you have suggestions for avoiding cross-contamination when chopping enriched plant samples? Why fabric bags? Would paper bags be satisfactory?

This line was adjusted as follows:

5.2 Following the principles described in steps 3.1 and 3.2, chop V8 and R1 aboveground biomass (≤5 cm by ≤5 cm); a yard waste chipper is a satisfactory option. Place chopped biomass in labeled fabric or paper bags and dry in a forced-air oven at 60 ºC until constant mass. Record the biomass dry weight (Figure 3B). Line 185-188

Line 185: Explain why flags are adjusted.

This line has been modified to explain why flags are adjusted.

5.4 Within the microplot, cut all corn stalks at the soil surface, tie into a bundle, label according to plot, and remove from the field (Figure 3C). Adjust microplot corner flags to be nearly flush with the soil surface to minimize the risk of removal by the combine during harvest or tillage post-harvest. Line 194-197

Line 204: One would assume only a small subsample is placed in the coin envelope. Is the subsample collected before or after grinding? How do you ensure the subsample is representative of the whole? What precautions are recommended to avoid cross-contamination between samples during grinding?

Section 5.10 was modified to address to questions you raised. Line 216-220 It now reads as follows:

5.10 Following the principles of steps 3.1 and 3.2, thoroughly mix and grind 100 to 200 g of dried plant material to pass through a 2-mm sieve. Thoroughly mix the ground material and store a subsample in a labeled coin envelope for further processing. A Thomas Wiley mill is a satisfactory option for plant tissue grinding while a Perten Laboratory Mill 3610 is a satisfactory option for grinding grain.

Line 241: This seems to need more explanation. Why is the 60-90 depth designated as an 'additional depth'? Is there something about it that separates it from the shallower sample depths? Why can't it be added to the depth sequence in the previous sentence? Was the equipment used limited to taking a 60-cm core? What if someone uses equipment that can collect a single core to 120 cm deep? or are you recommending that each depth be extracted separately? If so, why?

These are insightful questions. We have edited this section as follows: (Lines 228-237)

6.1 Collect first-year soil samples 8 days after 15N enriched fertilizer application, V8, R1, and post-harvest before tillage. Collect second-year soil samples at pre-plant and post-harvest. Due to logistical sampling constraints, the authors collected in-season soil samples at 0- to 15-, 15- to 30-, and 30- to 60-cm depths, post-harvest soil samples at 0- to 15-, 15- to 30-, 30- to 60-, and 60- to 90-cm depths, and second-year pre-plant soil samples at 0- to 30-, 30- to 60-, 60- to 120- cm depths.

NOTE: If a soil probe is unable to collect a soil core to the deepest desired depth as a single core, collect deeper depth cores from the same boreholes as the upper depths discarding the top 1-cm of soil to avoid contamination from soil falling from upper depths.

Line 309: The meaning here is unclear, especially regarding the 'replicate'. Replicate of what?

The line (324-326) was rewritten as:

8.2 Organize like-samples from lowest to highest expected 15N enrichment. Duplicate every eighth to twelfth sample in each run to check sample precision. Include at least one check sample per run16.

Line 340: This procedure is accomplished using forceps? Not done with gloved hands, right?

That is correct. The line was edited to read:

8.8 Using forceps, slowly crimp the top third of the capsule and fold over to seal. Using forceps, continue to fold and compress the capsule into a spherical shape taking care not to puncture or tear the tin.

Line 355-357

Line 455: Does this value come from Fig 4B? It's source should be indicated here.

Done Line 447

Line 461: Does this account for FDN removed in grain? I assume the difference in bar heights from YR1 to YR2 is N removed in grain. This should probably be explained somewhere.

This value does account for FDN removed in grain. This was clarified as follows:

This study indicates that by the end of the first and second years, only 41 and 29%, respectively of first-year FDN was accounted for within the soil-corn system (including FDN exported in the grain) while the remainder was either lost to the environment or leached below the 90 cm soil sampling depth. Line 454-457

Line 467: In the procedure section it is emphasized that it is critical to avoid contaminating samples with enriched material, which is of course, true. In this hypothetical example, the sample is contaminated with unenriched material. Should this risk be emphasized in the procedure section?

This risk should be emphasized in the procedure section as well. This correction was made in Section 3.1 that reads as follows:

3.1 Contamination of unenriched materials (fertilizer, soil, or plant) by enriched materials and vice versa can drastically affect results. Use dedicated equipment and processing areas for unenriched and enriched materials. Line 124-126

Line 483: This is a nice diagram and sampling scheme. However, I don't see the location of the preplant soil samples in year 2.

Thank you for noticing this error. The diagram has been corrected.

Line 488: Was labeled N in this study applied at planting or in-season? Wouldn't there be a significant risk of foliar absorption of labeled N, skewing results?

Labeled N was only applied at planting for this paper. Foliar absorption could skew results unless the urea solution was rinsed off the plants. To minimize confusion, this image was removed.

Line 489: Should this say 'six representative plants' instead of 'six representative samples'? I am assuming six plants were collected and composited into one sample.

This line was modified to read:

Collect and chop a six-aboveground corn plant composite sample from within the sampling area (15N unenriched) and a six-aboveground corn plant composite sample from the 15N enriched microplot at the pre-determined sampling times. Line 485-488

Line 497: Should 3A and 3B be changed to 4A and 4B? Could this be formatted as in caption for Fig 3 (i.e., (A), (B) instead of 4A, 4B)?

Thank you for catching this error. This caption has been corrected. Line 493-501

Line 501: How were cobs handled in yr 2? Were they included with the stover?

They were included with the stover in year 2. The caption has been edited as follows in line 496-501.

Figure 4: Example of aboveground biomass N partitioned into fertilizer derived N (FDN) and soil derived N (SDN) fractions. Total aboveground biomass N was separated into its individual sources of FDN (solid color) and SDN (hashed color) in (A) and (B). Error bars represent the standard error of the mean. (A) Aboveground biomass N was measured at the V8 and R1 corn physiological development stages and at physiological maturity in the year of 15N fertilizer application (PMY1) and the year following 15N fertilizer application (PMY2). The value above each column represents the percentage of the total N that was FDN. (B) Aboveground biomass N measured at PMY1 and PMY2 is shown in its individual parts of cob (only Year 1), stover (stalk and leaves; includes cob for PMY2), and grain for FDN and SDN.

Line 506: It is a little confusing that PHY1 is used here and R6 Yr1 is used in Fig 4. Can the same abbreviation be used throughout?

Thank you for this comment. We decided to change references to PHY* and R6 to physiological maturity (PMY*). The text, Figure 4, and Figure 5 have the same abbreviations that should improve clarity for the entire paper.

Line 530: I think edge effects should be pretty much zero. Would 'eliminate' or 'avoid' be better words than 'minimize'?

Done Line 531