NREC #2016-2-360190-386

Dissimilatory Nitrate Reduction to Ammonium: An Unexplored Microbial Pathway for Nitrate Retention in Agricultural Soils

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Kent & Yang

BACKGROUND

Economic and regulatory factors are increasing the pressure on Illinois producers to improve nutrient management, with nitrogen (N) run-off presenting a major challenge. Options like precision agriculture practices and denitrifying buffer zones are being used and improved, but may provide only partial solutions. Microbially mediated dissimilatory nitrate reduction to ammonium (DNRA) can lead to nitrogen retention by returning NO_3^- to the less mobile form of inorganic N, NH_4^+, rather than losing NO_3^- to leaching or gaseous nitrous oxide and dinitrogen via denitrification. DNRA can thus mitigate the water pollution and climate change impacts of fertilizer N inputs to agricultural systems while also potentially increasing crop yields by improving N retention for crop uptake. Despite the important role DNRA could play in creating sustainable agricultural systems, it has been understudied in agricultural soils, due to the prevailing conceptual model which suggests that DNRA occurs only under highly reducing conditions such as found in flooded soils. However, mounting evidence indicates that DNRA rates can be comparable to or even many times greater than NO_3^- leaching and denitrification rates in unsaturated soils, likely due to the activity of facultative anaerobes within anoxic soil microsites. Therefore, DNRA should no longer be ignored in assessments of soil N cycling.

The long-term goal of the research team is to reduce NO_3^- losses from agricultural systems in the Midwest U.S., thereby improving water and air quality while possibly increasing crop yields. DNRA is an unexplored pathway for NO_3^- retention in agricultural soils that may be optimized through management practices. However, the environmental and genetic potential for DNRA to occur in agricultural soil is currently unknown.

The *overall goal* of this project is to improve understanding about the importance of and controls on DNRA in Illinois agricultural soil. This goal is being achieved through the following *specific objectives*:

Objective 1: Quantification of DNRA and denitrification rates to determine if DNRA is an important process in Illinois agricultural soils,
Objective 2: Characterization of abundance and diversity of microbial communities capable of DNRA and denitrification in Illinois agricultural soils, and
Objective 3: Determination of drivers of DNRA rates and microbial community composition to identify treatments and management practices that affect DNRA rates in these soils.

To address these objectives, we are collecting and analyzing soil samples from a diversity of agricultural systems at the UIUC South Farms in Urbana, Illinois to identify which treatments

facilitate a meaningful level of DNRA potential, and of these, how management practices might be used to encourage increased DNRA rates within the system's soils.

PROJECT PARTICIPANTS

This interdisciplinary project employs the tools of molecular biology, biogeochemistry and community ecology to identify microbial functional groups and their activity in natural, engineered, and managed ecosystems. Dr. Angela Kent, (PI) is an Associate Professor of Microbial Ecology in the Department of Natural Resources and Environmental Sciences. Her microbial ecology research program is focused on characterizing and understanding the ecological drivers of microbial populations involved in key transformations in the nitrogen cycle that impact both plant productivity and environmental quality, as well as plant-microbe interactions. As the Primary Investigator of the project, Kent is overseeing the administration and management of the project, and is involved in all activities related to the characterization of microbial communities and their N-cycling activities. Dr. Wendy Yang, (co-PI) is an Assistant Professor of Global Change Ecology in the Plant Biology and Geology departments. She is a biogeochemist and ecosystem ecologist with expertise in the study of soil greenhouse gas emissions and nutrient cycling. She has developed new and also uses established stable isotope techniques to quantify dissimilatory nitrate reduction to ammonium (DNRA) and gross nitrogen cycling rates as well as soil nitrous oxide and dinitrogen emissions from denitrification. Yang is leading the efforts to measure N-cycling process rates using these stable isotope techniques. Sada Egenriether is a first-year Ph.D. student in the UIUC Program in Ecology, Evolution, and Conservation Biology who is co-advised by Kent and Yang. She joined the project in April 2016 and has been responsible for all of the experimental work.

SUMMARY OF ACTIVITIES TO DATE

The project is in its first year. Work began in April 2016 with the identification of relevant and accessible sites with differing treatments (fertilization regimes, crop rotations and types, tillage), and the development of protocols and sampling methods to measure DNRA potential using ^{15}N tracer methods.

Protocol development. Initial protocol plans requiring installed equipment for *in situ* ^{15}N tracer experiments were modified at the start of the 2016 field season in favor of soil core collection for laboratory experimentation. This more efficient method allowed for a greater number sampling

events during the field season, yielding a more comprehensive survey of available agricultural treatments and conditions.

Sampling locations. To compare potential DNRA rates across a variety of contrasting treatments, we selected five sampling sites, each with four replicate plots per treatment:

- Energy Farm: Plots include corn-soy rotation (fertilized during corn years, unfertilized during soy years), *Miscanthus x gigantus* (half fertilized, half unfertilized), and switchgrass (fertilized). The treatments at this site are being used to compare the effects of crop type and N application to *Miscanthus x gigantus* to DNRA potential.
- Perennial Buffers: We are using corn plots (cover cropped vs. non-cover cropped) and bioenergy grasses plots (complex vs. simple), to compare DNRA potential between simple and complex cropping systems and crop type (annual rotation vs. perennial).
- Biochar: All plots are planted with corn, with factorial design including treatments with the application of biochar vs. none, and fertilization vs. none. From this site we are comparing effects of biochar application, as well as fertilizer application within a single crop.
- Precision Zonal Management: All plots at this site are under conventional tilling regime. Fertilized corn and unfertilized soy plots are being used to compare DNRA potential between crops in a conventionally tilled system.
- No-till: This site is historically untilled corn-soy rotation, with half the plots planted in corn and half in soy annually. Corn plots are fertilized and soy are unfertilized. From this site we are comparing DNRA potential between crops in an untilled system, and, accounting statistically for site differences, between same crop in untilled vs. tilled systems planted in the same crop at other sites.

Sampling. During the 2016 field season, each site has been sampled twice, with the Energy Farm site having been sampled three times. For each sampling event, two soil samples from 0-10cm depth were collected at two random locations within each replicate plot and composited together for experimentation. Activities at each site are summarized in the table below.

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Table 1. Summary of activities at each sampling location.

Site	Activity
Energy Farm	• Crops: Perennial grasses (Miscanthus and switchgrass), and corn-soy rotation (soy year in 2016) with conventional tillage practices • Pre-fertilization samples collected 5/19/16 • 50lbs N as urea applied to switchgrass and fertilized miscanthus plots on 5/23/16 • Post-fertilization samples collected 6/6/16 • Peak-growth samples collected 8/11/16
Perennial Buffers	• Corn-soy rotation (corn year in 2016) grown with conventional tillage practices, including "complex" (cover cropped) and "simple" (non-cover cropped), and bioenergy ("simple" with switchgrass alone, "complex" with mixed switchgrass + big bluestem + Indiangrass + Prairie Cordgrass) • 200lbs N/acre as urea applied to corn plots 5/25/16 • Post-fertilization samples collected 6/2/16 • Peak-growth samples collected 8/18/16
Biochar	• Corn grown with conventional tillage practices • 240lbs N applied as UAN 4/24/16 • Post-fertilization bulk soil samples collected 5/16/16 • Peak-growth samples collected 7/27/16
No-till	• Corn and soy grown with conservation tillage (no-till) practices • 180lbs N applied to corn plots 5/16/16 • Post-fertilization samples collected 6/15/16 • Peak-growth samples collected 8/4/16
Precision Zonal Management	• Corn and soy grown with conventional tillage practices • 180lbs N applied to corn plots 4/23/16 • Post-fertilization samples collected 6/15/16 • Peak-growth samples collected 8/4/16

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Processing and analysis. Over the course of the 2016 field season, a total of 160 soil samples were collected for basic soil chemistry analysis, ^{15}N tracer experimentation, and DNA extraction. Immediately following sample collection, a 30 g subsample was taken from the composited soil sample from each plot and destructively sampled for extraction in KCl, another 10 g was set aside for soil moisture measurements, 5 g was used for pH measurements, and a 15 mL Eppendorf tube was filled with soil and immediately frozen for later DNA extraction. Two 150 g subsamples of soil were removed to fresh gas-permeable bags for addition of either 1 mL ^{15}N-labeled KNO_3 or NH_4Cl for tracer experiments carried out in parallel. From these, another 30 g subsample was removed from each bag for KCl extraction at time points 15-minutes, 4-hours, and 24-hours.

Filtration of soil KCl extractions

Shortly after extraction, a 5 mL aliquot was removed and frozen for later colorimetric analysis for NO_3^- and NH_4^+ concentrations, and the remainder of the sample was frozen for later analysis on an isotope ratio mass spectrometer (IRMS). In addition to the KCl extraction, an additional pair of subsamples were collected at time 4-hour for K_2SO_4 extraction and chloroform fumigation to assess microbial uptake of the ^{15}N isotope labels, as well as a 75 mL gas sample for analysis for N_2O, CH_4, and CO_2 on a gas chromatograph and $^{15}N_2O$ on an IRMS, and a 15 mL gas sample for $^{15}N_2$ analysis on an IRMS.

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Colorimetric analyses on Lachat

The reserved 5 mL aliquots were analyzed colorimetrically on a Lachat flow injection auto-analyzer. The 75 mL gas samples were analyzed on a gas chromatograph and IRMS within two weeks of experimentation to avoid deterioration of sample quality. The KCl extractions for ^{15}N analysis were diffused in batches of ~250 samples throughout fall and winter (ongoing) and run on an IRMS interfaced to an elemental analyzer in the Yang lab.

A batch of samples diffusing

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Diffusion disks prepared for encapsulation prior to IRMS analysis

Microbial DNA was extracted from 5 mg frozen soil for each sample and extracted using the FastDNA extraction kit for soil and purified using established protocols. Prepared DNA was sent to the Keck Center for Functional Genomics at UIUC for Illumina HiSeq sequencing of 16S rRNA genes (to examine all bacteria) and functional genes that are diagnostic for key N transformations. In addition, qPCR to quantify N cycling functional genes across agricultural management scenarios was carried out using the Fluidigm Biomark HD high throughput amplification system.

PRELIMINARY RESULTS

Objective 1: Determine if DNRA is an important process in Illinois agricultural soils

Because calculation of DNRA rates requires data from samples that are yet to be processed and run on the IRMS, we are unable to report rates normalized to units of μg-N/g-soil at this time. However, the raw data for production of $^{15}NH_4$ from $^{15}NO_3$ label addition (an observation indicative of DNRA activity) that we have measured so far suggest a strong likelihood meaningful DNRA rates. Among these, there appears to be a treatment effect favoring DNRA in perennial-planted plots, regardless of fertilization conditions, with an atom-percent ^{15}N enrichment in NH_4^+ of typically 2 to 3.5-fold that of background natural abundance ^{15}N enrichment over the 4-hour incubation period.

Objective 2: Characterization of microbial communities capable of DNRA

DNA sequencing and qPCR analyses reveal trends in microbial community composition that

supports preliminary treatment-related DNRA activity estimates. We find that the composition of communities associated with perennial crops are significantly different than annual cropping rotations (Fig. 1). In addition, there is evidence of a marked effect of tillage on community composition, with the communities associated with both soy and corn at the no-till site being significantly different from communities with the same crop at other, conventionally-tilled sites (Fig 1). Interestingly, there is no significant difference in community composition in fertilized vs. unfertilized treatments, or sampling time points (immediately post-planting vs. peak-growth).

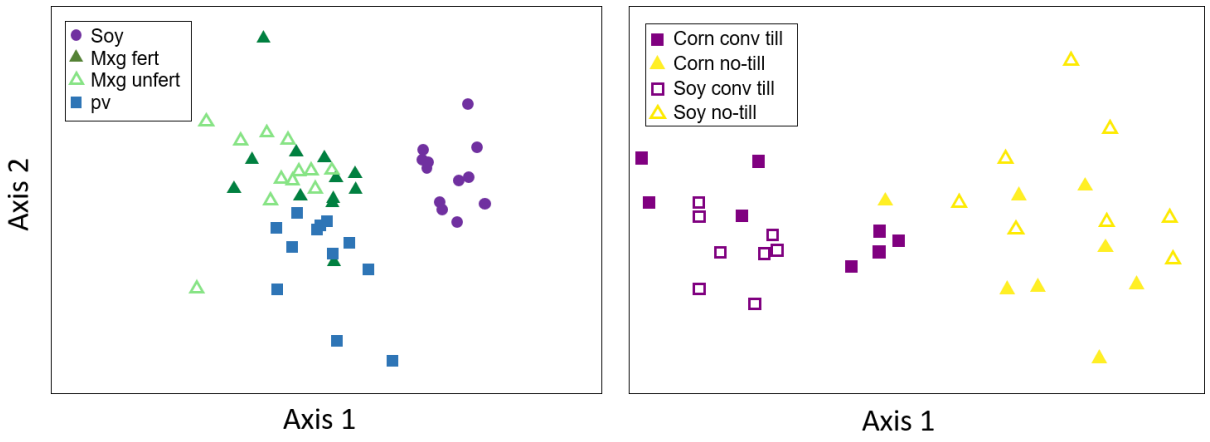

Figure 1. Nonmetric multidimensional scaling (NMDS) plots demonstrating community differences between annual and perennial crop types (Energy Farm, left), and between tilled and untilled (annual) treatments (right).

Given the unique communities and likely different rates in perennial versus annual crop systems, we anticipated differences among these treatments in abundance of *nrfA*, the gene encoding the nitrite reductase used in DNRA. However, qPCR analysis indicated that abundance of this gene was not significantly different between crop types. However, abundance of *nirK*, the gene associated with nitrite reduction to NO in denitrification, and *nosZ*, the gene associated with NO reduction to N_2O in denitrification, tended to be significantly *lower* in treatments exhibiting elevated DNRA activity. This seems to imply that elevated DNRA rates are not actually due to an increased genetic potential for DNRA in these treatments, so much as to a decreased potential for denitrification. Because these two processes compete for NO_3^-, a decrease in denitrification would allow an increase in DNRA rates.

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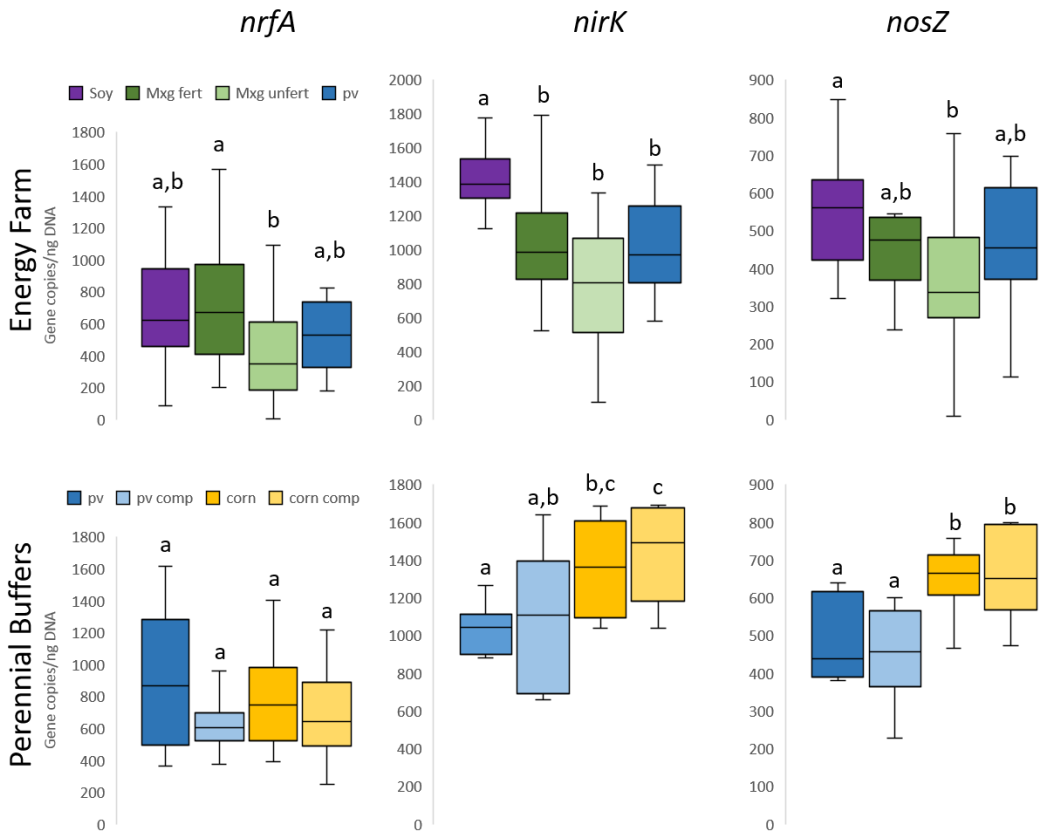

Figure 2. Gene abundance differences between perennial and annual crops for denitrification genes (nirK, nosZ) at Energy Farm and Perennial Buffers sites, but not for DNRA gene (nrfA).

Objective 3: Determination of drivers of DNRA and microbial community composition

Our preliminary analyses suggest that crop type, and possibly tillage, are the primary factors shaping microbial community composition and N-cycling behavior in our study sites. Once sample processing is completed this spring, we will perform multivariate analyses to further assess relationships between DNRA and denitrification rates and their potential drivers (e.g., soil temperature, moisture, C:N ratio, etc.). Similar analyses will be performed to determine drivers of abundance and composition of microbial communities.

Challenges: Primary challenges to date have included difficulty coordinating with multiple site managers to schedule sampling dates around planting and fertilization, and the establishment of an experimental protocol that allows for analysis of a wide breadth of treatment types and sites. We had initially hoped to compare DNRA potential in pre- and post-fertilized systems; however,

due to multiple sites being planted and fertilized without advance notice, this time point was missed at all sites except the Energy Farm.

Budget: The budget for this project remains unchanged since the renewal submitted in September.