Final Report for AFREC Project 755664

Controls over fundamental soil N cycling processes in Minnesota cropping systems: Nitrification, Nitrosation, and Mineralization

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Background and abbreviations: The nitrification process consists of two distinct steps. The first step transforms ammonium to nitrate and is catalized by ammonia-oxidizing bacteria (AMOaB) and archaea (AMOaA); the second step transforms nitrite into nitrate is carried out by nitrite-oxidizing bacteria (NOB). Nitrite and nitrate are very mobile forms of nitrogen and are often lost through soil leaching. Therefore, these two different groups of micro-organisms and their interactions will determine the fate of nitrogen soil movement and possible plant acquisition. In Minnesota soil, very little is known about this nitrification process and how soil types, soil farming history, and soil fertilizer management influence these microbe communities and the N cycling.

Objective: This project is divided into three objectives. The first objective is the determination of the key factors regulating nitrification and nitrite (NO_2^-) accumulation in Minnesota soils by quantifying inorganic N dynamics and the separate activities of the ammonium-oxidizing microbes (AOMs) and the nitrite-oxidizing microbes (NOMs). The second consists of the determination of the key factors regulating the incorporation of NO_2^- into soil organic matter during nitrification (i.e., nitrosation), and lastly, the third objective is the determination of the susceptibility of the incorporated N to mineralization.

Q2 2014 Progress (Apr 1 – Jun 30, 2014)

1-Personnel

Upon notice of the award, we advertised for a post-doc position having the required background and skills. In the meantime, we proceeded with establishing protocols with the support of personnel funded through other sources. Although we received dozens of applications for the post-doc position, very few had the appropriate experience with molecular microbiology.

Eventually we received an application from Dr. Florence Sessoms who was already working in another position here at the University. Dr. Sessoms started on this project on 1 Aug 2014.

2-Preparing lab area and purchasing supplies for molecular work

The first part of this project was to set up a designed place area for DNA extraction and qPCRs. Several lab supplies were bought for the DNA extraction, such as a centrifugation machine, soil DNA extraction kits, Eppendorf tubes, tips and freezer to store the DNA dilution and qPCRs supplies. The qPCR analysis is being run using the SYBR green protocol. The pipettes were also calibrated at the beginning of the study.

3-Primers design and testing

Several set of primers were ordered to amplify prokaryotic 16RNA, the bacterial and archaea ammonium oxydase and nitrobacter nitrite oxydase. All the primers tested were already designed, used for qPCR analysis, and published. The prokaryotic 16RNA, the archaea ammonium oxidase, and the nitrobacter nitrite oxydase set of primers had their efficiency positively tested (100%, 100% and 92 % efficiency respectively). The prokaryotic 16RNA and the nitrobacter nitrite oxydase set of primers were tested on soils already available in the lab. The dissociation curves of the amplified DNA were studied and the qPCR tubes were run on agarose gel to verify that these set of primers amplify a correct single band similar in size to the standard one. The remaining sets of primers are currently being tested but should not be problematic.

4-Soil sampling

While working on our qPCRs conditions, we are also deciding which Minnesota soils to sample and analyze. The sampling will occur in September at the end of the growing season. We would like to test different types of soil that possess different contrast (Ph and soil texture) but an identical crop rotation (soybean/corn). Soils will be sampled from the St Paul campus, Rosemount, Becker, Waseca and Cedar Creek and two different farmer fields. For each location two types of moisture condition (wet and not wet) will be tested. The soil samples will be divided in two parts, one for DNA extraction and qPCRs analysis; the second part will be used for N^{15} experiment. Already, soils sample from the St Paul and Becker location were harvested during spring and DNA

was successfully extracted. These previous samplings could give to our investigation an estimation of a temporal evolution of the nitrification process in these soils.

Q3 2014 Progress (Jul 1 – Sep 30, 2014)

1-Primers testing

Several sets of DNA primers were used to amplify prokaryotic 16RNA, the bacterial and archaea ammonium oxidase (AOB and AOA, respectively) and nitrobacter nitrite oxidase (NXRA). All sets of primers had their efficiency positively tested and were used on extractions of several soil types and treatments as described below.

2-Soil sampling

Samples of several contrasting soil types were sampled from the St Paul campus (2 different experimental fields), Rosemount field station (2 different experimental treatments, conventional tillage and no tillage), and from the Becker, Waseca, Lamberton and Crookston field stations. Where possible and available, soil samples were collected from the corn phase of corn/soybean rotation and from areas of field that did not receive nitrogen fertilizer in 2014. In addition we were able to obtain soil from Cedar Creek Reserve collected in November 2013. The soil samples were divided in two parts, one for fresh DNA extraction and the second part for incubation experiments. The moisture content, bulk density, pH, and extractable NH_4 and total NO_3-NO_2 were determined. For all soils, a subsample of them was air dried, sieved and conserved at room temperature before their use in incubation experiments (below).

3-Incubation experiment

Each soil is being tested in incubation experiments consisting of adding increasing amounts of urea and observing soil chemical and microbial responses over periods of 25 to 35 days with sampling intervals of 3-4 days each with 3 biological replicates. At each sampling time point, gas samples are extracted to measure the production of CO_2 and N_2O. Soils sub-samples are harvested for determination of the pH, NH_4, NO_2 and total NO_3. Also a small fraction of the samples is kept aside for possible further DNA extraction. Two soils, St Paul (Venterea field) and Becker, were

already started October 6 2014 and seven time points are already harvested. Two news soils, Crookston and Lamberton, will be started November 10 2014.

4-DNA extraction and qPCR

The incubation experiment allows the observation of the chemical soil response to different amount of urea added. The concentration of NH_4, NO_2, and NO_3 permit the visualization of these responses. Based on the evolution of these concentrations, several time points were chosen to have their DNA extracted to observe the microbial (AOA, AOB and NXRA) response of these soils. So far four time points were chosen, DNA extracted, and tested.

Q4 2014 Progress (Oct 1 – Dec 31, 2014)

1-Incubation experiments

Our experiments are underway and being completed as planned. We have completed the initial round of 30-day incubation experiments using soils from Becker, Saint Paul (corn system), Lamberton and Crookston; and experiments are underway using soils from Rosemount (conventionally tilled) and Rosemount (no-till). With each soil, 5 levels of N addition (including a zero-N) control were examined, with sampling intervals of 3-4 days and with 3 biological replicates on each sampling date. At each sampling time point, gas samples were collected to measure production of CO_2 and N_2O (and O_2 was measured every 1-2 weeks). Soils sub-samples were harvested for determination of pH, NH_4, NO_2 and NO_3. Additional sub-samples were taken for DNA extraction and qPCR analysis. Following the Rosemount soils, the same experiments are planned using soil from Waseca and Saint Paul (soybean system). These experiments are planned to be set up upon completion of the Rosemount experiment (after Feb 5, 2015).

2-DNA extraction and qPCR

Several subsamples from the incubation experiments have been extracted for DNA and successfully analyzed using qPCR for gene copy numbers of 16S ribosomal RNA (16S rRNA), ammonia monooxygenase (bacteria) (*amo*A-b), ammonia monooxygenase (Archaea) (*amo*A-a) and nitrite oxidoreductase (*nxr*A). We have also established the procedures quantify an additional gene that codes for nitrite oxidation (*nxr*B).

3-Additional soil analysis

We are also quantifying basic chemical and physical properties in all the soils, including total soil carbon and nitrogen, textural analysis, cation exchange capacity, and ammonium sorption capacity.

Overall Results

First objective: As previously described, 8 Minnesota soils were tested. Six of these soils were sampled from a corn-soybean rotation or from continuous corn system . Crookston was the only location with a rotation of wheat and soybean; interestingly, one field in St Paul had a soybean-only cropping system for more than 50 years and was therefore added to the study. For all soils, the texture was analyzed and revealed that these 8 soils could be divided in 4 categories (see table 1). Waseca and Lamberton possessed clay loam texture with a high percentage of Clay. Rosemount (Conventional tillage and no-till) and St Paul (long-term soybean and continuous corn) possessed a silt loam texture. Crookston possessed a loam texture and finally Becker presented a Sandy loam texture with high percentage of sand (80%)

Sample:	Waseca	Lamberton	Rosemount non tilled	Rosemount tilled	St Paul long term soytbean	St Paul Corn	Becker	Crookston
Cropping system	corn/soybean rotation	corn/soybean rotation	corn/soybean rotation	corn/soybean rotation	Longterm soybean	corn/soybean rotation	corn/soybean rotation	wheat/soybean rotation
Tillage	conventional	conventional	No tilled	conventional	conventional	conventional	conventional	conventional
%Clay:	29.54	27.05	10.42	15.54	16.92	14.90	11.54	19.06
%Silt:	33.23	30.43	55.59	58.28	50.76	59.61	7.69	38.12
%Sand:	37.22	42.53	33.98	26.18	32.32	25.48	80.77	42.81
Texture:	Clay Loam	Clay Loam	Silt Loam	Silt Loam	Silt Loam	Silt Loam	Sandy Loam	Loam

Table 1: Identification and texture analysis of 8 Minnesota soils

Taken together, the texture analysis confirms that we were able to sample very different types of soil. Therefore, we will be able to observe how soil texture influences the nitrification process. In addition, the presence of different types of tillage practice (Rosemount soils) and different cropping systems (Long term soybean versus corn) within a single texture of soil (Silt Loam) will allow us to evaluate how these parameters influence the microbial community (AOM and NOM) involved in the nitrification processes

All of these soils were tested in a series of incubation experiment consisting of 5 different urea amendments (0, 100, 250 500 and 1000 mg N per Kg of soil). Each incubation consisted of 10 time points with a 3-4 days interval between each time point. For each time point the extractable NH_4, NO_2 and NO_3 and pH were measured.

First of all, the extractable NH_4 curve shows a rapid increase after urea amendment, revealing that the urea hydrolysed to ammonium. Several days after urea amendment, total extractable NH_4 decreases for all treatments in each soil indicating that ammonium enters the first step of the nitrification.

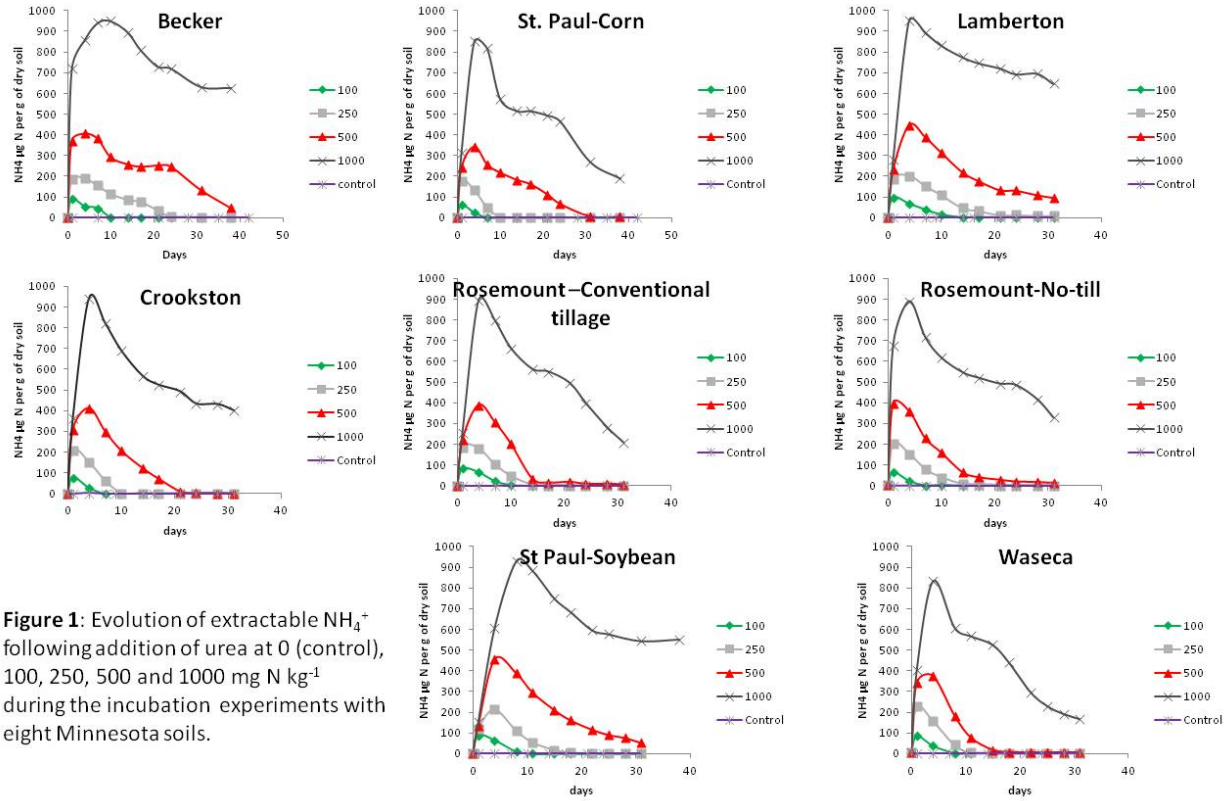

Figure 1: Evolution of extractable NH_4^+ following addition of urea at 0 (control), 100, 250, 500 and 1000 mg N kg^{-1} during the incubation experiments with eight Minnesota soils.

The observation of the nitrite curves (Figure 2) allows us to separate the soils into three distinct groups. The first group consisting of Becker and Crookston soils presented a nitrite accumulation for the 250, 500 and 1000 urea amendments; Becker had a greater accumulation of nitrite for the 250 treatment compared to Crookston. The second group consisted of St Paul-corn, St Paul-soybean and Rosemount conventional tillage which all displayed accumulation of nitrite for the 500 and 1000 treatments. However St Paul-corn had the highest nitrite accumulation. The third

group consisted of Lamberton, Rosemount no till and Waseca, with only an accumulation of nitrite found in the 1000 urea treatment.

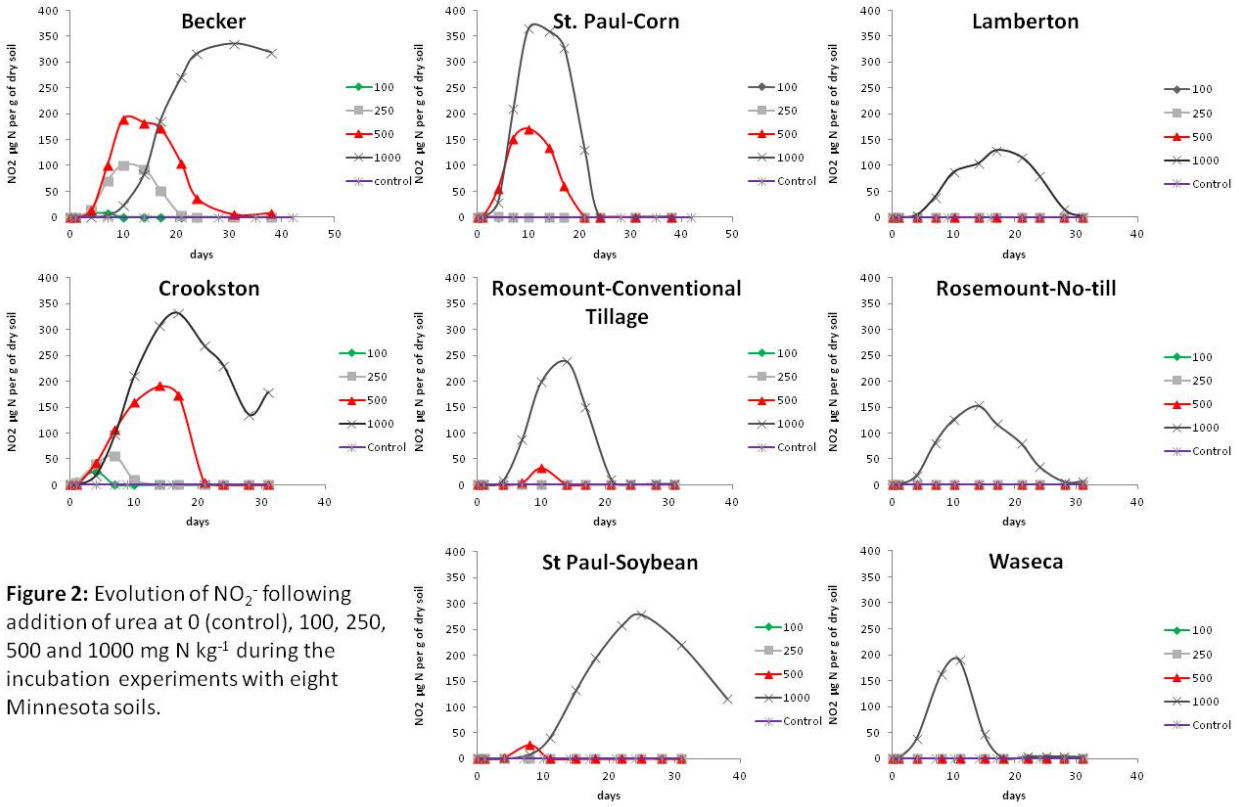

Figure 2: Evolution of NO_2^- following addition of urea at 0 (control), 100, 250, 500 and 1000 mg N kg^{-1} during the incubation experiments with eight Minnesota soils.

The nitrate curves (Figure 3) reveal a similar separation of the soils based on how fast the nitrate reaches its maximum accumulation. For Becker and St Paul, all the treatments reach their maximum nitrate accumulation at different times. For St Paul-corn, St Paul-soybean and Rosemount conventional tillage, the 100 and 250 treatments reach their maximum accumulation at the same time. However, the St Paul soils present different curves for the highest urea amendment. For Lamberton, Rosemount no till and Waseca, the 100, 250 and 500 treatments reach their maximum nitrate accumulation almost at the same time.

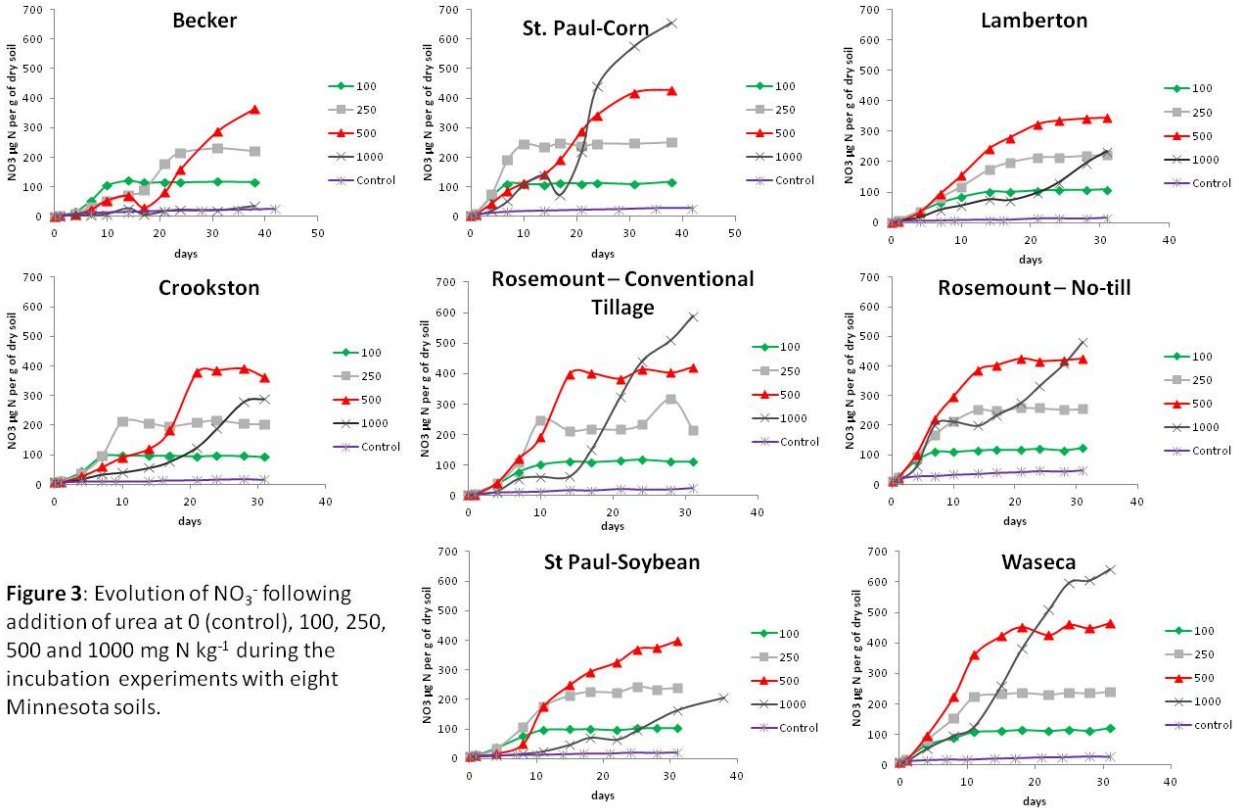

Figure 3: Evolution of NO_3^- following addition of urea at 0 (control), 100, 250, 500 and 1000 mg N kg^{-1} during the incubation experiments with eight Minnesota soils.

Altogether, a higher nitrite accumulation and slower nitrate accumulation indicate possible inhibition of the nitrifications process. Crookston and especially Becker are the most sensitive soils to low (100), medium (250) and high (500-1000) urea amendments. The St Paul soils are more resistant to the urea amendment but still behave differently. This indicates that the type of cropping system may influence the nitrification process. Finally, the soils from Waseca, Rosemount (no-till) and Lamberton are the most resistant to high urea amendment. The clear difference between the two Rosemount soils points out the importance of tillage practice on the nitrification process.

Actual N_2O production was different for all the soils, as seen in Figure 4. Becker and Crookston showed N_2O production for all the urea amendments. St Paul (Corn), Rosemount (Conventional tillage) and St Paul (Soybean) displayed N_2O accumulation for the 500 and 1000 urea amendments. However, N_2O production in the 1000 treatment was lower in St Paul (Soybean) despite high nitrite production (Figure 2) and slow nitrate accumulation (Figure 3). Lamberton, Waseca and Rosemount (no-till) displayed N_2O production only for the highest urea amendment; and for all soils treated with urea, N_2O production was the highest compared to the 5 other soils.

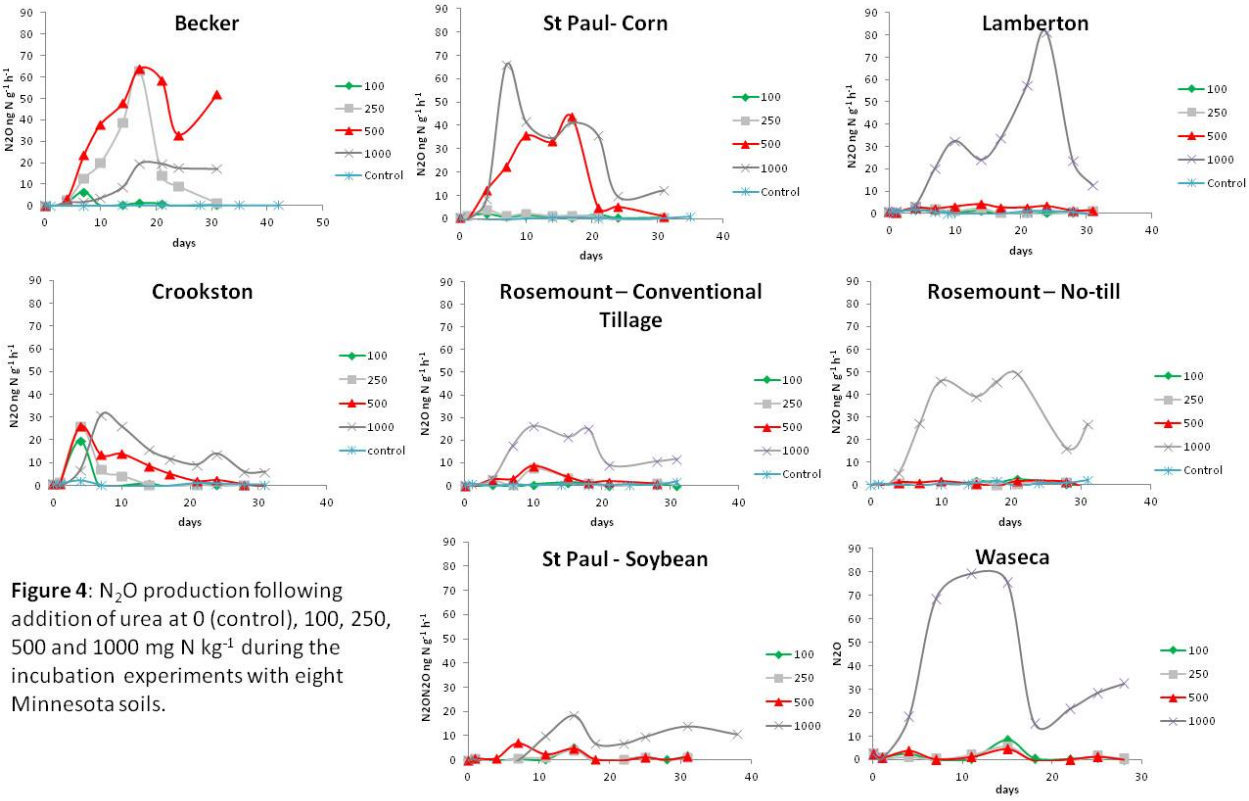

Figure 4: N_2O production following addition of urea at 0 (control), 100, 250, 500 and 1000 mg N kg^{-1} during the incubation experiments with eight Minnesota soils.

The hydrolysis of urea into ammonium leads to soil pH increase. Figure 5 shows that for the majority of soils, urea amendment leads to a pH increase that is proportional to the amount of urea added. However, the pH variation is very subtle in Crookston soil. Nitrification then leads to acidification of the environment due to the release of free hydrogen. For the majority of soils, pH is found to slowly decrease over time. Again, Crookston shows a very stable pH for all urea treatments.

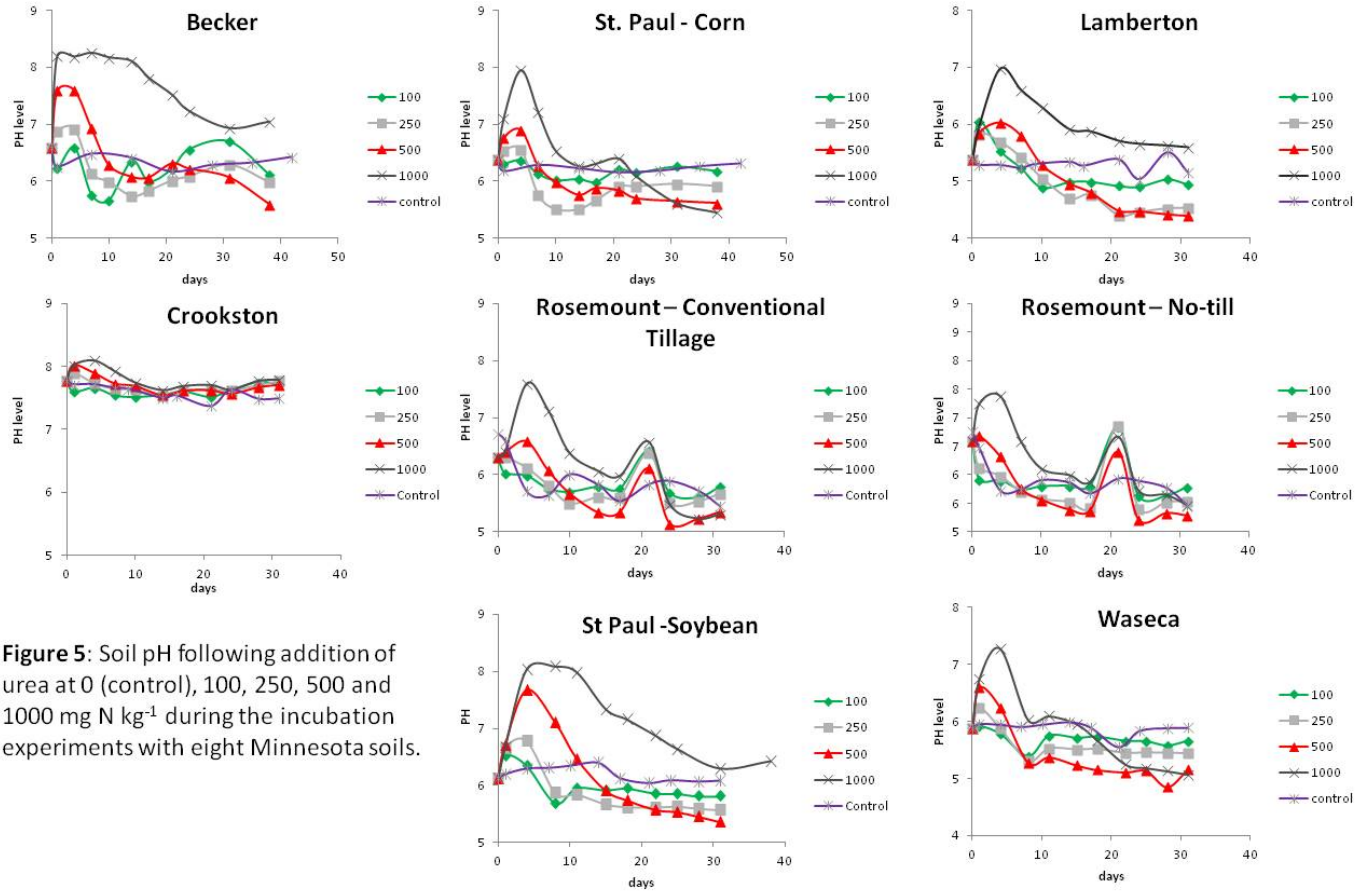

Figure 5: Soil pH following addition of urea at 0 (control), 100, 250, 500 and 1000 mg N kg^{-1} during the incubation experiments with eight Minnesota soils.

Several genes involved in the nitrification processes were tested on Lamberton and Crookston soils (see Figure 6). The normalized gene copy number of AOB and NXRA are different for the two tested soils and correlate well with the observed ammonium levels, nitrite production and nitrate accumulation.

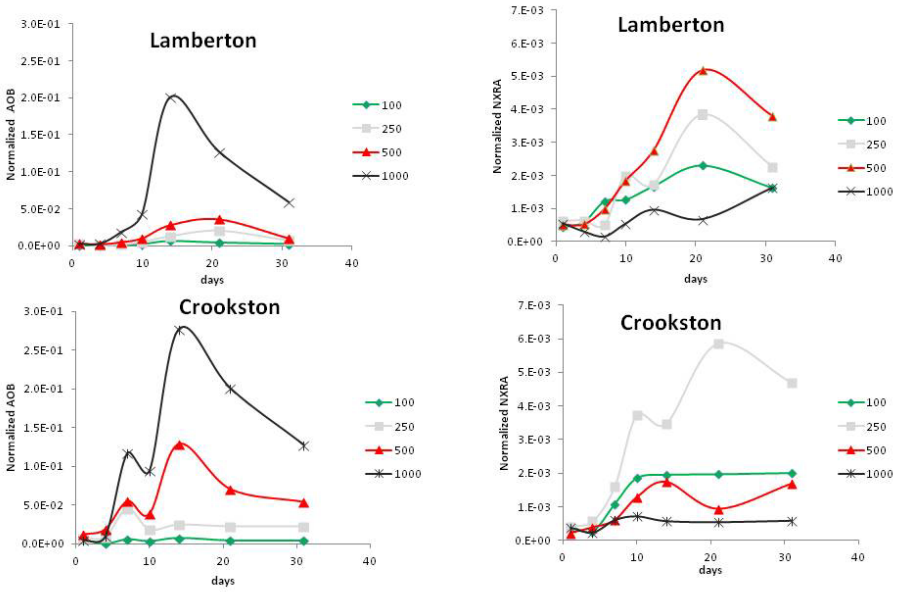

Figure 6: Gene copy abundances of *amo*A-b and *nxr*A following addition of urea at 100, 250, 500 and 1000 mg N kg^{-1} Lamberton and Crookston soils.

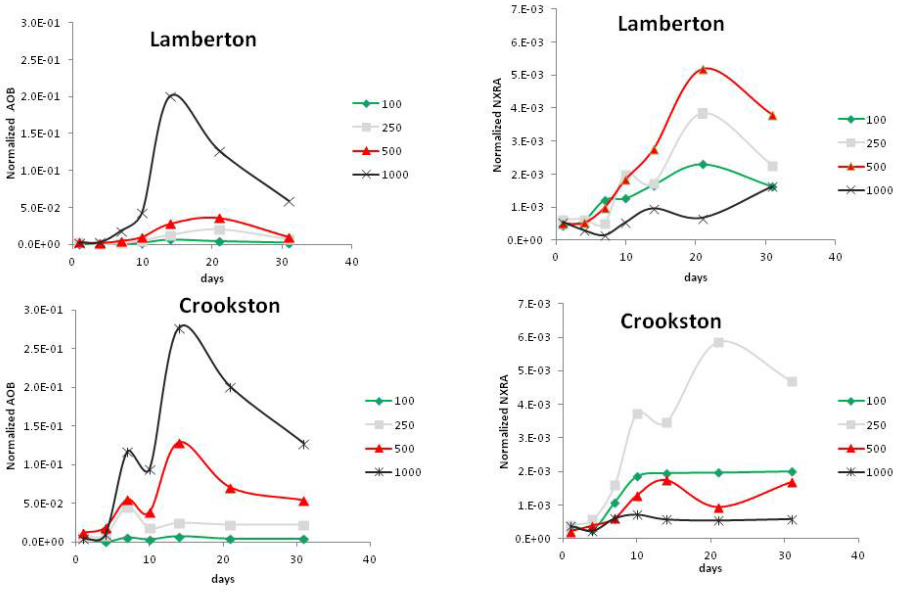

Figure 6: Gene copy abundances of *amo*A-b and *nxr*A following addition of urea at 100, 250, 500 and 1000 mg N kg^{-1} Lamberton and Crookston soils.

Q1 2015 Progress (Jan 1 – Mar 31, 2015)

1-Primer design and testing

Several set of primers including prokaryotic 16S RNA, the archaea ammonium oxidase, the bacteria ammonium oxidase and the *Nitrobacter* nitrite oxidase were successfully tested on Minnesota soils. Because the nitrite oxidizing bacteria are composed of multiple genera, we have decided to test an additional set of primers able to amplify the *Nitrospira* nitrite oxidase. Procedures for analysis of all 5 genes are now established and being used for qPCR analysis (see item 4 below).

2-Incubation experiments

As previously described, eight different soils have been sampled from the University of Minnesota field research fields in St. Paul (2 locations), Rosemount (2 locations), Becker, Waseca, Lamberton, and Crookston. Incubation experiments (10 time points) consisting of 5 urea treatments (0, 100, 250, 500 and 1000) were successfully performed on these soils. The last incubation experiment ended April 16, 2015.

3-Soil analyses

In parallel with the incubation experiments, other basic soils characterization analyses have been completed on these 8 soils including, Carbon and Nitrogen content, particle size analysis, pH, ammonium sorption capacity, and cation exchange capacity. We are also planning to determined organic matter content using the Loss on Ignition procedure.

4-DNA extraction and qPCR analysis

DNA was successfully extracted for 6 of the incubation experiments and the last two sets of DNA extraction are currently in progress. The quantitative PCRs of 5 different genes for 10 time points, and for each incubation, are presently being analyzed.

Q2 2015 Progress (Apr 1 – Jun 30, 2015)

1-Technician.

We hired a new technician, Nancy Rolstad; her starting date was July13 2015. She will partially assist in finishing the DNA extraction and dilution and participate in the following nitrosation experiments.

2-Soil analyses.

In parallel to the incubation experiments, other analyses were performed on the 8 Minnesota soils, including: particle size analysis, carbon and nitrogen content, cation exchange capacity and ammonium sorption capacity.

4-DNA extraction and qPCR analysis.

The DNA extractions and qPCR analyses for the lab experiment with Crookston, Lamberton, Rosemount (tilled and untilled), and Becker soils have been completed. DNA extractions for the experiments with Waseca and St. Paul-soybean soils are currently underway and should be finished by the end of July 2015, and the corresponding qPCR analyses should be finished by August 28, 2015. The DNA extractions for the St. Paul-corn soil are completed and the corresponding qPCRs are started and should be completed by Aug 7, 2015.

5-Field experiment.

A small plot experiment was started in a field on the St Paul campus. The objectives of this experiment are to 1) quantify the same chemical and biochemical dynamics being measured in the lab experiments and also examine the effects of rhizosphere and corn crop development phase. The experiment consists of two urea treatments (0 or 200 kg N/ha) manually added to a corn planted area and non-planted area. At several time points, soil sampling is being performed in the non-planted and planted areas, including soil from the rhizosphere and non- rhizosphere areas with measurement of soil moisture, pH, NH4 and total NO2-NO3, DNA/qPCRs as in the lab experiments.

Q3 2015 Progress (Jul 1 - Sep 30, 2015)

1. **Technician.**

We hired a new technician, Nancy Rolstad, who started on July13, 2015. She finished the last incubation experiment and finished all the DNA extraction and dilution. Unfortunately, she found a permanent position and left the project at the beginning of October. We are deciding whether or not to hire a new technician.

2. **Soil analyses.**

The particle size analysis, Carbon and Nitrogen content, Ammonium sorption and an estimation of soil organic carbon were determined for all soils.

3. **DNA extraction and qPCR analyses.**

The qPCRs for Crookston, Lamberton, Rosemount (two soils, tilled and no-till), St Paul (two soils, corn and soybean) and Becker were successfully completed. We also decided to repeat one of the incubation experiments using the St Paul-Corn soil, in order to test the effect of aeration technique and soil storage time, during the month of August (control and four urea treatments). DNA was extracted and diluted and qPCR are currently running.

4. **Field experiment.**

A small-plot experiment was started in research plots on the St Paul campus. The experiment consisted of four treatments, no urea or urea (200 kg N/ha) added to both corn-planted area and non-planted areas. At several time points (once every week for two months), soil samples were collected from the rhizosphere and non-rhizosphere areas to coincide with different corn development phases (V18, VT, R1 and R5). For all samples, soil moisture, pH and NH_4 and total NO_2-NO_3 were determined. Soil sub-samples are also stored in -80C for future DNA extractions and qPCRs on nitrification-associated genes. The goals of this experiment are to 1) observe the nitrification process after urea amendment in the field, and 2) examine the effect of corn rhizosphere on nitrification and corresponding gene abundances.

5. **Effects of long-term tillage and rotation management on gene copies of ammonium-oxidizing bacteria and archaea**

Soils from different depth intervals (0-5, 5-10, 10-20, 20-30 and 30-45cm) were collected from replicated plots at the University of Minnesota Research and Outreach Center in Rosemount. These plots have had different tillage practices (conventional and no tillage) and different crop systems (continuous corn or corn/soybean rotation) imposed for nearly 25 years (since 1997). Soils from adjacent, non-cultivated (wooded) areas were also sampled. Soil pH, carbon and nitrogen content were assessed and DNA was extracted to evaluate the distribution of the nitrifying (bacteria and archaea) populations in function of the soil depths. The primers used to quantify bacterial AOB in the previous soils tested were found to be non-specific in the forest samples. Thus, another pair of primers was identified in the literature from denaturating gradient gel electrophoresis experiments. This pair of primers is only specific to the β –subclass of AOB proteobacteria. The second δ-subclass of AOB proteobacteria is only found currently in marine environments. First, the primers were modified for standard PCR and tested on several cultivated and non-cultivated soils. Then, the pair of primers was tested on qPCR and melt curves analysis which showed the presence of only one amplicon. The DNA sequence for the standards is in process to be synthesized (gBlocks Gene Fragments, IDTdna).

6. **Data analysis.**

The extensive data set generated by the completed incubation experiments was analyzed. Clear differences have been observed in the patterns of nitrite accumulation and dynamics of nitrifier gene copy abundances among the different soil types. We continue to analyze these results to determine if these differences can be explained by any of the measured soil properties.

Q4 2015 Progress (Oct 1 – Dec 31, 2015)

1-Completion of analyses

The qPCR analyses for Crookston, Lamberton, Rosemounts (2 soils), St Paul (2 soils Corn and soybean cultivated) and Becker were successfully finished for the 4 different genes (prokariotic 16SRNA, Bacterial *amoA*, Nitrobacter *nxrA* and nitrospira *nxrB*). Data from the incubation and mineralization experiments, particle size analysis, C and N content, pH, moisture and bulk density were compiled for further statistical analysis. We have met to discuss the types of statistic analyses to perform.

2-Nitrite recovery experiments

For all eight soils, nitrite recovery experiments were performed. Soils were subjected to 4 different amount of KNO_2 addition. After 2 hours of incubation, N_2O production rate and extractable NH_4, NO_2 and NO_3 were determined. Linear regression analysis between N_2O production and NO_2 concentration were done and compared to the data obtained during the incubation experiments.

3-**Rosemount experiment**

DNA was extracted to evaluate the distribution of the nitrifying (Bacteria and Archea) populations as a function of soil depth. The use of the normal bacterial AOB primers in PCR resulted in the appearance of two bands. We extracted and cloned the two bands for sequencing. The results of the sequencing revealed that the second band was non-specific; therefore, this pair of primers could not be used with our type of soil. Another pair of primers, targeting the 16sRNA of the ammonia-oxidizing bacteria was found through the literature and tested on the different Rosemount soils. The PCR results were encouraging, even for the problematic soils, so we decided to use this new pair of primers for this experiment. All QPCRs were finished for the 3 genes: Procaryotic 16sRNA, Archeal amoA and Ammonia-Oxidizing Bacteria 16s RNA and data are currently being analyzed.