ADVANCING SOIL HEALTH: LINKING BELOWGROUND MICROBIAL PROCESSES TO ABOVEGROUND LAND MANAGEMENT

A Dissertation
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

Heather Lynn Dose

In Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

Major Department:
Soil Science

March 2016

Fargo, North Dakota

North Dakota State University
Graduate School

Title

ADVANCING SOIL HEALTH: LINKING BELOWGROUND MICROBIAL PROCESSES TO ABOVEGROUND LAND MANAGEMENT

By

Heather Lynn Dose

The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

SUPERVISORY COMMITTEE:

Thomas DeSutter
Chair

Francis Casey

Jack Norland

Robert Brueggeman

Approved:

04/05/2016
Date

Francis Casey
Department Chair

ABSTRACT

Advancing soil health lies at the intersection of belowground microbial processes and aboveground land management. However, linking microbial processes to land use is difficult. Understanding the response of soil microbes to management factors will provide agricultural producers and land managers with information regarding best management practices that not only improve soil health, but also maximize profitability. This study advances the understanding of microbial responses to land management by measuring microbial response to a) amendment application and tile drainage to remediate sodic soils, b) cover crop growth and tile drainage to ameliorate saline soils and c) tillage and fertilizer management factors on *Bradyrhizobium japonicum*, a symbiotic bacteria needed for biological N fixation in soybeans (*Glycine max* L.). Multiple time point measurements of soil microbial enzymes and functional gene copy numbers from three field experiments were used to determine microbial responses to land management. Key findings indicate that gypsum amendment applications, although effective at reducing sodicity, reduce soil enzyme activity levels in the short-term while tile drainage has no effect on microbial response to sodic soils. This work also demonstrates that the quantity of nitrifiers and denitrifiers can be used as either short–term or long–term indicators of soil health which reflect overall ecosystem health in sodic soils. In contrast to sodic soils, nitrifiers and denitrifiers are not useful indicators of soil health in saline soils as these microbial communities exhibit salinity induced community tolerance. Finally, tillage system and N availability have the greatest effect on *B. japonicum* numbers and activity in the soil. The lower amount of N obtained from biological N fixation in soybeans grown under no till systems reflects the reduced reliance on *B. japonicum* for plant N needs. Inoculated populations of *B. japonicum* are important for early season biological N fixation, but persistent and diverse populations of *B. japonicum* play an

important role in late season N fixation in the lateral soybean root nodules. Overall, this work demonstrates that monitoring soil microbial activity can be useful for producers and land managers looking to improve soil health.

ACKNOWLEDGEMENTS

Numerous individuals have contributed and supported me during the completion of this dissertation. Dr. Tom DeSutter graciously mentored me through the many obstacles I faced. His guidance and support through this journey ensured my success. I would like to thank my dissertation committee, Dr. Frank Casey, Dr. Jack Norland, and Dr. Robert Brueggeman for their assistance as I progressed through my studies. In addition, Dr. Ann-Marie Fortuna for her guidance on laboratory methods and Dr. Larry Cihacek for his additional support.

During data collection and laboratory analysis Joel Bell and Brandon Dahl provided countless hours of entertainment and encouragement. The dedication of Nate Derby and Kevin Horsager in managing the field research plots certainly did not go unnoticed. David Burkland and Steve McLean were kind to agree to hosting research on their farms.

I would also like to thank my family, friends and "group" for their constant encouragement and support. Last, but not least, I would like to thank the staff and faculty in the Department of Soil Science for providing me with an opportunity and environment to thrive.

DEDICATION

To Mark, my constant support and fountain of laughter, and to my parents for always encouraging me to do my best.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A.....Adenine

AOA.....Ammonia Oxidizing Archaea

AOB.....Ammonia Oxidizing Bacteria

BNF.....Biological Nitrogen Fixation

C.....Carbon

C.....Cytosine

Ca.....Calcium

cDNA.....DNA copy synthesized from mRNA

Cl^-.....Chlorine

Ct.....Threshold Cycle

DNA.....Deoxyribonucleaic Acid

ESP.....Exchangeable Sodium Percentage

EC.....Electrical Conductivity

ECe.....Electrical Conductivity of the Saturation Extract

ESP.....Exchangeable Sodium Percentage

FA.....Factorial Analysis

G.....Guanine

gDNA.....Genomic DNA

IC.....Inorganic Carbon

K.....Guanine or Thymine

M.....Adenine or Cytosine

MDS.....Minimum Data Set

Mg^{2+}.....Magnesium

Mo.....Molybdenum

MPN.....Most Probable Number

MPRR .....Multiresponse Permutation Procedure

mRNA.....Messenger Ribonucleic Acid

N.....Nitrogen

Na^+ .....Sodium

NGS.....Next Generation Sequencing

NMS.....Nonmetric Multidimensional Scaling

P .....Phosphorus

PCA.....Principal Component Analysis

perMANOVA .....Permutation Multivariate Analysis of Variance

pHe.....pH of the Saturation Extract

PMN.....Potentially Mineralizable Nitrogen

qPCR.....quantitative Polymerase Chain Reaction

R.....Adenine or Guanine

RT-PCR.....Reverse Transcription Polymerase Chain Reaction

S .....Cytosine or Guanine

S .....Sulfur

SAR.....Sodium Adsorption Ratio

SARe.....Sodium Adsorption Ratio of the Saturation Extract

SICT.....Salinity Induced Community Tolerance

T .....Thymine

TC .....Total Carbon

V.....Adenine, Cytosine or Guanine

W.....Adenine or Thymine

Y.....Cytosine or Thymine

LIST OF APPENDIX TABLES

LIST OF APPENDIX FIGURES

GENERAL INTRODUCTION

Soil health is defined as "the capacity of soil to function within ecosystems and land-use boundaries to sustain biological productivity, maintain environmental quality, and promote plant, animal and human health" (Doran and Parkin, 1994). Two of the major functions of soil is to 1) support biodiversity and habitat for soil organisms and 2) supply nutrients to plants by recycling organic compounds. In one teaspoon of soil, there are more microbes than there are people on Earth. The term soil microbe includes bacteria, archaea, fungi, actinomycetes, algae, protozoa and nematodes. Soil microorganisms are important and provide many ecological benefits and processes to the soil such as stabilizing aggregates, improving water holding capacity, and making plant nutrients available through fixation or decomposition. By focusing on the N cycle as one of soils' main functions, scientists can connect aboveground management to belowground microbially mediated processes. Nitrogen is needed in large quantities for maximizing plant yields, but N is typically limited in the soil. Specific groups of microorganisms are responsible for converting N between its various chemical forms through the secretion of enzymes. These enzymes speed up the decomposition rate and make plant nutrients available. There are many types of enzymes, and each enzyme is specific to a substrate. Additionally, each enzyme is controlled by a set of genes. There are several important groups of organisms in the N cycle including nitrifiers, denitrifiers, and N fixing bacteria (Fig. 1). The nitrifiers convert ammonia to nitrate through a two-step process and encompass a group of ammonia oxidizing bacteria and archaea that have the *Amo* genes which encodes for the production of ammonia monooxygenase-the enzyme that converts ammonia to hydroxylamine, which is further converted to nitrite. Another important N transformation is denitrification. Denitrifying bacteria convert nitrate to elemental N through a multi-step process involving several enzymes and genes as shown in

Figure 1. Finally, *Rhizobium*, or N fixing bacteria convert atmospheric N to ammonia either through a symbiotic relationship with legumes or as free-living bacteria. *Rhizobium* fix N by producing the nitrogenase enzyme which is encoded by the *nif* genes.

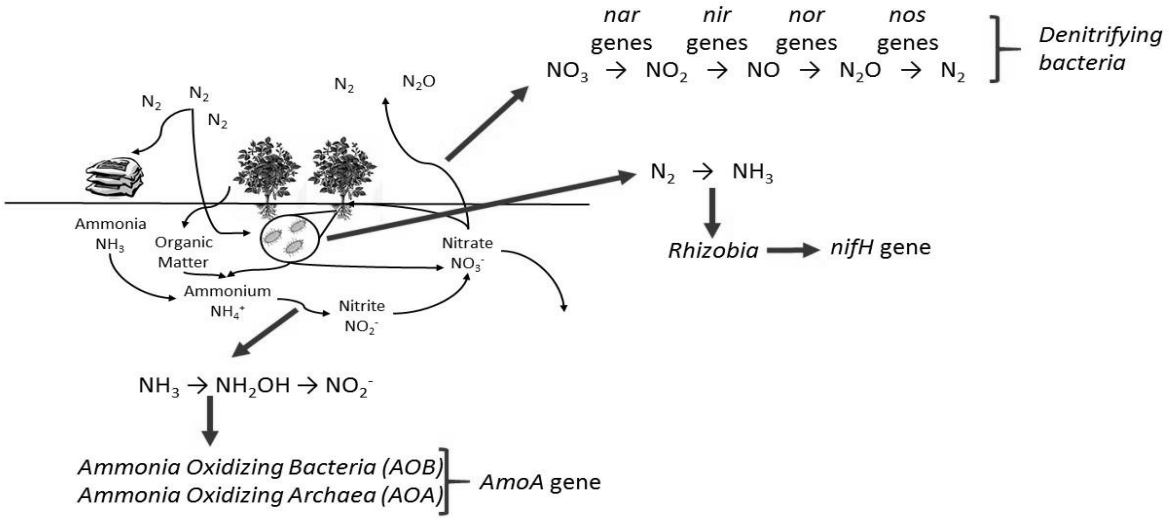

Figure 1. Nitrogen in the soil is cycled through various chemical forms as a result of genetically regulated enzymes produced by microorganisms.

The quantity, activity and diversity of these N transforming organisms can be assessed through both enzyme assays and by molecular approaches which target genes. These measurements can be linked to soil chemical and physical properties and aboveground management.

This dissertation is organized into a literature review and three research chapters. Each research chapter is written in paper publication format with the intent to publish in peer-reviewed journals.

References

Doran, J.W. and Parkin, T. B. 1994. Defining and assessing soil quality. In: Defining soil quality for a sustainable environment. In: Doran, J.W., D.C. Coleman, D.F. Bezdicek, B.A. Stewart (eds.) Defining soil quality for a sustainable environment. Soil Science Society of America special publication. Madison, WI, USA.

LITERATURE REVIEW

Soil health: linking aboveground land management to belowground microbial processes

Linking microbial processes to soil health and land management is a vital question in soil science and is necessary to provide agricultural producers with information regarding best management practices. Soil health is defined as "the capacity of soil to function within ecosystems and land-use boundaries to sustain biological productivity, maintain environmental quality, and promote plant, animal and human health" (Doran and Parkin, 1994). As such, soil health should "maximize the benefits of natural cycles, reduce dependence on non-renewable resources, and help producers identify long-term goals for sustainability that also meet short-term needs for production" (Doran and Zeiss, 2000). Soil health is measured through the use of indicators which link complex soil property relationships to land use and management. Soil health indicators using physical, chemical and biological properties have been outlined by the United States Department of Agriculture-Natural Resources Conservation Service (USDA, 2014) and agreed to globally by the Food and Agriculture Organization of the United Nations (FAO, 2014). Soil health indicators need to be sensitive to changes in management, but not so sensitive to in season fluctuations that preclude their use as indicators. Indicators also need to be correlated to beneficial soil functions, be useful for explaining ecosystem processes, be useable and understandable to land managers, and finally easy and inexpensive to measure (Doran and Zeiss, 2000). Soil physical and chemical properties have been the focus of many soil health indices that have been created for specific soil series or land management strategies. However, biological indicators of soil health can be used as an effective means to monitor the impacts of changing land use, soil remediation strategies, and best management practices in soil conservation (Bezdicek et al., 1996). Table 1 contains suggested physical, chemical and

biological indicators as well as the percentage of studies indicating the measure is a preferred soil health indicator across 11 studies. Although soil physical and chemical measures have been traditionally used as soil health indicators, there is interest in incorporating biological soil health indicators (Pankhurst et al., 1997; de la Paz Jimenez et al., 2002; Gil-Sotres et al., 2005; Imaz et al., 2010; Lima et al., 2013) as changes in biological indicators can be detected in less time than traditional indicators such as soil organic matter (Paul, 1984; Rice et al., 1996). Across these 11 studies, several indicators show potential to be applied across a wider range of studies and physiographic areas and include physical measures of aggregate stability, porosity and hydraulic conductivity, and the biological measures of total organic C and potentially mineralizable N. Several enzyme activities such as acid phosphatase, acid phosphomonoestrase, acid-hydrolizable carbohydrates, dehydrogenase, arylsulfatase and β-glucosidase show potential to be incorporated into soil health measurements. Informing and educating land owners on the importance of biological indicators can increase producers' understanding and acceptance of using soil organisms to gauge management decisions. The ability to assess changes in soil health quickly can help limit land managers from investing in practices that may not improve soil health, or can give a positive indication of improvement during a management transition (Bezdicek et al., 1996).

Table 1. Several proposed physical, chemical and biological soil health indicators. A dash (-) indicates the measure was not a statistically significant indicator

Measure	Reference	Percent of studies referenced in which the measure was a statistically significant soil health indicator
Physical		
A horizon depth	Brejada et al., 2000	-
A horizon color	Brejada et al., 2000	-
Water properties		
Available water	Andrews et al., 2004; Lima et al., 2013	50%
Field capacity	Govaerts et al., 2006	-
Hydraulic conductivity	Shipper and Sparling, 2000; Imaz et al., 2010	50%
Infiltration rate	Govaerts et al., 2006	-
Macroporosity	Lima et al., 2013	-
Microporosity	Lima et al., 2013	-
Permanent wilting point	Govaerts et al., 2006	100%
Time to ponding	Govaerts et al., 2006	100%
Total porosity	Schipper and Sparling, 2000; Roussesau et al., 2012	100%
Susceptability to runoff	Govaerts et al., 2006	100%
Aggregation		
Aggregate distirbution	Govaerts et al., 2006	100%
Water stable aggregates	Andrews et al., 2004. Imaz et al., 2010; Brejada et al., 2000a; Brejada et al., 2000b; Lima et al., 2013	80%

Table 1. Several proposed physical, chemical and biological soil health indicators (continued).

Measure	Reference	Percent of studies referenced in which the measure was a statistically significant soil health indicator
Compaction		
Compaction	Govaerts et al., 2006	-
Penetration resistance	Imaz et al., 2010	100%
Other		
Texture	Schipper and Sparling, 2000; Brejada et al., 2000a; Brejada et al., 2000b; Imaz et al., 2010; Rousseau et al., 2012;	-
Chemical		
Nutrient status		
Al	Lima et al., 2013	-
Ca	Andrews et al., 2004; Lima et al., 2013	-
Cu	Rousseau et al., 2012; Lima et al. 2013	50%
Fe	Rousseau et al., 2012; Lima et al. 2013	-
K	Imaz et al., 2010; Rousseau et al., 2012; Lima et al., 2013	-
Mg	Andrews et al., 2004; Rousseau et al., 2012; Lima et al., 2013	-
Mn	Rousseau et al., 2012; Lima et al., 2013	50%
P	Schipper and Sparling, 2000; Brejada et al., 2000a; Brejada et al., 2000b; de la Paz Jimenez et al., 2002; Andrews et al., 2004; Govaerts et al., 2006; Imaz et al., 2010; Rousseau et al., 2012; Lima et al., 2013	22%
Zn	Rousseau et al., 2012; Lima et al., 2013	50%
Total N	Bolinder et al., 1999; Brejada et al., 2000a; Brejada et al., 2000b; de la Paz Jimenes et al., 2002; Govaerts et al., 2006; Imaz et al., 2010	16%

Table 1. Several proposed physical, chemical and biological soil health indicators (continued).

Indicator	Reference	Percent of studies referenced in which the measure was a statistically significant soil health indicator
Base saturation	Schipper and Sparling, 2000	100%
Sum of bases	Rousseau et al., 2012	100%
C:N	Imaz et al., 2010; Rousseau et al., 2012	-
$CaCO_3$	Imaz et al., 2010.	-
Cation-exchange capacity	Brejada et al., 2000a; Brejada et al., 2000b; Schipper and Sparling, 2000; Govaerts et al., 2006; Rousseau et al., 2012; Lima et al., 2013	-
EC	Andrews et al., 2004; Govaerts et al., 2006; Imaz et al., 2010	33%
Exchangeable acitity	Brejada et al., 2000a; Brejada et al., 2000b; Rosseau et al., 2012	-
Exchangeable bases	Brejada et al., 2000a; Brejada et al., 2000b	-
pH	Schipper and Sparling, 2000; Brejada et al., 2000a; Brejada et al., 2000a; de la Paz Jimenez, 2002; Andrews et al., 2004; Govaerts et al., 2006; Imaz et al., 2010; Rosseau et al., 2012; Lima et al., 2013	33%
Sodium adsorption ratio	Andrews et al., 2004	100%
Biological		
Enzyme activity		
Acid phosphatase	Lima et al., 2013	-
Acid phosphomonoestrase	de la Paz Jimenez et al., 2002	100%
Acid-hydrolizable carbohydrates	Bolinder et al., 1999	100%

Table 1. Several proposed physical, chemical and biological soil health indicators (continued).

Indicator	Reference	Percent of studies referenced in which the measure was a statistically significant soil health indicator
Alkaline phosphatase	Lima et al., 2013	-
Dehydrogenase activity	de la Paz Jimenes et al., 2002; Gil-Sotres et al., 2005	50%
Arylsulphatase	de la Paz Jimenez et al., 2002	100%
Urease	de la Paz Jimenes et al., 2002; Gil-Sotres et al., 2005	-
β-glucosidase activity	de la Paz et al., 2002; Gil-Sotres et al., 2005; Lima et al., 2013	33%
Fauna		
Arthropods	Rousseau et al., 2012	-
Earthworms	Rousseau et al., 2012; Lima et al., 2013	50%
Macrofauna	Rousseau et al., 2012	100%
Biological Functions		
Microbial biomass C	Bolinder et al., 1999; Shipper and Sparling, 2000; Brejada et al., 2000a; Brejada et al., 2000b; Andrews et al., 2004; Gil-Sotres et al., 2005; Govaerts et al., 2006;	43%
Potentially mineralizable C	Brejada et al., 2000a; Brejada et al., 2000b	-
Potentially mineralizable N	Brejada et al., 2000a; Brejada et al., 2000b; Schipper and Sparling, 2000; Andrews et al., 2004; Gil-Sotres et al., 2005; Imaz et al., 2010; Lima et al., 2013	43%
Respiration	Schipper and Sparling, 2000; de la Paz et al., 2002; S Imaz et al., 2010	33%
Organic matter measures		
Macro-organic matter C	Bolinder et al., 1999	100%

Table 1. Several proposed physical, chemical and biological soil health indicators (continued).

Indicator	Reference	Percent of studies referenced in which the measure was a statistically significant soil health indicator
Macro-orgainc N	Bolinder et al., 1999	100%
Total organic C	Bolinder et al., 1999; Brejada et al., 2000a; Brejada et al., 2000b; de la Paz Jimenes, 2002; Andrews et al., 2004; Govearts et al., 2006; Imaz et al., 2010; Lima et al., 2013	88%

Soil enzymes as potential biological soil health indicators

Several indicators of biological soil health have been suggested (Pankhurst et al., 1997) and employed to measure soil health such as phospholipid fatty acid profiles (Zelles, 1999; Kaur et al., 2005), microbial biomass C and respiration (Anderson, 2003). Soil enzymes and functional gene copy numbers that encode for enzyme production (Nannipieri et al., 2012) show promise as biological soil health indicators (Dick, 1994; Halvorson et al., 1996; Pankhurst et al., 1997; Bolinder et al., 1999; de la Paz Jimenes et al., 2002; Gil-Sotres et al., 2005; Lima et al., 2013; Bowles et al., 2014). There are currently over 15,000 published articles that link enzyme activity with soil properties and management with over 100 assay methods published (Dick and Burns, 2011). The first enzyme detected in soils was the catalase enzyme in 1896 and currently much focus has been placed on nitrogen cycling enzymes (Dick and Burns, 2011). Soil enzymes catalyze important nutrient transformations within the soil and have received attention as biological soil health indicators because enzyme activity levels are related to organic matter, soil physical properties, microbial biomass and are key processes which link microbial communities to nutrient cycling (Sinsabaug and Moorhead, 1994; Schimel and Weintrab, 2003). Additionally, soil enzymes can be used to detect changes in soil health in as little as 1 to 2 years (Dick, 1994). For example across several studies the incorporation of manure and green manure into the soil increased average nitrogen cycling enzymes (amidase, urease, protease, histidase) by 55% and carbon cycling enzymes (α-galactosidase, α-glucosidase, β-galactosidase, β-glucosidase, invertase and dehydrogenase) by 79% compared to a control. Adding N alone only increases these enzymes by an average of 34% and 6%, respectively, with some studies showing a negative effect of added N fertilizer on N cycling enzymes (Fauci and Dick, 1994; Bandick and Dick, 1999; Kanchikerimath and Singh, 2001; Zhang et al., 2015). Additions of inorganic N have

a negative effect on amidase enzyme activity as amidase decreases at a constant rate of 7.34 µg NH_4 g^{-1} soil 4 hr^{-1} for every kg ha^{-1} yr^{-1} of added inorganic N while urease enzyme decreases at a rate of 3.94 µg NH_4 g^{-1} soil 4 hr^{-1} for every kg ha^{-1} yr^{-1} of added inorganic N (Dick et al., 1988).

Soil enzymes can exist in the soil in many forms, including intercellularily, either within the cytoplasm or periplasmic space. For example, the dehydrogenase enzyme is a class of enzymes involved in the oxidation of many organic molecules produced during cellular respiration and is only produced by living organisms (Skujiņš, 1967). However, many other enzymes can attached to outer surfaces of cells, in soil water, attached to cell debris, or adsorbed to clay minerals and humic colloids where they are stabilized (Burns, 1982). Similar enzymes catalyzing the same reaction may be produced by not only bacteria, but also fungi and plants in the soil. For example, sulfur cycling enzymes are produced by microorganisms, plant roots and soil fauna (Klose et al., 2006), phosphatase enzymes are produced from soil microorganisms, plants, and also fungi (Acosta-Martínez and Tabatabai, 2010) while the ammonia monooxygenase enzyme is only produced by a class of bacteria (*Nitrosomonas, Nitrosospira* and *Nitrosovibrio*) and archaea (Kandeler et al., 2010), and nitrate reductase is produced by 10-50% of the total identified bacteria (Philippot, 2005).

Soil enzyme assays are easily adaptable to standard laboratory equipment. In the laboratory, soil enzyme assays are run at a fixed pH, with known substrate concentrations, and a constant incubation temperature. In standardizing the conditions, comparisons of different maximum potentials of enzyme activity can easily be made (Dick, 2011). Due to the large diversity of enzymes and their locations in the soil, enzymes have been extensively used as soil health indicators. In addition, measuring a suite of soil enzyme activity levels involved in either multiple nutrient cycles or multiple classes of enzymes involved in the decomposition of organic

matter are preferred soil health indicators over using one or two enzyme assays. Not only do soil enzymes measure the potential activity of soil organisms (Caldwell, 2005; Nannipieri et al, 2012), but they can also be used to elucidate soil microbial diversity resulting from substrate specificity and nutrient resources (Caldwell, 2005).

Despite the popularity of using enzymes as soil health indicators, soil enzyme activity levels can be impacted by factors such as temperature, pH, and soil water content because these factors can change the population levels of organisms producing enzymes, or change the conformation and effectiveness of stabilized enzymes (Henry et al., 2005; Allison and Treseder, 2008; Niemi and Vepsalainen, 2005). For example, the activity of phosphodiesterase in the soil can double when lime is applied to a low pH soil and the pH is raised (Acosta-Martínez and Tabatabai, 2001; Ekenler and Tabatabai, 2003). Soil enzyme activities have been shown to have a seasonal fluctuation with activity levels being highest in the spring and fall and decreasing in the summer and winter due to moisture and temperature limitations (Burns, 1978). The spatial variability of enzymes must also be considered. Some enzymes, such as dehydrogenase, urease and β-glucosidase do not have a spatial distribution component, but phosphatase activity is dependent on the distribution of C and P in the soil (Bergstrom et al. 1998). Additionally, sample storage and analysis of field-moist frozen or air-dried soils results in differences in observed enzyme activities. For example soils frozen for 1 week had 109, 108, 113 and 99% of the values of fresh soil for arylsulfatase, α-glucosidase, β-glucosidase, and chitenase, respectively, while soil allowed to air-dry for 1 week resulted in 159, 107, 94 and 83% for the same enzymes, respectively (Wallenius et al., 2010). Measuring soil enzyme activity using laboratory incubations which controll temperature, contain excess substrate and are buffered at an optimum pH can be used to standardize the conditions in which the enzyme activities are measured. Due

to potential limitations of applying soil enzyme activities as sole biological indicators of soil health, the integration of functional gene copy numbers into soil health indices has been recommended (Nannipieri et al., 2012).

Functional gene copy numbers as potential biological soil health indicators

Functional gene copy numbers are a reflection of the number of organisms capable of producing an enzyme. Unlike soil enzyme assays which are run under controlled laboratory conditions, gene copy numbers give a snapshot of the *in situ* soil microbial community assemblage at the time of sampling. Gene copies are assessed through quantitative Polymerase Chain Reaction (qPCR) using primers specified for the targeted gene. Using primers that are specific to a target gene, the template DNA is denatured into one strand and the primers begin to bond complimentary strands of the target region followed by an extension of bases to form double stranded DNA in the length of the primer. For example, there are over 33 primers documented for amplification of the *amoA* gene, which regulates nitrification and the production of ammonia monooxygenase (Junier et al., 2008), while there are 86 primer pairs designed to target the nitrogenase *nifH* gene in nitrogen fixation (Gaby and Buckley, 2012). A reaction containing specified primers and genomic DNA from a known organism to have the target gene is used to create a standard curve of known copy numbers can then be used to determine the number of copies in an unknown sample.

Although functional gene copy numbers require specialized equipment, trained personnel, and specialized reagents, these molecular techniques have become easier and less expensive. Rapid changes in copy numbers can occur as organisms respond and reproduce quickly to nutrient availability changes. Gene copy numbers can be used as either short–term or long–term indicators of soil health which reflect overall ecosystem health.

One example of using gene copy numbers proposed by Wessén and Hallin (2011) is the ratio of ammonia oxidizing archaea (AOA) to ammonia oxidizing bacteria (AOB) (AOA:AOB), which can be related to soil properties and management. Many studies indicate that AOA and AOB communities occupy overlapping niches but their growth and activity vary from one another with changes in N concentration, edaphic properties, ecosystem, and changes in land management (Di et al., 2010; Di et al., 2009; Fortuna et al, 2012; Taylor et al., 2010; Wessén et al., 2010). Ammonia oxidizing bacteria proliferate under high NH_4^+ additions (200-400 µg N g^{-1} soil) (Jia and Conrad, 2009; Verhamme et al., 2011), whereas AOA populations increase under low fertilization (<20 µg N g^{-1} soil), where the NH_4^+ is supplied via organic matter (Zhang et al., 2010; Verhamme et al., 2011). In agricultural soils, AOA:AOB ratios were 2.8 where barley (*Hordeum vulgare*) was planted, 232 in non-fertilized soils, 78 in mineral fertilized soils, 147 in soils receiving mineral and organic fertilizers, 70 in no-till soils and 93 in plowed soils (Leininger et al., 2006). In contrast, AOA was found to be more numerous than AOB under the fallow portion of a crop-fallow system (~2.2 $x10^8$ AOA AmoA gene copies g^{-1} soil and 2.0 $x10^6$ AOB *amoA* gene copies g^{-1} soil, respectively) where inorganic fertilizer inputs are low (Taylor et al., 2010). However, during the crop phase of the rotation, AOB were more numerous following fertilization (~$9x10^7$ and ~$5x10^7$ *amoA* gene copies, respectively). A 23-fold increase in AOB was observed where urea was applied on an acid soil where accumulation of nitrate occurs (Hai et al., 2009). In contrast, the application of urea fertilizer can have either little effect or detrimental effects on populations of AOA that are favored in oligotrophic environments low in ammonium concentrations (Hai et al., 2009; Martens-Habbena et al., 2009; Schleper, 2010; Fortuna et al., 2012; Sims et al., 2012). However, across agronomic treatments AOA tends to dominate over AOB with a general range between $7x10^6$ and $1x10^8$ copies g^{-1} soil (Leininger et

al., 2006), as AOA have two to three nearly identical copies of the *amoA* gene in their genome as compared to one *amoA* gene copy in AOB (Norton et al., 2002; Hatzenpichler, 2012). Greater copy numbers of AOB are measured at mid pH ranges while AOA exist in a wide pH range and outnumber AOB in acid conditions (He et al., 2007; Prosser and Nicol, 2012). There is evidence that AOB are negatively correlated with increasing salt concentrations in sediments (Li et al., 2011) and that AOA copy numbers are constant across a wide salt gradient in water systems ranging from rice patties, estuaries and open oceans (Erguder et al., 2009).

Although gene copy numbers cannot always be related back to enzyme levels, copy numbers are valuable as an indicator of the size and activity of the microbial population and are often more sensitive than enzyme assays. While copy numbers of extracted DNA represent the total community present *in situ* during sampling, analyzing the mRNA, which is actively produced during transcription, is a valuable tool as mRNA corresponds to actual *in situ* activity of organisms which respond quickly to changes in substrate availability. As substrates' availability declines, the production of mRNA decreases, leaving an *in situ* snapshot of the microbial community at the time of sampling which can be related to soil health. For example, Bollmann et al., (2005) cultured the AOB *Nitrosospira breiensis* under starvation, and when NH_4^+ was added to the cultures 100% activity in mRNA *amoA* genes was observed.

In conjunction with gene copy numbers, next generation sequencing (NGS) using gene specific primers can be used to identify the microbial strains and community composition of a soil sample. Applying NGS to soil samples taken from various managements and land use, Roesch et al. (2007) concluded that agricultural soils are species rich, but phylum poor and agricultural management can influence the bacterial and archaeal diversity. Moreover, sequence information such as species, diversity and abundance obtained from microorganisms in the soil

can be linked to abiotic and biotic factors (Roh et al., 2010) in which changes in soil health can be linked to land management.

Salt affected soils

Nearly 40% of agricultural land throughout the world has been degraded through soil erosion, over-grazing, land clearing, salinization and desertification (Oldeman, 1994). However, improving soil health can reduce, and in some cases reverse, the impacts of human induced land degradation. Biological activity, including enzyme activity levels and functional gene copy numbers as metrics for soil health, may be even more relevant in marginal salt affected soils where inherent edaphic properties may be constraining soil biological activity, nutrient cycling, and overall soil health.

Worldwide, salt affected soils encompass roughly 10% of Earth's land surface (Pessarkli and Szabolcs, 1999). Salt affected soils can be classified as sodic, saline, or saline-sodic, with each having unique chemical and physical characteristics. Sodic soils have an excess of exchangeable Na^+ ions that severely degrade soil structure (He et al., 2015; Rengasamy and Olsson, 1991) and reduce plant growth and nutrition and affect 955 x 10^6 ha (Szobolcs, 1994). As such, sodic soils are a poor rooting medium for plant growth.

Upon wetting, the swelling 2:1 montmorillonitic clays in sodic soils swell as Na^+ in solution causes the interlayer space between clays to increase (van Olphen, 1963). Upon further wetting, clay minerals can disperse due to the repellency of the negative charges on clay surfaces due to the electric double layer (Tan, 1982). The double layer consists of two layers, the first layer is a swarm of only positive charges on the negatively charged surfaces of clays (Stern layer) as caused by isomorphous substitution of Al^{3+} for Si^{4+} in the silica tetrahedral on Mg^{2+} or Fe^{2+} for Al^{3+} in the octahedral coordination (Essington, 2004) while the second layer consists of

positively charged cations but in a concentration that decreases as distance increases from the Stern layer. The distance in which the concentration of cations is greater than that of the bulk soil solution makes up the double layer.

In the United States, sodic soils are classified as having a sodium adsorption ratio (SAR) (ratio of Na^+ to Ca^{2+} and Mg^{2+}) of 13 or greater, exchangeable Na^+ percentage (ESP) greater than 15, electrical conductivity (EC) of less than 4 dS m^{-1}, and pH of 8.5 or greater (Richards, 1954). When Na^+ is the dominant cation in the double layer and the electrical conductivity of the solution is low, the thickness of the double layer is large which causes clay particles to disperse when their double layers overlap, especially in the swelling 2:1 clays (Essington, 2004). However, the classification of sodic soils by the USDA-NCRS has been questioned as there is evidence that swelling and dispersion are affected by not only sodium content, but electrolyte content as well. Swelling and dispersion is more pronounced in montmorillonite clays (Sumner, 1993) and 50% of clay particles dispersed can occur at an electrical conductivity of 4 dS m^{-1} when the SAR is 24. However, the same level of clay dispersion can occur at <1 dS m^{-1} when the SAR is 1 (He et al., 2013). Also, the ratio of Ca:Mg for montmorillonite and kaolinite at the same SAR did not influence clay dispersion (He et al., 2013). The hydraulic conductivity of soils has been shown to decrease by 32% and 89% where the ESP is 10 and 30, respectively and leached with a 10 meq L^{-1} NaCl-$CaCl_2$ solution. However, these reductions in hydraulic conductivity were observed for a low clay content, montmorillinitic soil (3-18%) (Frenkel et al., 1978). It is likely that hydraulic conductivity would be impacted to a greater degree at the lower ESP content for a soil with greater clay content.

In sodic soils, organic matter can interact with and also cause clay particles to disperse by altering the linking between clay and organic matter (Churchman et al., 1993). In addition,

organic matter itself can disperse in the soil similar to clays as both contain negative surface charges, especially when leached with water of low electrolyte concentration. Organic matter also affects the tensile strength of aggregates in sodic soils. As the organic matter decreased, aggregate tensile strength also decreased, however the reduction in tensile strength was greatest for high organic matter soils and lowest for low organic matter soils (Rahimi et al., 2010). For instance, at high organic matter soils (10.32% organic carbon) where the EC was 0.5 dS m^{-1} and SAR was 0, the tensile strength was ~ 50 kPa for the same soil with an EC of 4 and SAR of 15, the tensile strength was reduced to ~38 kPa. At low organic matter soils (2.13% organic carbon) where the EC was 0.5 dS m^{-1} and SAR was 0 the tensile strength was ~33 kPa and the same soil at an EC of 4 and SAR of 15 had a tensile strength of ~29 kPa (Rahimi et al., 2000).

In addition to high Na^+ concentrations which reduce Ca^{2+} and Mg^{2+} availability (Bernstein, 1975), high pH of sodic soils affects the availability of many important plant nutrients (Abrol, 1988). The high soil pH increases ammonia volatilization losses, and decreases N availability by affecting the habitat of soil microorganisms (Haynes 1986). Additionally, the high pH of sodic soils can reduce Zn^{2+}, Cu^{2+}, Fe^{3+}, Fe^{2+} and Mn^{2+} , where Fe^{3+}, Fe^{2+} and Mn^{2+} are lowest in availability at pH 9 (Lindsay, 1979).

In contrast to sodic soils, saline soils have an excess of soluble salts in the soil profile (Abrol, 1988). In the US, saline soils are classified opposite of sodic soils and have an EC of greater than 4 dS m^{-1}, SAR of less than 13, ESP of less than 15, and a pH less than 8.5. These salts are typically Cl^- and SO_4^- or HCO_3^- salts as the anions and Na^+, Mg^{2+} and Ca^{2+} as the cations. Unlike with sodic soils, clays within saline soils are generally flocculated in saline soils and the hydraulic conductivity is much better. However, Na-Mg solutions average 5.1% less in saturated hydraulic conductivity than Na-Ca solutions at an SAR value near 0, and the relative

hydraulic conductivities remain between 90 and 110% between solution electrolyte concentrations of 50 to 800 meq L^{-1} (McNeal et al., 1986). Chloride and Na^+ toxicities in sensitive crops are common in saline soils where these salts can dominate (Bernstein et al. 1974). The ion flux of Cl^- and Na^+ were greatest in barley xylem sap (~500 and ~380 nmol m^{-2} s^{-1}, respectively) at an external NaCl concentration of ~75 mol m^{-3} (Munns and Termaat, 1986). Reduction of plants' osmotic potential can be observed within the leaves by measuring pre-dawn leaf water potential and stomatal conductance. Significant decreases in leaf water potential and stomatal conductance in sugar beets (*Beta vulgaris)*, tomato (*Solanum lycopersicum*), chickpea (*Cicer arietinum*) and lentils (*Lens culinaris*) were reported as the soil EC increased (Katerji et al., 2000). In order to retain the osmotic gradient, plant roots also need to increase internal salt concentrations which is done through either the uptake of additional solutes from the soil solution, or through the production of organic solutes within the plant itself (Chapman, 1960). Proline is an osmoregulator found in a wide variety of plants undergoing stress such as drought or salinity stress (Delauney and Verma, 1993). For example, the observed proline fold increase for several agronomically important crops ranges from 8 in alfalfa (*Medicago saltiva* L.) at NaCl concentration of 150 mM (Fougére et al., 1991) to 11 in soybeans (*Glycine max* L.) at NaCl concentration of 200 mM (Moftah and Michel, 1967). When the osmotic balance is not maintained in plant roots, plants can essentially suffer from drought and yield declines. Table 2 contains information at which common crop yield begins to decline as the soil EC surpasses the threshold EC. The threshold EC is used to determine the salinity tolerance rating class of several important crops.

Table 2. The salt tolerance of several important crops (adapted from Tanji and Kielen, 2002).

Common name	Scientific name	Threshold, ECe dS m^{-1}	Rating	References
Canola	*Brassica campestris* L.	9.7	Tolerant	Francois, 1994
Corn	*Zea mays* L.	1.7	Moderately sensitive	Bernsein and Ayars, 1949, Kaddah and Ghowail, 1964
Rye	*Secale cereale* L.	11.4	Tolerant	Francois et al., 1989
Sorghum	*Sorghum biocolor* L.	6.8	Moderately tolerant	Francois et al., 1984
Soybean	*Glycine max* L.	5	Moderately tolerant	Bernstein et al., 1955; Abel and McKenzie, 1964; Bernstein and Ogata, 1996
Sugar beet	*Beta vulgaris* L	7	Tolerant	Bower et al., 1954
Sunflower	*Helianthus annuus*L	4.8	Moderately tolerant	Cheng, 1983; Francois, 1996
Triticale	*X Triticosecale*	6.1	Tolerant	Francois et al., 1988
Wheat	*Triticum aestivum*	6	Moderately tolerant	Hayward and Uhvits, 1944; Ayers et al., 1952
Alfalfa	*Medicago sativa*	2	Moderately sensitive	Gauch and magistad, 1943; Brown and Hayward, 1956, Bernstein and Ogata, 1966; Bower et al., 1969; Bernstein and Francois, 1973; Hoffman et al., 1975
Barely (forage)	*Hordium vulgare*	6	Moderately tolerant	Drenge, 1962; Hassan et al., 1970

Plant toxicity of P can occur under saline soil conditions at levels that would not affect plant growth under non saline conditions (0.5-2 mM P) (Berstein et al., 1974). In saline soils, imbalances in Ca^{2+}, Na^{+}, and K^{+} can occur. Due to the higher solubility of Na salts, Na^{+} remains in solution and these soils are more prone to becoming sodic or saline-sodic.

Saline-sodic soils exhibit properties of both saline and sodic soils. In order for a soil to be classified as saline-sodic, the EC is greater than 4 dS m^{-1}, SAR less than 14, ESP less than15, and pH less than 8.5 and SAR greater than 13 (Richards, 1954). Saline-sodic soils can exhibit

reduced structural stability and reduced hydraulic conductivity and can affect nutrient availability and toxicity to plants in similar ways to saline and Na affected soils (Qadir and Oster, 2004).

Salt affected soils and microbial responses

In addition to reducing plant growth, salinity affects microbial distribution (Bernhard et al., 2007) and biologically mediated nutrient transformations (Frankenberger and Bingham, 1982; McClung and Frankenberger, 1985; Saleem and Ahmed, 1988). Frankenberger and Bingham (1982) documented declines in dehydrogenase, catalase, amidase, urease, acid and alkaline phosphatase, phosphodiesterase, inorganic pyrophosphate, arylsulfatase, rhodanese, α-galactosidase and α-glucosidase increases in soil salinity. In general, these authors found NaCl salts were most detrimental, followed by CaCl2 salts and Na2SO4 salts having a lesser effect on enzyme levels. For instance, dehydrogenase activity declined 30, 30 and 81% for at an ECe level of 20 to 22 dS m-1, respectively for Na2SO4, CaCl2 and NaCl salts. However, arylsulfatase activity remained relatively unaffected by increases in ECe, where at very slightly saline levels, arylsulfatase declined by 0.3% compared to an 11.2% decline at strongly saline levels. Soil salinity limits the biological steps of the N cycle by suppressing amidase and urease enzyme activities (Akhtar et al., 2012; Irshad et al., 2005; Laura, 1977) and reducing nitrification and denitrification (Gandhi and Paliwal, 1976; McClung and Frankenberger, 1985; Irshad et al., 2005; Akhtar et al., 2012). The proposed level whereby nitrification and denitrification is impacted by salinity is at an EC1:5 of 1.5 dS m-1 (ECe 7.8 dS m-1, He et al., 2013) (Zeng et al., 2006). However, these studies were conducted on non-saline soils in which varying levels of salts were added to the soil. The addition of salts in this manner can be a shock to the microbial

community and without sufficient time to adjust to the changing environmental conditions, microbial enzymes and processes are likely to be depressed.

Salinity, in addition to reducing microbial N transformations, can affect the diversity of soil organisms. For example, Canfora et al., (2014) found distinct patchiness and response of several bacterial phyla to salinity gradients across multiple sites. These authors also identified several bacterial phyla inhabiting extreme saline soil environments that had not been previously identified as being associated with saline soils (Nitrospira, Deferribacteres, Cyanobacteria/Chloroplast, Tenericutes and Spirochaete). The authors also stress the microbial community of saline soil environments is of interest for researchers who may find useful biotechnological applications relating to the restoration of these unique environments or to be studied as a model system for bacterial adaptation to selective environmental conditions. Microbial diversity in saline soil environments is understudied and it is estimated that less than 50% of the archaeal and 25% of bacterial species have been described in these environments (Ma and Gong, 2013). Reducing the effects of primary soil salinity therefore should have an impact on microbial function and structure as the soil solution changes in ion concentration.

Sodic soils also have reduced soil biological functioning due in part to the interaction of organic C materials that can be used as substrates in the soil. Differences in C mineralization have been observed for both saline and sodic soils. For example, when sodic soils were amended with organic matter, the CO2 respiration of a soil at pH 10 was similar to that of an amended soil at pH 8. In a saline soil, sesbania (*Sesbania cannabina*) green manure was added as an organic amendment which improved CO2 respiration when soil ECe < 26 dSm-1 (Rao and Pathak, 1996). The reduced C mineralization under saline conditions can be caused by decreases in enzyme activities such as dehydrogenase, α-galactosidase and α-glucosidase, as a result of salt

inhibition of microbial growth (Frankenberger and Bingham, 1982). Dispersion of clays, caused by sodicity, can also disperse organic matter and may be responsible for decreased enzyme activity in these soils as enzymes that are usually protected by organic matter can become degraded (Garcia-Gil et al., 2000). The dispersed organic matter can be available as a C substrate, hence increased C oxidation in sodic soils (Nelson et al., 1996). The total microbial biomass in sodic soils has decreased at a rate of 9.0 mg kg soil-1 for each unit increase in ESP (Rietz and Haynes, 2003). Overall, there is much less information available on the effects of sodicity on soil microbial properties.

Remediation strategies for sodic and saline soils

Remediating the effects of excess Na^+ in sodic soils can be accomplished with differing soil amendments and land managements. The goal of remediation of sodic soils is to replace the Na^+ on the soil exchange sites with Ca^{2+} followed by leaching of excess Na^+ below the rooting zone. Chemical application of Ca^{2+} amendments such as gypsum or lime have been shown to reduce the effects of sodicity (Keren and Shainberg, 1981; Ilyas et al., 1993; Ilyas et al., 1997; Amezketa et al., 2005). Calcium flocculates clay particles leading to improvements in soil structure (Frenkel et al., 1989), replaces Na^+ on soil exchange sites and reduces the thickness of the double layer and thus limiting like charge repellency of the clay particles which in turn improves hydraulic conductivity (Mahanta et al., 2014). For example, a study conducted on a silty clay loam soil in Pakistan showed a 25% improvement in the surface saturated hydraulic conductivity after 6 months where 25 Mg gypsum ha^{-1} was applied and a 150% increase in hydraulic conductivity 1 year after gypsum application. However, no improvement or a decrease in hydraulic conductivity was noted below 0.6 m soil depths (Ilyas et al., 1993) indicating that both rate of Ca applied and having adequate water for leaching are important. Calcium

amendments are also correlated to an increase in Na^+ in solution as the Na^+ is removed from the exchange sites (Ilyas et al., 1997). The solubility and mesh size of the applied gypsum amendment dictates the length of reclamation time. For example, industrial gypsum also known as phosphogypsum, is a byproduct of phosphate fertilizer has nearly a 10-fold increase dissolution coefficients in the 1.0-2.0 mm size range over mined gypsum, however, at larger sizes (4.0-5.7 mm), industrial gypsum has an 8 fold increase in its dissolution coefficient. As such, the industrial gypsum has been found to be more effective at improving soil infiltration rate than mined gypsum (Keren and Shainberg, 1981; Chawla and Abrol, 1982). Rates of gypsum application can be calculated using several methods that take into account the soils cation exchange capacity, target SAR and current SAR values to determine the amount of soluble Ca^{2+} needed to remove Na^+ from the soil exchange sites (Oster and Frenkel, 1980; Ashworth et al., 1999). In addition to Ca amendments, sulfuric acid amendments have been shown to be more effective at improving water infiltration of sodic soil as infiltration rate doubled where sulfuric acid was applied compared to gypsum amendments (Amezketa et al., 2005), likely due to the increase in soil solution EC, the dissolution of carbonates due to lower soil pH, or both. However, sulfuric acid is impractical for improving large areas of sodic soil due to cost, application and transportation. After chemical treatment, other land managements that leach soluble Na^+, such as subsurface tile drainage and or the addition of irrigation water need to be utilized to remove excess Na^+ from the rooting zone (Pessarakli and Szabolcs, 1999). Tile drainage also can prevent salt accumulation due to fluctuations in water table depth, capillary rise, and evaporation (Chang et al., 1991). Leaching of the Na^+ from the soil profile can occur by using water with successively lower EC as low electrolyte concentration of water can easily disperse soils with low ESP and SAR (Shainberg et al., 1981). Additionally, the availability and

rate of suitable water for leaching will impact infiltration, transmission, and gypsum solution which will impact the rate of reclamation (Shainberg et al., 1981) as gypsum dissolution is roughly 1.5 times greater in 0.1N NaCl than water (Keren and Shainberg, 1981).

Crop rotations and organic amendments have also been shown to be effective in remediating sodic soils. After 1 year following gypsum application (25 Mg ha^{-1}), saturated hydraulic conductivity was 170% and 130% greater when alfalfa (*Medicago sativa*) and a rotation of sesbania (*Sesbania grandiflora*)-wheat (*Triticum aestivum*)-sesbania was grown, respectively (Ilyas et al., 1993). Organic amendments and plant roots release acids that help dissolve Ca^{2+} compounds (Ca and Mg carbonates) commonly found in soils (Abrol et al., 1988), which may improve flocculation. Additions of organic amendments such as crop residues, mulch, manures, and composts have been shown to speed the rate of Na^{+} leaching in sodic soil leading to improved water infiltration, water holding capacity, aggregate stability (El-Shakweer et al., 1998), and reduced ESP (Tejada et al., 2006). Incorporation of 7.5 Mg ha^{-1} of wheat straw to sodic soils under alfalfa improved surface water infiltration rate by 30% after 6 months and by 11% after 1 year, while the same addition of wheat straw to a sesbania-wheat-sesbania rotation improved the surface water infiltration by 58% after 6 months and 13% after 1 year (Ilyas et al., 1993). Theoretically, organic amendments and subsequent microbial responses would lead to increases in soil atmospheric CO_2 concentrations whereby this CO_2 produced can interact with water to produce carbonic acid which lowers the soil pH and dissolves lime, increasing Ca^{2+} availability (Nakayama 1969; Robbins, 1985) and replacing Na^{+} on the exchange sites (Robbins, 1986). Hydrogen ions also produced from organic acids during the decomposition of organic amendments can have similar dissolution effects of lime materials. Applications of municipal solid waste and compost can increase the nutrient content of soil, specifically N, C, P, and Ca^{2+}

but may decrease K and Mg while increasing heavy metal concentrations (Ouni et al., 2013). However, municipal solid waste application in conjunction with gypsum was effective at lowering the SAR to 11.1 after 2 years while the control SAR was 111 (Avnimelech et al., 2004). Many amendments, including composts, may increase soil EC but soil microbes have been shown to be able to adapt to changes in osmotic stress (Sparling et al., 1989; Wichern et al., 2006).

The remediation strategies for saline soils is to leach excess salts from the rooting zone. Leaching of salts is accomplished through applications of water to the soil surface in excess of the evapotranspiration (water evaporation from soil surface + transpiration of water through plants) (Qadir et al., 2000). The quantity of water needed to remove salts from the rooting zone without increasing soil salinity is known as the leaching requirement (Richards, 1954). However, spatial variability of salt distribution within the soil complicates the calculation of the leaching requirement (Hillel, 2000). Soil texture is an important consideration when determining the amount of water to sufficiently leach soils. To remove roughly 80% of initial salts, the depth of leaching water per unit depth of silty loam soil would be roughly 0.5, whereas the depth of leaching water per unit depth of soil for a clay soil to remove the same amount of salt is about 1.5 (Dieleman, 1963). In addition to the quantity of water needed, water quality used for leaching needs to be carefully considered. The leaching of divalent cations may leave the soil saturated with monovalent Na^+ cations and leaching saline soils with water of low salinity can cause a dispersal of the soil particles and reduced infiltration. Reeve and Bower (1960) recommend leaching saline soils with decreasing EC to limit potential dispersal and physical decline of soil during. Application of a gypsum amendment can help limit soil dispersal (Hillel, 2000) as its dissolution increases EC. Finally, adequate drainage and removal of salts from the soil profile is

needed for successful remediation of saline soils (Qadir et al., 2000). Organic amendments have also been shown to improve saline soils. The application of 4.5 and 9.1 Mg ha^{-1} of crushed cotton gin compost and poultry manure not only reduced ESP to a range of 7.5-10.5 whereas the control soil had an ESP of 15.5, but these organic amendments increased the microbial biomass C anywhere from 400 to 900%, increased the β-glucosidase activity 45 to 100% and the alkaline phosphatase activity 50 to 120% (Tejada et al., 2006).

Importance of monitoring soil health on marginal soils in North Dakota

In the northern Great Plains, USA, the risk of salinization and sodification has drastically increased due to a 20+ year wet climactic cycle that allowed for the movement of inherent soluble salts into the rooting zone (Lobell et al., 2010). The risk of salinization and sodification in this region is exacerbated by land use changes where grasslands are being converted to annual row cropping fields (Stubbs, 2007). Soil health will play an ever increasingly important role with greater risks of land degradation through land use conversion. Using biological, chemical, and physical soil indicators to monitor changes in soil health can aid producers in making sound decisions for not only current but future production needs that take advantage of healthy, functioning soils.

Statistical analysis for identifying soil health indicators

The development of a soil health index is outlined in the Soil Management Assessment Framework Design (Andrews et al., 2004). As the number of physical, chemical, and biological indicators that can be measured for soil health indices is vast, reducing the number of measured variables to a minimum data set (MDS) is required. Differences in inherent soil properties, climate, landscape position and cultural management practices have resulted in multiple MDS being identified to measure soil health. Multivariate analysis tools such as principal component

analysis (PCA) (Schipper and Sparling, 2000; Andrews et al., 2004; Marinari et al., 2006; Rousseau et al., 2012) and factor analysis (FA) (Shulka et al., 2006; Imaz et al., 2010) have been used to create an MDS. Both PCA and FA are statistical techniques that aim to reduce the number of variables (Rencher and Christensen, 2012). However, PCA and FA are linear combinations of variables or linear combinations of factors. Principal component analsysis is used to explain total variance whereas FA is used to explain covariances among the variables. As such, FA is not appropriate for many datasets. Although PCA and FA are commonly used, soil variables behave in a non-linear fashion over the scale of which they can be measured.

McCune and Grace (2006) describe nonmetric multidimensional scaling (NMS) as a data reduction ordination tool that currently is "one of the most defensible techniques during peer review." Nonmetric multidimensional scaling does not have assumptions of normality or linearity in the underlying model and is better adapted to be used more in soil science. Nonmetric multidimensional scaling works by adjusting the spatial distribution of samples over *k* axes through a dissimilarity matrix based on the number of samples and number of variables collected. The distance between the samples in *k* space is calculated. Multiple iterations of adjusting samples in *k* space are performed until the distance forms a monotonic line. The stress of the line, which is opposite of the "goodness of fit" is calculated and subsequent iterations moving points over *k* axes is performed until stress is minimized. Nonmetric multidimensional scaling is used as a tool to visualize relationships among variables and samples. Coupling NMS with hypothesis testing elevates the strength of NMS as a statistical tool.

Hypothesis testing to determine differences in management factors can be completed using either perMANOVA or multi-response permutation procedures (MRPP) for unbalanced designs. The perMANOVA test is semi-analogous to multivariate analysis of variance

(ANOVA), except perMANOVA does not have assumptions of normality and can be used in conjunction with any distance measure (Anderson, 2001). In contrast, MRPP can be used for hypothesis testing of difference between groups based on within-group similarities (Peck, 2010). Both perMANOVA and MRPP rely on multiple permutations based on a randomization procedure. Following either perMANOVA or MRPP, pairwise comparisons using Bonferonni correction can be completed to test for significant differences between experimental factors.

Choosing the proper statistical model is important in determining soil health indicators and their responses to changes in soil health due to land use and management. Improper statistical model choice can result in a less than desirable soil health indicators, where proper model choice will result in the identification of robust soil health indicators. Soil management activities like tillage and fertilizer applications can also influence the beneficial symbiotic relationships between microbes and plants, such as biological N fixation.

Soil health and biological nitrogen fixation

World demand for food resources in 2050 is estimated to be double current production levels (Tillman et al., 2011). Ensuring adequate food supply in the face of high input costs and limited supply of inorganic fertilizer sources will become even more crucial as agriculture production intensifies on marginal lands (Strijker, 2005). Soil health improvements can be realized through the contributions of biological N fixation to crop production by maximizing natural nutrient cycles while simultaneously decreasing dependence on non-renewable resources (Doran and Ziess, 2000; Keyser and Li, 1992). Soil microorganisms, such as bacteria, form symbiotic relationships with plants to help improve aboveground plant production (Ferguson and Mathesius, 2003) and management systems. Impacts on these beneficial bacterial populations need to be assessed for their contribution to soil health. Using bacterial species involved in

biological N fixation has been suggested as an indicator of soil health (Ferreira de Araújo et al., 2008). Providing producers with information regarding bacterial N fixing populations can aid producers in making decisions for current and future crop production and soil health.

Biological N fixation (BNF) can account for 25 to 75% of plant N accumulation (Zapata et al., 1987). Rhizobium is a collective term to describe the N fixing bacteria and includes the genera *Rhizobium, Bradyrhizobium,* and *Azorhizobium* (Fischer, 1994). Biological nitrogen fixation results from symbiotic *Rhizobium* species infecting root nodules in leguminous plants. Rotating crops with legumes can contribute to sustainable crop production from BNF and the additional plant available N (Bohlool et al., 1992). *Bradyrhizobium japonicum* is used extensively as an inoculant to promote BNF in soybean (*Glycine max* L.) production in the northern United States. The United States is a major producer and consumer of soybeans (Shiro et al., 2013), a high N demand crop which worldwide contributes to 77% of legume crop N fixed with an estimated contribution of 16.44 Tg N yr^{-1} fixed (Herridge et al., 2008). Therefore, incorporation of high value legumes such as soybeans into crop rotations will contribute to sustainable crop production by reducing synthetic fertilizer needs (Bohlool et al., 1992).

Increases of up to 45% seed protein have been attributed to BNF by inoculating several strains of *rhizobium* in soils where soybean has not been previously grown (Abel and Erdman, 1964); where soybeans have been historically grown ~ 5 to 20% of nodules on the roots are formed from inoculated strains (Caldwell and Vest, 1970; Kuykendall and Weber, 1978). However, establishing strains of *B. japonicum* through inoculation that have high BNF capacities is a challenge due to the presence of naturalized rhizobium (previously inoculated, yet differing strains of *B. japonicum*) (Thies et al., 1991). Within individual *rhizobium* species, strain level differences in BNF exist. For example, *B japonicum* strains USDA 110, 129 and 122 were found

to have greater N fixing effectiveness than the *B. japonicum* K961, 2 and K902 strains (Sloger, 1969). Differences in strain level effectiveness were also identified for clones of *B. japonicum* USDA 110 with the clones I-110 and S-110 exhibiting 5 to 10-fold greater effectiveness than the L1-110 and L2-110 clones (Kuykendall and Elkan, 1976). Inoculated *Rhizobium* strains are selected to fix higher levels of N than competing naturalized rhizobium that may have equal ability to infect legume roots, but may not fix high levels on N (Singleton and Tavares, 1986). Studies of *Rhizobium* in Brazil have found that inoculant strains can diversify both through adaptation to local climate and crop production practices, but also by horizontal gene transfer of the nitrogen fixing genes which may impact their infectiveness and effectiveness (Hungria et al., 1996; Nishi et al., 1996; Boddey and Hungria 1997, Hungria et al., 1998).

Rhizobium signaling to initiate nodulation

Legume nodulation is initiated through a series of biochemical signals between the plant and the bacteria (Dénarié et al., 1992). In soybeans the nodulation process is very specific and is limited to *Bradyrhizobium japonicum, B. elkanii, Sinorhizobium fredii, B. liaoningense, S. xinjiangense,* and *Mesorhizobium tianshanense* (Jordan, 1982; Scholla and Elkan, 1984; Chen et al, 1988; Kuykendall et al., 1992; Chen et al., 1995; Xu et al., 1995; Tan et al., 1997; Peng et al., 2002; Yao et al., 2002; Vinuesa et al., 2008). Evidence shows that the ability to infect legumes is controlled genetically by the host plant, with *Rhizobium* being the control mechanism that initiates nodulation (Dénarié and Roche, 1992). The isoflavones, genestein and daidzein secreted by plants are the major inducers of nodulation for *B. japonicum*. Additionally, apigen, a flavone, and kaempferol, a flavanol have been shown to induce nodulation by *B. japonicum* (Dénarié et al., 1992). Once the signaling molecules have been detected, the induction of the *nodABC* genes, which are found in all symbiotic N fixing bacteria (Martinez et al., 1990), and *B. japonicum*

specific *nodZ* and *nodVW* initiate root hair curling, infection thread formation, and nodulation in soybeans (Göttfert et al., 1990). Many *Rhizobium* are host legume particular, and soybean nodulation by strains of *B. japonicum* is narrow and specific, but mutations in genes have been shown to cause cross infection with other legume species (Barbour et al., 1992; Göttfert et al., 1990). Rotations involving additional legume crops, such as edible beans (*Phaseolus vulgaris*), peas (*Pisum sativum),* or alfalfa which require different *Rhizobium* inoculating species may have a negative effect on *B. japonicum*.

Quantifying *Rhizobium* in the soil

Rhizobium populations are larger in the plant rhizosphere, or the area of soil surrounding a root, as compared to the bulk soil (Dénarié et al., 1992). *Rhizobium* populations have previously been quantified by the Most Probable Number (MPN) method (Weaver and Frederick, 1972), a population enumeration technique based on statistical probabilities. The most probable number is assessed by growing soybeans in clear plastic pouches, inoculating with a replicated series of soil dilutions, determining nodule formation at each dilution, and using a statistical table that estimates probable population sizes (Cochran, 1950). Most Probable Number methods are time and space intensive in that they require large space in growth chambers and nodulation is assessed three weeks post inoculation. Individual strains of *Rhizobium* can be identified using serology techniques (Means et al., 1964). However, advances in molecular microbiology techniques (i.e. quantitative Polymerase Chain Reaction (qPCR) and high throughput sequencing with Sanger PCR) can be used to quantify the population levels and community structure of rhizobium (Saeki et al., 2006; Mao et al., 2011). The Polymerase Chain Reaction is advantageous relative to MPN for *Rhizobium* population estimates because soil can be stored frozen long-term and results can be obtained in a matter of hours (Furseth et al., 2010).

Additionally, MPN is based on the assumption that one nodule is one cell (Weaver and Frederick, 1972). Quantitative PCR is a more accurate way to measure copy numbers and be used to identify in-situ rhizobium independent of culturing.

Rhizobium persistence in the soil

In order to ensure adequate population levels for nodulation, *Rhizobium* inoculants are added to legume seeds during planting to facilitate root infection from desirable *Rhizobium* strains. These inoculated Rhizobium populations are generally able to persist in the soil. For example, *B. japonicum* USDA 110 and USDA 123 were inoculated with seed and survived at a quantity of 10^3 g^{-1} of soil for 17 years without a host plant (Narożna et al., 2015). However, crop management strategies such as tillage and fertilizer can affect *Rhizobium* persistence. Tillage distributes *Rhizobium* in the soil more efficiently and uniformly that percolating water or wind-blown deposition (Lowther et al., 1987). In contrast, no till is a system that does not disturb the soil and seeds are planted directly through the previous crop residues which allows for increased soil organic matter and higher soil microbial biomass (Hungria and Vargas, 1996), but the potential for greater stratification of inoculated and persistent *Rhizobium* populations is increased. The diversity of *Rhizobium* populations has also been shown to be greater under no till soils with diverse cropping patterns involving soybean relative to plowed and disked soils without soybean in the crop rotation (Ferreira et al., 2000). Soil organic matter is important for carryover of *Rhizobium* populations as they can exist in the soil in a saprophytic stage using organic C as energy substrates (Siqueira et al., 2014).

Rhizobium infectiveness

A range of 10 to 100 individual *Rhizobium* cells results in 63 to 100% nodulation of plants (Perkins, 1925), but in order to have successful taproot nodulation, thousands of

Rhizobium would need to be added per seed (Burton and Curley, 1967). Applications of inoculant at a rate of 10^6 cells g^{-1} soil resulted in highest soybean yields, with a recommendation of applying a minimum of 10^5 cells g^{-1} soil (Hume and Blair, 1992). Levels of N in the soil also impact *Rhizobium* infectiveness in forming nodules. Where 0.50 and 1.0 mM of NO_3-N was applied, the time of initial nodulation was delayed by 3 to 6 days, respectively, contained 40 fewer nodules and fixed less N_2 by the acetylene reduction assay (Gibson and Harper, 1985). The negative relationship between inorganic N and nodulation infectiveness has been confirmed elsewhere (Lyons and Earley, 1952; Döbereiner et al., 1966;; Thies et al., 1991).

***Rhizobium* effectiveness**

Different strains of *B. japonicum* and *B. elkanii*, which is applied as an inoculant in the southern US and areas like Brazil, have been shown to have different N fixing abilities (effectiveness) (Wright, 1925a; Wright 1925b; Boyes and Bond, 1942), and different strains are more efficient for different soybean varieties (Boyes and Bond 1942; Damirgi, 1963). For example, table 3, adapted from Hungria et al. (1998), studied the nodule infectiveness and effectiveness of several strains of *B. japonicum and B. elkanii* and found that across two different soybean varieties grown in Leonard jars with N-free solution that *B. japonicum* USDA 110 showed the greatest nodule efficiency while *B. elkanii* USDA 97 was the least efficient. Interestingly, non-efficient strains have been shown to have similar nodule counts as efficient strains and efficient *Bradyrhizobium* strains may actually have fewer nodules than less effective strains (Caldwell et al., 1968; Hungria et al., 1998).

Table 3. Nodule occupancy and effectiveness of several studied *Bradyrhizobium* reference strains. Adapted from Hungria et al. (1998).

Species and strain	Nodule number plant^{-1}	Nodule dry weight, mg nodule plant^{-1}	Total N accumulated by plants, mg of N plant^{-1}	Nodule efficiency, mg of N mg^{-1} of nodules
Bradrhizobium japonicum USDA 110	58.2	200.5	96.5	0.481
Bradyrhizobium japonicum USDA 122	61.1	225.4	77.7	0.345
Bradyrhizobium japonicum USDA 123	62.8	231.1	77.1	0.334
Bradyrhizobium sp. mixed genotype USDA 73	50.4	188.2	75	0.398
Bradyrhizobium elkanii USDA 31	70.4	283.8	50.4	0.178
Bradyrhizobium elkanii USDA 76	75.6	263.5	60.2	0.228
Bradyrhizobium elkanii USDA 94	62.1	223.6	31.1	0.139

Nitrogen fixing capabilities of nodules can be measured in several ways. The first is through the use of stable N isotope ^{15}N as described in Peoples et al. (2009). There are several methods using ^{15}N, the first use is growing legumes in a chamber in which $^{15}N_2$ is supplied to the atmosphere. The uptake of ^{15}N in soybean plant tissues is then determined by mass spectroscopy. Another ^{15}N isotope method for determining N fixation is the natural abundance technique as the atmosphere has a constant composition of 0.3663 atoms% (Höberg, 1997). The ^{15}N composition of soil N also needs to be determined, and is commonly 0.001-0.007 atoms% above atmospheric ^{15}N (Peoples et al., 2002) and the differences between ^{15}N accumulated within plant tissue originating from the atmosphere and the soil N pools can be used to determine biological fixation (Shearer and Kohl, 1986; Unkovich and Pate, 2000; Boodey et al., 2000). However, ^{15}N methods are limiting due to costs high cost of running samples (Peoples et al., 2009). The acetylene reduction method is another way in which plant biological nitrogen fixation can be determined as the nitrogenase enzyme not only coverts N_2 to NH_3 in the nodules, but also can reduce acetylene

(C_2H_2) to ethylene (C_2H_4) (Hardy et al., 1973). The acetylene reduction method requires nodules or roots with nodules attached to be sealed in an air-tight container with an acetylene enriched atmosphere and the rate of C_2H_4 accumulation is measured via a gas chromatograph (Vessey, 1994). Although this method is inexpensive and many samples can be run in a day, the acetylene reduction method only represents the N fixing activity as a single point in time (Peoples et al., 2009). A third method to measure N accumulation from fixation is by plant ureide-N concentrations (Herridge, 1982). Nitrogen fixed from N_2 in soybean and several other tropical legumes, is transported from within the nodules into the xylem sap as ureide compounds-allantoin and allantoic acid (Fujihara and Yamaguchi, 1978). Ureide concentration in the stems is greater when biological N_2 fixation is occurring, while nitrate and amino-N concentrations are greater in the stems when soil N is being utilized by legumes (Peoples and Herridge, 1990; Herridge and Danso, 1995). As such, the ratio of ureide-N to plant nitrate-N can be used to determine the percentage of N from fixation in dried plant tissues. A new method for ureide N concentration was developed by Goos et al. (2015) in which the allantoin and allantoic acids in the xylem sap or aqueous extracts are converted to urea prior to analysis. This method is quick and suitable for in-field determinations of BNF as up 100-150 plots can be sampled in a day and the ureide content determined in several more days (Unkovich et al., 2008; Peoples et al., 2009). With recent advances in molecular techniques, the ability to quickly assess nodule activity through quantification of messenger RNA (mRNA) may be possible. Messenger RNA is produced by active organisms and is used in the production of amino acids. In theory, by extracting mRNA and using the *NifH* gene in qPCR, active N fixation gene correlations with ureide N content can be used to assess nodule effectiveness.

Relating biological nitrogen fixation to soil health

Biological N fixation can be related to soil health, fertilizer, and tillage management through statistical analysis. Similar to identifying soil health indicators, nonmeteric multidimensional scaling (NMS) can be utilized to relate specific soil properties and management to BNF. For instance, the influence of *in situ* available soil N, soil organic matter, pH, etc. can be related to nodule counts, total plant N derived from BNF, *Rhizobium* diversity, nodule N fixation potential, and free-living N_2 fixing bacteria gene copy numbers. Furthermore, measured soil and plant variables can be related to N fertilizer management and tillage practices thereby clearly linking soil health to crop production systems.

In the north central Great Plains, USA, soybean acreage has grown by nearly 45% from 1990 to 2014 (NASS, 2015). Understanding the mechanisms underpinning *Rhizobium* population dynamics can improve soil health and sustainability of crop production through efficient capture and use of N. Crop management strategies, including tillage, rotations, and fertilizer additions all work in concert to modify the habitat and activities of soil bacteria. Molecular microbiology techniques, such as the PCR and genetic sequencing allow for rapid quantification of rhizobium populations, both specific and promiscuous, in the soil and within infected nodules of soybean roots. Relating aboveground soil management to the belowground activities of beneficial microbial populations and activities will be paramount in managing soil health for the future.

Conclusions

Identifying and relating sensitive biological indicators to changes in soil health as a result of land use and management is a challenge that needs to be addressed. Changes in the landscape and cropping systems in the northern Great Plains necessitates careful monitoring of soil health and the roles soil microbes play in cropping system sustainability and long term agricultural

production. Simultaneously, the short-term production needs of the region need to still be achieved. To accomplish these simultaneous goals, sensitive and interpretable biological soil health indicators need to be identified and related to shifts in land use and management, especially on degraded, salt affected soils. Differences in soil amendment applications and tile drainage treatments are expected to result in changes in soil health. Additionally, using BNF as a soil health indicator by contributing new information on rhizobium population dynamics and the ecology of rhizobium by reexamining the infectiveness and effectiveness of inoculant and naturalized rhizobium in soybean nodules utilizing new molecular techniques. The contributions of promiscuous rhizobium communities will be assessed in relation to N fixing rhizobium specific to soybean nodule infection, formation and effectiveness. Understanding soil N dynamics and changes in soil health under different management systems is important for crop production to ensure sustainable ecosystems in the future.

References

Abel, G.H., A.J. MacKenzie. 1964. Salt tolerance of soybean varieties (*Glycine max* L. Merrill) during germination and later growth. Crop Sci. 4:157-161. doi:10.2135/cropsci1964.0011183X00400020010x

Abrol, I.P., J.S.P. Yadav, and R.I.Massoud. 1988. Salt-affected soils and their management. Soil Resources, Management and Conservation Service, Food and Agricultural Organization of the United Nations. Land and Water Development Division. Rome, Italy.

Acosta-Martínez, V. M.A. Tabatabai.2001. Enzyme activities in a limed agricultural soil. Biology and Fertility of Soil. 31:85-91. doi:10.1007/s00374.007-0251-5

Acosta-Martínez, V. M.A. Tabatabai. 2011. Phosphorus cycle enzymes. *In*: Dick, R.P. (ed). Methods of soil enzymology. Soil Science Society of America Book Series, no. 9. Soil Science Society of America, Madison, WI, USA.

Allison, S.D. and K.K. Treseder. 2008. Warming and drying suppress microbial activity and carbon cycling in boreal forest soils. Global Change Biol. 14:2898-2909. doi:10.1111/j.1365-2486.2008.01716.x

Amezketa, E., R. Aragües, R. Gazol. 2005. Efficiency of sulfuric acid, mined gypsum, and two gypsum bi-products in soil crusting prevention and sodic soil reclamation. Agron. J. 97:983-989. doi:10.2134/agronj2004.0236

Anderson, M. J. 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecology. 26:32-46. doi:10.1111/j.1442-9993.2001.01070.pp.x

Anderson, T-H. 2003. Microbial eco-physiological indicators to assess soil quality. Agric. Ecosyst. Environ. 98:285-293. doi:10.1016/S0167-8809(03)00088-4

Andrews, S.S., D.L. Karlen and C.A. Cambardella. 2004. The soil management assessment framework: A quatitative soil quality evaluation method. Soil Sci. Soc. Am. J. 68:1945-1962. doi:10.2136/sssaj2004.1945

Ashworth, J. D. Keyes, and J-M. Crépin. 1999. A comparison of methods for gypsum requirement of brine-contaminated soils. Can. J. Soil Sci. 79:449-455. doi:10.4141/S99-001

Avnimelech, Y., Shkedy, D., Kochva, M., Yotal Y. 2004. The effect of municipal solid waste compost (Msw) on the replacement of sodium in sodic soil models. Soil Sci. 169:567-572. doi:10.1097/01.ss.0000138417.37793.7b

Ayers, A.D., J.W. Brown, C.H. Wadleigh. 1952. Salt tolerance of barley and wheat in soil plots receiving several salinization regimes. Agron. J. 44:307-310. doi: 10.2134/agronj1952.00219620040006006x

Bandick, A.K., R.P. Dick. 1999. Field management effects on soil enzyme activites. Soil Biol. Biochem. 31:1471-1479. doi:10.1016/S0038-0717(99)00051-6

Barbour, W.M., S.P. Wang, G. Stacey. 1992. Molecular genetics of *Bradyrhizobium* symbiosis. In: G. Stacey, H.J. Evans and R.H. Burris (eds). Biological Nitrogen Fixation. Chapman and Hall, New York, NY, USA.

Bengtson, P., A. E. Sterngren, and J. Rousk. 2012. Archaeal Abundance across a pH Gradient in an Arable Soil and Its Relationship to Bacterial and Fungal Growth Rates. Appl. Environ. Microbiol.78:5906–5911. doi:10.1128/AEM.01476-12.

Bergstrom, D.W., C.M. Monreal and D.J. King. 1998. Sensitivity of soil enzyme activities to conservation practices. Soil Sci. Soc. Am. J. 62:1286-1295. doi:10.2136/sssaj1998.03615995006200050020x

Bernstein, L. 1975. Effects of salinity and sodicity on plant growth. Annu. Rev. Phytopathol. 13:295-312. doi:10.1146/annurev.py.13.090175.001455

Bernstein, L., A.D. Ayers. 1949. Salinity tolerance of sweet corn. *In*: United States salinity laboratory report to collaborators, Riverside, CA, USA.

Bernstein, L., L.E. Francois. 1973. Leaching requirement studies: sensitivity of alfalfa to salinity of irrigation and drainage waters. Proc. Soil Sci. Soc. Am. 37:931-943. doi:10.2136/sssaj1973.03615995003700060038x

Bernstein, L., L.E. Francois, and R.A. Clark. 1974. Interactive effects of salinity and fertility on yields of grains and vegetables. Agron. J.. 66:412-421. doi:10.1007/BF00270750

Bernstein, L., H.E. Hayward. 1958. Physiology of salt tolerance. Annu. Rev. Plant Physiol. 9:25-46.

Bernstein, L., A.J. MacKenzie, B.A. Krants. 1955. Salt tolerance of field crops-soybeans. *In*: United States salinity laboratory report to collaborators, Riverside, CA, USA.

Bernstein, L., G. Ogata. 1966. Effects of salinity on nodulation, nitrogen fixation, and growth of soybeans and alfalfa. Agron J. 58:201-203. doi:10.2134/agronj1966.00021962005800020025x

Bezdicek, D.E., R.I. Papendick, and R. Lal. 1996. Introduction: Importance of soil quality to soil health and sustainable land management. In Doran, J.W. and A.J. Jones (eds). Methods for assessing soil quality. SSSA Special Publication Number 49. Soil Science Society of America, Inc. Madison, WI, USA.

Blagodatsky, S.A. and O. Richter. 1998. Microbial growth in soil and nitrogen turnover: a theoretical model considering the activity state of microorganisms. Soil Biol. Biochem. 30:1743–1755. doi:10.1016/S0038-0717(98)00028-5

Boddey, L.H. and M. Hungria. 1997. Phenotypic grouping of Brazilian Bradyrhizobium strains which nodulate soybeans. Biol. Fertil. Soils. 25:407-415. doi:10.1007/s003740050333

Boddey, R.M., M.B. Peoples, B. Palmer, P.J. Dart. 2000. Use of the $_{15}$N natural abundance technique to quantify biological nitrogen fixation by woody perennials. Nutr. Cycl. Agroecosys. 57:235-270. doi:10.1023/A:1009890514844

Bohlool, B. B., J. K. Ladha, D. P. Garrity, T. George. 1992. Biological nitrogen fixation for sustainable agriculture: a perspective. Plant and Soil. 141:1-11. doi:10.1007/BF00011307

Bollmann, A., I. Schmidt, A.M. Saunders, M.H. Nicolaisen. 2005. Influence of starvation on potential ammonia-oxidizing activity and *amoA* mRNA levels of *Nitrosospira briensis*. Appl. Environ. Microbiol. 71:1276-1282. doi:10.1128/AEM.71.3.1276-1282.2005

Bower, C.A., C.D. Moodie, P. Orth, F.B. Gschwent. 1954. Correlation of sugar beet yields with chemical properties of a saline-alkali soil. Soil Sci. 77:443-451.

Bower, C.A., G. Ogata, J.M. Tucker. 1969. Rootzone salt profiles and alfalfa growth as influenced by irrigation water salinity and leaching fraction. Agron. J. 61:783-785. doi:10.2134/agronj1969.00020061000500039x

Bowles, T.M., V. Acosta-Martínez, F. Calderón, L.E. Jackson. 2014. Soil enzyme activities, microbial communities, and carbon and nitrogen availability in organic agroesystems across an intensively-managed agricultural landscape. Soil Biol. Biochem. 68:252-262. doi:10.1016/j.soilbio.2013.10.004

Boyes, J. and Bond, G. 1942. The effectiveness of certain strains of soybean nodule organism when associated with different varieties of the host plant. Ann. Appl. Biol 29: 103-108.

Brown, J.W., H.E. Hayward. 1956. Salt tolerance of alfalfa varieties. Agron. J. 48:18-20. doi:10.2134/agronj1956.00021962004800010005x

Burns R.G., 1978. Soil Enzymes. Academic Press, London, England.

Burns, R. G. 1982. Enzyme activity in soil: location and possible role in microbial ecology. Soil Biol. Biochem. 14:423-427. doi:10.1016/0038-0717(82)90099-2

Burton, J. C. and and R.L. Curley. 1967. Nodulation of the soybean. Glycine max, as influenced by rhizobia inoculum and growth conditions. Agro. Abstracts: 89:1.

Caldwell, B.E. 2005. Enzyme activities as a component of soil biodiversity: A review. Pedobiologia. 49:637-644. doi:10.1016/j.pedobi.2005.06.003

Caldwell, B. E. and Vest, Grant. 1968. Modulation interactions between soybean genotypes and serogroups of Rhizobium japonicum. Crop Sci. 8: 6 80-6 82. doi:10.2135/cropsci1968.0011183X000800060012x

Chang, M.H., S.M. Yaseen, and M. Naeem. 1991. Reclamation of saline-sodic soils by gypsum under tile drainage system. Irrig. Drain. 3:25-32.

Chapman, V.J. 1960. Salt marshes and salt deserts of the world. London: Leonard Hill.

Chawla, K.L. and I.P. Abrol. 1982. Effect of gypsum fineness on the reclamation of sodic soils. Agric. Water Manage. 5:41-50. doi:10.1016/0378-3774(82)90037-3

Chen, W.X., G.H. Yan, L.J. Li. 1988. Numberical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. Int. J. Syst. Bacteriol. 38:392-397. doi:10.1099/00207713-38-4-392

Chen, W., E. Want, S. Wang, Y. Li, X. Chen, Y. Li. 1995. Characteristics of *Rhozibium tianshanense* sp. nov. a moderately and slowly growing root nodule bacterium isolated from an arid saline environment in Xinjiang, People's Republic of China. Int. J. Syst. Bacteriol. 45:153-153. doi:0020-7713/95/$04.00 + 0

Cheng, S.F. 1983. Effect of salinity on sunflower production and mineral concentration. J. Chinese Agric. Chem. Soc. 21:231-237.

Churchman, G.J., J.O. Skjemstad, and J.M. Oades. 1993. Influence of clay minerals and organic matter effects of sodicity on soils. Aust. J. Soil Res. 31:779-800. doi:10.1071/SR9930779

Cochran, W.G. 1950. Estimation of bacterial densities by means of the "most probable number". Biometrics. 6:105-116.

Cole, C.V., S.R. Olsen, and C.O. Scott. 1953. The nature of phosphate sorption by calcium carbonate. Soil Sci. Soc. Am. J. 17:352-356. doi:10.2136/sssaj1953.03615995001700040013x

Damirgi, Salih M., Frederick, L. R. and Anderson, I. C. 1967. Serogroups of Rhizobium japonicum in soybean nodules as affected by soil types. Agron. J. 59: 10-12. doi:10.2134/agronj1967.00021962005900010004x

Delauney, A.J., D.P.S. Verma. 1993. Proline biosynthesis and osmoregulation in plants. The Plant J. 4:215-223. doi:10.1046/J.1365-313X.1993.04020215.x

Dénarié, J., F. Debellé, and C. Rosenberg. 1992. Signaling and host range variation in nodulation. Annu. Rev. Microbiol. 46:497-531. doi:0066-4227/92/1001-0497$02.00

Dénarié, J. and P. Roche. 1992. Rhizobium nodulation signals. In: Verma, D.P.S. (ed). 1992. Molecular signals in plant-microbe communication. CRC Press, Boca Raton, LA, USA.

Di, H.J., K.C. Cameron, J.P. Shen, C.S. Winefield, M. O'Callaghan, S. Bowatte, and J.Z. He. 2009. Nitrification driven by bacteria and not archaea in nitrogen rich grassland soils. Nat. Geosci. 2:621–624.Dick, R.P. 1994. Soil enzyme activities as indicators of soil quality. *In* Defining soil quality for a sustainable environment, eds. J.W. Doran, D.C. Coleman, D.F. Bezdicek, B.A. Stewart. Madison, WI: Soil Science Society of America.

Di, H.J., K.C. Cameron, J.-P. Shen, C.S. Winefield, M. O'Callaghan, S. Bowatte, and J.-Z. He. 2010. Ammonia-oxidizing bacteria and archaea grown under contrasting soil nitrogen conditions. FEMS Microbiol. Ecol. 72:386–394. doi:10.1111/j.1574-6941.2010.00861.x

Dick, R. P. 1994. Soil enzyme activities as indicators of soil quality. In: Doran, J.W., D.C. Coleman, D.F. Bezdicek, B.A. Stewart (ed.) Defining soil quality for a sustainable environment. Soil Science Society of America special publication. Madison, WI, USA.

Dick, W. A. 2011. Development of a soil enzyme reaction assay. *In*: Dick, R. P. (ed). Methods of soil enzymology. Soil Science Society of America Book Series 9. Madison, WI, USA.

Dick, R.P., R. G. Burns. 2011. A brief history of soil enzymology research. *In*: Dick, R. P. (ed). Methods of soil enzymology. Soil Science Society of America Book Series 9. Madison, WI, USA.

Dick, R.P., P.E. Rasmussen, E.A. Kerle. 1988. Influence of long-term residue management of soil enzyme activities in relation to soil chemical properties of a wheat-fallow system. Biol. Fertil. Soils. 6:159-164. doi:10.1007/BF00257667

Dieleman, P.J. (ed). 1963. Reclamation of salt affected soils in Iraq. International Institute for Land Reclamation and Improvement. Publication no. 11. Wageningen, the Nedtherlands

Dighton, J. 1983. Phosphatase production by mycorrhizal fungi. In: Atkinson, D., Bhat, K.K.S., M.P. Coutts, P.A. Mason, and D.J. Read (Eds). Tree root systems and their mycorrhizas. Developments in Plant and Soil Sciences. Volume 7.

Döbereiner, J., N.B. deArruda, and A. de Figueiredo Penteado. 1966. Availiacao da fixa cao do nitrogenio, em leguminosas, pela regressao do nitrgenio total das plantas sobre o peso dos nodulos. Pesq. Agropec. Bras. 1: 233-237.

Doran, J.W. and Parkin, T. B. 1994. Defining and assessing soil quality. In: Defining soil quality for a sustainable environment. In: Doran, J.W., D.C. Coleman, D.F. Bezdicek, B.A. Stewart (Eds.) Defining soil quality for a sustainable environment. Soil Science Society of America special publication. Madison, WI, USA.

Doran, J.W. and M.R. Zeiss. 2000. Soil health and sustainability: managing the biotic component of soil quality. Appl. Soil Ecol. 15:3-11. doi:10.1016/S0929-1393(00)00067-6

Dreng, H.E. 1962. Effects of various salts on barley growth. New Mexico Agriculture Experiment Station Research Report 64.

Ekenler, M., M.A. Tabatabai. 2003. Response of phosphatases and arylsulfatase in soils to liming and tillage systems. J. Plant Nutr. Soil Sci. 166:281-290. doi:10.1002/jpln.200390045

El-Shakweer, M.H.A., El-Sayad, E.A., Ejes, M.S.A., 1998. Soil and plant analysis as a guide for interpretation of the improvement efficiency of organic conditioners added to different soils in Egypt. Commun. Soil Sci. Plant Anal 29, 2067–2088. doi:10.1080/00103629809370094

Erguder, T.H., N. Boon, L. Wittebolle, M. Marzorati, and W. Verstraete. 2009. Environmental factors shaping the ecological niches of ammonia-oxidizing archaea. FEMS Microbiol Rev. 33:855-869. doi:10.111/j.1574-6976-2009-00179.x.

Essington, M.E. 2004. Soil and water chemistry, an integrative approach. CRC Press, Boca Raton, London, New York, Washington D.C.

FAO [Food and Agriculture Organization of the United Nations]. 2014. Global soil health indicators and assessment. http://www.fao.org/soils-portal/soil-degradation-restoration/global-soil-health-indicators-and-assessment/en/. Accessed on 27 October 2014.

Fauci, M.F., R.P. Dick. 1994. Soil microbial dynamics: short- and long-term effects of inorganic and organic nitrogen. Soil Sci. Soc. Am. J. 58:801-806. doi:10.2136/sssaj1994.03615995005800030023x

Ferguson, B.J. and U. Mathesius. 2003. Signaling interactions during nodule development. J. Plant Growth Regul. 22:47-72. doi:10.1007/s00344-003-0032-9

Ferreira, M. C., D. de S. Andrade, L. M. de O. Chueire, S. M. Takemura, and M. Hungria. 2000. Tillage method and crop rotation effects on the population sizes and diversity of Bradyrhizobium nodulating soybean. Soil Biol. Biochem. 32:627-637. doi:10.1016/S0038-0717(99)00189-3

Ferreira de Araújo, A. S., M. do Vale Barreto Figueiredi, and R.T. Rosim Monteiro. 2008. Potential of biological nitrogen fixation as indicator of soil pollution. In: Couto, G.N. (ed). Nitrogen fixation research progress. Nova Science Publishers, Inc., Hauppauge, NY, USA.

Fischer, H.-M. 1994. Genetic regulation of nitrogen fixation in Rhizobia. Microbiol. Rev. 58:352-386.

Fortuna, A-M., C.W. Honeycutt, G. Vandemark. T.S. Griffin, R.O. Larkin, Z. He, B.J. Wienhold, K.R. Sistani, S.L. Albrecht, B.L. Woodbury, H.A. Torbert, J.M. Powell, R.K. Hubbard, R.A. Eigenberg, R.J. Wright, J.R. Alldredge, J.B. Harsh. 2012. Links among nitrification, nitrifier communities, and edaphic properties in contrasting soils receiving dairy slurry. J. Environ. Qual. 41:262-272. doi:10.2134/jeq2011.0202

Fougére, F. D.L. Rudulier, J.G. Streeter. 1991. Effects of salt stress on amino acid, organic acid, and carbohydrate composition of roots, bacteroides and cytosol of alfalfa (*Medicago sativa* L.). Plant Physiol. 96:1228-1236. doi:0032-0889/91/96/1228/09/$01.00/0

Frankenberger, W.T. Jr. and F.T. Bingham. 1982. Influence of salinity on soil enzyme activities. Soil Sci. Soc. Am. J. 46:1173-1177. doi:10.2136/sssaj1982.03615995004600060011x

Francois, L.E. 1994. Growth, yield, and oil content of canola grown under saline conditions. Agron. J. 86:233-237. doi:10.2146/agronj1994.00021962008600020004x

Francois, L.E. 1996. Salinity effects on four sunflower hybrids. Agron. J. 88:215-219. doi:10.2134/agronj1996.00021962008800020016x

Francois, L.E., T.J. Donovan, K. Lorenz, E.V. Maas. 1989. Salinity effects on rye grain yield, quality, vegetative growth, and emergence. Agron. J. 81:707-712. doi:10.2134/agronj1989.00021962008100050001x

Francois, L.E., T.J. Donovan, E.V. Maas. 1984. Salinity effects on seed yield, growth, and germination of grain sorghum. Agron. J. 76:741.744. doi:10.2134/agronj1984.00021962007600050008x

Francois, L.E., T.J. Donovan, E.V. Maas, G.L. Rubenthaler. 1988. Effect of salinity on grain yield and quality, vegetative growth, and germination of triticale. Agron. J. 80:642-647. doi:10.2134/agronj1988.00021962008000400l9x

Frenkel, H., Z. Gerstl, and N. Alperovitch. 1989. Exchange-induced dissolution of gypsum and the reclamation of sodic soils. J. Soil Sci. 40:599–611. doi:10.111/j.1365-2389.1989.tb01301.x

Frenkel, H., J.O. Goertzen, J.D. Rhoades. 1978. Effects of clay type and content, exchangeable sodium percentage, and electrolyte concentration on clay dispersion and soil hydraulic conductivity. Soil Sci. Soc. Am. J. 42:32-39. doi:10.2136/sssaj1978.03615995004200010008x

Fujihara, S. and M. Yamaguchi. 1978. Probable site of allantoin formation in nodulating soybean plants. Phytochemistry. 17:1239-1243. doi:10.1016/S0031-9422(00)94564-3

Furseth, B.L., S.P. Conley, and J.-M. Ané. 2010. Enumeration of soybean-associated rhizobia with quantitative real-time polymerase chain reaction. Crop Sci. 50:2591–2596. doi:10.2135/cropsci2010.03.0141

Gaby, J.C., D.H. Buckley. 2012. A comprehensive evaluation of PCR primers to amplify the *nifH* gene of nitrogenase. PLoS ONE 9:393883. doi:10.1371/journal.pone.0093883

García, C. and T. Hernández. 1996. Influence of salinity on the biological and biochemical activity of a calciorthid soil. Plant and Soil. 178:255-263. doi:10.1007/BF00011591

Garcia-Gil, J.C., C. Plaza, P. Soler-Rovira, and A. Polo. 2000. Long-term effects of municipal solid waste compost application on soil enzyme activities and microbial biomass. Soil Biol. Biochem. 32:1907-1913. doi:10.1016/S0038-0717(00)00165-6

Gauch, H.G., O.C. Magistad. 1943. Growth of strawberry clover varieties and of alfalfa and ladino clover as affected by salt. Agron. J. 35:871-880.

Gibson, A. H. and J. E. Harper. 1985. Nitrate effect on nodulation of soybean by Bradyrhizobium japonicum. Crop Sci. 25:497-501. doi:10.2135/cropsci1985.0011183X002500030015x

Goos, R.J., N. Abdraimova, and B.E. Johnson. 2015. Method for determination of ureides in soybean tissues. Commun. Soil Sci. Plant Anal. 46:242-429. doi:10.1080/00103624.2014.981276

Göttfert, M., P. Grob, H. Hennecke. 1990. Proposed regulatory pathway encoded by the nodV and nodW genes, determinants of host specificity in Bradyrhizobium japonicum. Proc. Natl. Acad. Sci. USA 87:268-284.

Hai, B., N. H. Diallo, S. Sall, F. Haesler, K. Schauss, M. Bonzi, K. Assegbetse, J.-L. Chotte, J.C. Hunch, S. Schloter. 2009. Quantification of key genes steering the microbial nitrogen cycle in the rhizosphere of sorghum cultivars in tropical Agroecosystems. Appl. Environ. Microbiol. 75:4993-5000. doi:10.1128/AEM.02917-08

Halvorson, J.J., J.L. Smith, R.I. Papendick. 1996. Integration of multiple soil parameters to evaluate soil quality: a field example. Biol. Fertil. Soils. 30:207-214. doi:10.1007/BF00335937

Hardy, R.F.W., R.C. Burns, R.D. Holsten. 1973. Application of the acetylene-ethylene assay for measurement of nitrogen fixation. Soil Biol. Biochem. 5:47-81. doi:10.1016/0038-0717(73)90093-X

Hassan, N.A.K., J.V. Drew, D. Knudsen, R.A. Olson. 1970. Influence of soil salinity on production of dry matter and uptake and distribution of nutrients in barely and corn. I. Barley (*Hordium vulgare*). Agron. J. 62:43-45. doi:10.2134/agronj1790.00219620062000100l4x

Hatzenpichler, R. E.V. Lebedeva, E. Spieck, K. Stoecker, A. Richter, H. Daims. 2008. A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring. Proc. Natl. Acad. Sci. U.S.A. 105:2134-2139. doi:10.1073/pnas.0708857105

Haynes, R.J. 1986. Mineral nitrogen in the plant-soil system. Academic Press. Syndey, Australia.

Hayward, H.E., R. Uhvits. 1944. The salt tolerance of wheat. *In*: United States salinity laboratory report to collaborators, Riverside, CA, USA.

He, J., J. Shen, L. Zhang, Y. Zhu, Y. Zheng, M. Xu, H. Di. 2007. Quantitative analyses of the abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea of a Chinese upland red soil under long-term fertilization practices. Environ. Microbiol. 9:2364-2374. doi:10.1111/j.1462-2920.2007.01358.x

He, Y., T.M. DeSutter, and D.E. Clay. 2013. Dispersion of pure clay minerals as influenced by Calcium/Magnesium ratios, sodium adsorption ratio, and electrical conductivity. Soil Sci. Soc. Am. J. 77:2014-2019. doi:10.2136/sssaj2013.05.0206n

Henry, H.A.L., J.D. Juarez, C.B. Field, and P.M. Vitousek. 2005. Interactive effects of elevated CO_2, N deposition and climate change on extracellular enzyme activity and soil density fractionation in a California annual grassland. Global Change Biol. 11:1808-1815. doi:10.1111/j.1365-2486.2005.001007.x

Herridge, D. F. 1982. Relative abundance of ureides and nitrate in plant tissues of soybean as a quantitative assay of nitrogen fixation. Plant Physiol. 70:1-6. doi:10.1104/pp.70.1.1

Herridge, D.F., S.K.A. Danso. 1995. Enhancing from legume N_2 fixation through selection and breeding. Plant Soil. 174:51-82. doi:10.1007/BF00032241

Hillel, D. 2000. Salinity management for sustainable irrigation, integrating science, environment and economics. The International Bank for Reconstruction and Development. Washington, D.C., USA.

Höberg, P. 1997. ^{15}N natural abundance in soil-plant systems. New Phytol. 137:179-203. doi:10.1046/j.1469-8137.1997.00808.x

Hoffman, G.J., E.V. Maas, S.L. Rawlins. 1975. Salinity-ozone interactive effects on alfalfa yields and water relations. J. Environ. Qual. 4:326-331. doi:10.2134/jeq1975.00472425000400030008x

Hume, D.J., D.H. Blair. 1992. Effect of numbers of *Bradyrhizobium japonicum* applied in commercial inoculants on soybean seed yield in Ontario. Can. J. Microbiol. 38:588-593. doi:10.1139/m92-097

Hungria, M., L.H. Boddey, M.A. Santos, M.A.T. Vargas. 1998. Nitrogen fixation capacity and nodule occupancy by *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* strains. Biol. Fertil. Soils. 27:393-399. doi:10.1007/s003740050449

Hungria, M., C.Y.M. Nishi, J. Cohn, and G. Stacey. 1996. Comparison between parental and variant soybean *Bradyrhizobium* strains with regard to the production of lecithin nodulation signals, early stages of root infection, nodule occupancy and N_2 fixation rates. Plant and Soil. 186:331-341.

Hungria, M. and G. Stacey. 1997. Molecular signals exchanged between host plants and rhizobia: basic aspects and potential application in agriculture. Soil Biol. Biochem. 29:819-830. doi:10.1016/S0038-0717(96)00239-8

Hungria, M. and M.A.T. Vargas. 1996. Exploring the microbial diversity and soil management practices to optimize the contribution of soil microorganisms to plant nutrition. In Stacey, G., B. Mullin, and P.M. Gresshoff (Eds). Biology of Plant-Microbe Interactions. Society of Molecular Plant-Microbe Interactions. St. Paul, MN, USA

Ilyas, M., R.W. Miller, R.H. Qureshi. 1993. Hydraulic conductivity of saline-sodic soil after gypsum application and cropping. Soil Sci. Soc. Am. J. 57:1580-1585. doi:10.2136/sssaj0361599500570006003 1x

Ilyas, M, R.H. Qureshi, and M.A. Quadir. 1997. Chemical changes in a saline-sodic soil after gypsum application and cropping. Soil Technol. 10:247-260. doi:10.1016/S0933-3630(96)00121-3

Johnson, D.D. and W.D. Guenzi. 1963. Influence of salts on ammonium oxidation and carbon dioxide evolution form soil. Proceedings of the Soil Science Society of America. 27:663-666.

Jordan, D.C. 1982. Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing, root nodule bacteria from leguminous plants. Int. J. Syst. Bacteriol. 32:136-139. doi:10.1099/00207713-32-1-136

Junier, P. O.-K. Sun, V. Molina, P. Limburg, T. Junier, J.F. Imhoff, K.-P. Witzel. 2008. Comparative *in silico* analysis of PCR primers suited for diagnostics and cloning of ammonia monooxygenase genes from ammonia-oxidizing bacteria. FEMS Microbiol. Ecol.. 64:141-152. doi:10.111/j.1574-6941.2007.00437.x

Kaddah, M.T., S.I. Ghowali. 1964. Salinity effects on the growth of corn at different stages of development. Agron. J. 56:214-217. doi:10.2134/agronj.00021962005600020028x

Kandeler, E., C. Poll, W.T. Frankenberger Jr. 2011. Nitrogen cycle enzymes. *In*: Dick, R.P. (ed). Methods of soil enzymology. Soil Science Society of America Book Series, no. 9. Soil Science Society of America, Madison, WI, USA.

Kanchikerimath, M., D. Singh. 2001. Soil organic matter and biological properties after 26 years of maize-wheat-cowpea cropping as affected by manure and fertilization in a Cambisol in semiarid region of India. Agric. Ecosyst. Environ. 86:155-162. doi:10.1016/S0167-8809(00)00280-2

Katerji, N., J.W. van Hoorn, A. Hamdy, M. Mastrorilli. 2000. Salt tolerance classification of crops according to salinity and to water stress day index. Agric. Water Manage. 43:99-109. doi:10.1016/S0378-3774(99)00048-7

Kaur, A., A. Chaudhary, A. Kaur, R. Chudhary and R. Kaushik. 2005. Phospholipid fatty acid-a bioindicator of environment monitoring and assessment in soil ecosystem. Current Sci. 89:1103-1112.

Keren, R., I. Shainberg. 1981. Effect of dissolution rate on the efficiency of industrial and mined gypsum in improving infiltration of a sodic soil. Soil Sci. Soc. Am. J. 45:103-107. doi:10.2136/sssaj1981.03615995004500010022x

Keyser, H.H. and F. Li. 1992. Potential for increasing biological nitrogen fixation in soybean. Plant and Soil. 141:119-135. doi:10.1007/BF00011313

Kuykendall, L.D., B. Saxena, T.E. Cevine, S.E. Udell. 1992. Genetic diversity in *Bradyrhizobium* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. Can. J. Microbiol. 38:501-505. doi:10.1139/m92-082

Kuykendall, L.D., D.F. Weber. 1978. Genetically marked *Rhizobium* identifiable as inoculum strain in nodules of soybean plants grown in fields populated with *Rhizobium japonicum*. Appl. Environ. Microbiol. 36:915-919. doi:0099-2240/78/0036-0915$02.00/0

Lakdhar, A. C. Hafsi, M. Rabhi, A. Debez, F. Montermurro, C. Abdelly, N. Jedidi and Z. Ouerghi. 2008. Application of municipal solid waste compost reduces the negative effects of saline water in *Hordeum maritimum* L. Bioresour. Technol. 99:7160-7167. doi:10.1016/j.biortech.2007.12.071

Leininger, S., T. Urich, M. Schloter, J. Qi, G.W. Nicol, J.I. Prosser, S.C. Schuster, C. Schleper. 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature. 442:e04983. doi:10.1038/nature04983

Li, M., Cao, H., Hong, Y., & Gu, J. D. (2011). Spatial distribution and abundances of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) in mangrove sediments. Appl. Microbiol. Biotechnol. 89:1243-1254. doi: 10.1007/s00253-010-29259-0

Liang, Y., M. Nikolic, Y. Peng, W. Chen, and Y. Jiang. 2005. Organic manure stimulates biological activity and barley growth in soil subject to secondary salinization. Soil Biology & Biochemistry 37, 1185–1195. doi:10.1016/j.soilbio.2004.11.017

Lindsay, W. 1979. Chemical equilibria in soils. John Wiley. New York, NY, USA.

Lobell, D. B., S. M. Lesch, D. L. Corwin, M. G. Ulmer, K. A. Anderson, D. J. Potts, J. A. Doolittle, M. R. Matos, M. J. Baltes. 2009. Regional-scale assessment of soil salinity in the Red River Valley using multi-year MODIS EVI and NDVI. J. Environ. Qual. 39:35-41. doi:10.2134/jeq2009.0140

Lowther, W.L., M.D. Rumbaugh, D.A. Johnson. 1987. Populations of *Rhizobium meliloti* in areas with rangeland alfalfa. J. Range Manage. 40:468-271. doi:10.2307/3899095

Lyons, J. C. and Earley, E. B. 1952. The effect of ammonium nitrate applications to field soils on nodulation, seed yield, and nitrogen and oil content of the seed of soybeans. Soil Sci. Soc. of Am. Proc. 16: 259-263.

Mahanta, K.K, G.C. Mishra, and M.L. Kansal. 2014. Estimation of the electric double layer thickness in the presence of two types of ions in soil water. Appl. Clay Sci. 87:212-218. doi:10.1016/j.clay.2013.11.007

Malik, K.A. and K. Haider. 1997. Decomposition of carbon-14-labelled plant material in saline-sodic soils. In: Soil organic matter studies. Proceedings of a symposium organized by IAEA, FAO and Arochimica 1, 215-225. International Atomic Energy Agency, Vienna, Austria.

Mao Y., A. C. Yannarell, and R. I. Mackie. 2011. Changes in N-transforming Archaea and Bacteria in soil during the establishment of bioenergy crops. PLOS ONE 6(9): e24750.10.1371/journal.pone.0024750.

Marinari, S., R. Mancinelli, E. Campiglia, and S. Grego. 2006. Chemical and biological indicators of soil quality in organic and conventional farming systems in Central Italy. Ecol. Indic. 6:701-711. doi:10.1016/j.ecolind.2005.08.029

Martens-Habbena, W., P.M. Berube, H. Urakawa, J.R. de la Torre, and D.A. Stahl. 2009. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. Nature. 461:976-9. doi: 10.1038/nature08465

Martinez, E., D. Romero, and R. Palacios. 1990. The Rhizobium genome. Crit. Rev. Plant Sci. 9:59-93. doi:10.1080/07352689009382282

Means, U. M., H. W. Johnson, and R. A. Date. 1964. Quick serological method of classifying strains of rhizobium japonicum in nodules. J. Bacteriol. 87:547-553.

McCune, B. and J.B. Grace. 2006. Analysis of Ecological Communities. MjM Software Design. Glenden Beach, OR, USA.

McNeal, B.L., D.A. Layfield, W.A. Norvell, J.D. Rhoades. Factors influencing hydraulic conductivity of soils in the presence of mixed-salt solutions. Soil Sci. Soc.Am. J. 32:187-190. doi:10.2136/sssaj1968.03615995003200020012x

Moftah, A.E., B.E. Michel. 1968. The effect of sodium chloride on solute potential and proline accumulation in soybean leaves. Plant Physiol. 83:238-240. doi:10.1104/pp.83.2.238

Munns, R., A. Termaat. 1986. Whole-plant responses to salinity. Aust. J. Plant Physiol. 13:143-160. doi:10.1071/PP9860143

Nakayama, F.S. 1969. Theoretical consideration of the calcium sulfate-bicarbonate-carbonate interaction in soil solution. Soil Sci. Soc. Am. Proc. 33:668-672.

Nannipieri, P. L. Giagoni, G. Renella, E. Puglisi, B. Ceccanti, G. Masciandaro, F. Fornasier, M.C. Moscatelli, and S. Marinari. 2012. Soil enzymology: classical and molecular approaches. Bio Fertil Soils. 48:743-762. doi:10.1007/s00374-012-0723-0

Narożna, D. K. Pudelko, J. Króliczak, B. Golińska, M. Sugawara, C.J. Madrzak, M.J. Sadowsky. Survival and competitiveness of *Bradyrhizobium japonicum* strains 20 years after introduction into field locations in Poland. Appl. Environ. Microbiol. 81:5552-5559. doi:10.1128/AEM.01399-15

NASS [National Agricultural Statistics Service]. 2015. Statistics by subject-National statistics for soybean in North Daktoa. http://quickstats.nass.usda.gov/results/AC6583D7-1E57-32B6-89EF-37419FD44B15?pivot=short_desc. Accessed 16 Jan 2015.

Nelson, P.N., J.N. Ladd, and J.M Oades. 1996. Decomposition of ^{14}C-labelled plant material in a salt-affected soil. Soil Biol. Biochem. 28:433-441. doi:10.1016/0038-0717(96)00002-8

Nelson, P.N. and J.M. Oades. 1996. Organic matter, sodicity and soil structure. In: Sumner, M.E. and R. Naidu (Eds). Sodic soils: distribution, processes, management and environmental consequences. Oxford University Press, New York, NY, USA.

Niemi, R.M. and M. Vepsalainen. 2005. Stability of the fluorogenic enzyme substrates and pH optima of enzyme activities in different Finnish soils. J. Microbiol. Methods. 60:195-205.

Nishi, C.Y.M., L.H. Boddey, M.A.T. Vargas, and M. Hungria. 1996. Morphological, physiological and genetic characterization of two new *Bradyrhizobium* strains recently recommended in Brazilian commercial inoculants for soybean. Symbiosis. 20:147-162.

Norton, J.M., J.J. Alzerreca, Y. Suwa, M.G. Klots. 2002. Diversity of ammonia monooxygenase operon in autrotrophic ammonia-oxidizing bacteria. Arch. Microbiol. 177:139-149. doi:10.1007/s00203-001-0369-z

Oldeman, L.R. 1994. The global extent of soil degradation. In: Greenland, D.J. and I. Szabolcs (Eds.). Soil resilience and sustainable land use. CAB International, Wallingford, Oxon, UK.

Oster, J.D., H. Frenkel. 1980. The chemistry of the reclamation of sodic soils with gypsum and lime. Soil Sci. Soc. Am. J. 44:41-45. doi:10.2136/sssaj1980.03615995004400010010x

Ouni, Y, A. Lakhdar, R. Scelza, R. Scotti, C. Abdelly, Z. Varhouni and M.A. Rao. 2013. Effects of two composts and two grasses on microbial biomass and biological activity in a salt-affected soil. Ecol. Eng. 60:363-369. doi:10.1016/j.ecoleng.2013.09.002

Pankhurst, C.E., B.M. Doube, and V.V.S.R. Gupta. 1997. Biological indicators of soil health. CAB International. Wallingford, England, UK.

Paul, E. A. 1984. Dynamics of organic matter in soils. Plant Soil. 76:275-285. doi:10.1007/BF02205586

Peck, J. E. 2010. Multivariate analysis for community ecologists. MjM Software Design. Gleneden Beach, OR, USA.

Peng, G.X., Z.Y. Tan, E.T. Wang, B. Reinhold-Hurek, W.F. Chen, W.X. Chen. 2002. Identification of isolates from soybean nodules in Xinjiang region as *Sinorhizobium xinjiangense* and a genetic differentiation of *S. xinjiangense* from *Sinorhizobium fredii*. International Journal of Systematic and Evolutionary Microbiology. 52:457-462. doi:10.1099/ijs.0.01921-0

Peoples, M.B., R.M. Boddey, D.F. Herridge. 2002. Quantification of nitrogen fixation. *In*: Leigh G.J. (ed.) Nitrogen fixation at the millennium. Elsevier Science, Amsterdam, the Netherlands.

Peoples, M.B., D.F. Herridge. 1990. Nitrogen fixation by legumes in tropical and sub-tropical agriculture. Advances in Agronomy, Volume 44. Academic Press, London, United Kingdom

Peoples, M.B., M.J. Unkovich, D.F. Herridge. 2009. Measuring symbiotic nitrogen fixation by legumes. *In*: Emerich, D.W., H.B. Krishnan (Eds). Nitrogen fixation in crop production. Agronomy Monograph no. 52. American Society of Agronomy, Madison, WI, USA.

Perkins, A. T. 1925. The effect of bacterial numbers on the nodulation of Virginia soybeans. J. Agric. Res. 30: 95-96.

Pessarakli, M. and I. Szabolcs. 1999. Soil salinity and sodicity as particular plant/crop stress factors. In: Pessarkli, M. (ed) Handbook of plant and crop stress, second edition, revised and expanded. Marcel Dekker, New York, NY, USA.

Philippot, L., 2005. Tracking nitrate reducers and denitrifiers in the environment. Biochem. Soc. Trans. 33:200-204. doi:10.1042/BST0330200

Polacco, J.C. 1976. Nitrogen metabolism in soybean tissue culture. Plant Physiol. 58:350-357. doi:10.1104/pp.58.3.350

Prosser, J.I. and G.W. Nicol. 2012. Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. Trends Microbiol. 20(11), 523-531 doi:10.1016/j.tim2012.08.001

Qadir, M., J.D. Oster. 2004. Crop and irrigation management strategies for saline-sodic soils and water aimed at environmentally sustainable agriculture. Sci. Total Environ. 323:1-19. doi:10.1016/j.scitotenc.2003.10.012

Rahimi, H., E. Pazira, F. Tajik. 2000. Effect of soil organic matter, electrical conductivity and sodium adsorption ratio on tensile strength of aggregates. Soil Tillage Res. 45:145-153. doi:10.1016/S0167-1987(00)00086-6

Rao, D.L.N. and H. Pathak, 1996. Ameliorative influence of organic matter on biological activity of salt-affected soils. Arid Soil Res. Rehab. 10:311-319. doi:10.1080/15324989609381446

Reeve, R.C., C.A. Bower. 1960. Use of high salt waters as a flocculant and source of divalent cations for reclaiming sodic soils. Soil Sci. 90:139-144. doi:10.1097/00010694-196008000-00011

Rencher, A.C., W.F. Christensen. 2012. Methods of multivariate analysis 3rd edition. Wiley and Sons, Inc. Hoboken, NJ, USA.

Rengasamy, P. and K.A. Olsson. 1991. Sodicity and soil structure. Aust. J. Soil Res. 29:935-952. doi:10.1071/SR9910935

Rice, C. W., T. B. Moorman, and M. Beare. 1996. Role of microbioal biomass and carbon and nitrogen in soil quality. In: Doran, J.W. and A. J. Jones [eds.] Methods for assessing soil quality. Soil Science Society of America Special Publication Number 49. Madison, Wisconsin, 1996.

Richards, L.A. (ed) 1954. Diagnosis and Improvement of Saline and Alkali Soils, U.S. Salinity Laboratory Staff. Handbook 60. U.S. Dept. Agric., Washington, D.C.

Rietz, D.N. and R.J.Haynes. 2003. Effects of irrigation-induced salinity and sodicity on soil microbial activity. Soil Biol. Biochem. 35:845-854. doi:10.1016/S0038-0717(03)00125-1

Robbins, C.W. 1985. The $CaCO_3$-CO_2-H_2O system in soils. J. Agron. Edu. 14:3-7.

Robbins, C.W. 1986. Sodic calcareous soil reclamation as affected by different amendments and crops. Agron. J. 78:916-920. doi:10.2134/agronj1986.00021962007800050034x

Roh, S.W., G.C.J. Abell, K.-H. Kim, Y.-D. Nam, J.-W. Bae. 2010. Comparing microarrays and next-generation sequencing technologies for microbial ecology research. Trends Biotechnol. 28:291-299. doi:10.1016/j.tibtech.2010.03.001

Rousseau, G.X., O. Deheuvels, I. Rodrigues Arias, and E. Somarriba. 2012. Indicating soil quality in cacao-based agroforestry systems and old-growth forests: The potential of soil macrofauna assemblage. Ecol. Indic. 23:535:543. doi:10.1016/j.ecolind.2012.05.008

Saeki, Y., N. Aimi, S. Tsukamoto, T. Yamakawa, Y. Nagamoto, and S. Akao. 2006. Diversity and geographical distribution of indigenous soybean-nodulating Bradyrhizobium in Japan. J. Plant Nutr. Soil Sci.. 52:418-426. doi:10.1111/j.1747-0765.2006.00050.x

Saleem, T.M., and N. Ahmed. 1988. Soil fertility research and fertilizer use in Pakistan. *In*: Managing soil resources: Proceedings of the First National Congress on Soil Science, 93–97. Lahore, Pakistan: Soil Science Society of Pakistan.

Sarig, S. and Y. Steinberger. 1994. Microbial biomass response to seasonal fluctuation in soil salinity under the canopy of desert halophytes. Soil Biol. Biochem. 26:1405-1408. doi:10.1016/0038-0717(94)90224-0

Schipper, L.A. and G.P. Sparling. 2000. Performance of soil condition indicators across taxonomic groups and land uses. Soil Sci. Soc. Am. J. 64:300-311. doi:10.2136/sssaj2000.641300x

Schimel, J.P., M.N. Weintraub. 2003. The implications of exoenzyme activity on microbial carbon and nitrogen limitation in soil: a theoretical model. Soil Biol. Biochem. 35:549-563. doi:10.1016/S0038-0717(03)00015-4

Schleper, C. 2010. Ammonia oxidation: different niches for bacteria and archaea. ISME J. 4:1092-1094. doi:10.1038/ismej.2010.111

Scholla, M.H. and H.G. Elkan. 1984. *Rhizobium fredii* sp. nob. A fast-growing species that effectively nodulates soybeans. Int. J. System. Bacteriol. 34:484-486. doi: 10/1984; 34(4). doi:10.1099/00207713-34-4-484

Shearer, G., D.H. Kohl. 1986. N_2-fixation in field settings: estimations based on natural ^{15}N abundance. Aust. J. Plant Physiol. 13:699-756. doi:10.1071/PP9860699

Shukla, M.K., R. Lal, and M. Ebinger. 2006. Determining soil quality indicators by factor analysis. Soil Tillage Res. 87:194-204. doi:10.1016/j.still.2005.03.011

Singleton, P. W. and J. W. Tavares. 1986. Inoculation response of legumes in relation to the number and effectiveness of indigenous Rhizobium populations. Appl. Environ. Microbiol. 51:1013-1018.

Sims A., J. Horton, S. Gajaraj, S. McIntosh, R.J. Miles, R. Mueller, R. Reed and Z. Hu. 2012. Temporal and spatial distributions of ammonia-oxidizing archaea and bacteria and their ratio as an indicator of oligotrophic conditions in natural wetlands. Water Res. 46:4121-4129. doi:10.1371/journal.pone.0113515

Sinsabaugh, R.L., D. Moorhead. 1994. Resource allocation to extracellular enzyme production: a model for nitrogen and phosphorus control of litter decomposition. Soil Biol. Biochem. 26:1305-1311 doi:10.1016/0038-0717(94)90211-9

Siqueria, A.F., E. Ormeno_Orrilly, R.C. Souza, E.P. Rodrigues. L.G.P. Almeida, F.G. Barcellos, H.S.S. Batista, A.S. Nakatani, E.Martínez-Romero, A.T.R. Vasconcelos, M. Hungria. 2014. Comparative genomics of *Bradyrhizobium japonicum* CPAC 15 and *Bradyrhizobium diazoefficiens* CPAC 7: elite model strains for understanding symbiotic performance with soybean. BMC Genomics. 15:420. doi:10.1186/1471-2164-15-420

Skujiņŝ, J. 1967. Enzymes in soil. *In*: A.D. McLaren, G.H. Peterson (eds.) Soil biochemistry. Vol. 1. Marcel Dekker, New York, NY, USA.

Strihker, D. 2005. Marginal lands in Europe- causes of decline. Basic Appl. Ecol. 6:99-106. doi:10.1016/j.baae.2005.01.001

Stubbs, M. 2007. Land conversion in the Northern Plains. Congressional Research Service Report for Congress, April 5, 2007, order number RL33950. Washington, DC: Congressional Research Service.

Sumner, M.E. 1993. Sodic soils-new perspectives. Aust. J. Soil Res. 31:683-750. doi:10.1071/SR9930683

Sylvia, D.M., Fuhrmann, J.J., Hartel, P.G., and Zuberer, D.A., 2005. Principles and Applications of Soil Microbiology. 2nd Edition. 373 - 404.

Tan, K.H. 1982. Principles of soil chemistry. Marcel Dekker, Inc.

Tan, Z.Y., Z.D. Xu, E.T. Wang, J.L. Gao, E. Martinex-Romero, W.X. Chen. 1997. Phylogenetic and genetic relationships of *Mesorhizobium tianshanense* and related rhizobia. Int. J. System. Bacteriol. 47:874-879. doi:0020-7713/97/$04.00 + 0

Tanji, K.K., N.C. Kielen. 2002. Agricultural drainage water management in arid and semi-arid areas. FAO irrigation and drainage paper 61. Food and Agriculture Organization of the United Nations, Rome, Italy.

Taylor AE, L.H. Zeglin, T.A.Wanzek, D.D. Myrold and P.J. Bottomley. 2012. Dynamics of ammonia-oxidizing archaea and bacteria populations and contributions to soil nitrification potentials. ISME J. 6:2024–2032. doi:10.1038/ismej.2012.51

Taylor, A.E., L.H. Zeglin, S. Dooley, D.D. Myrold, and P.J. Bottomley. 2010. Evidence for different contributions of archaea and bacteria to the ammonia-oxidizing potential of diverse Oregon soils. Appl. Environ. Microbiol. 76:7691-7698. doi:10.1128/aem.01324-10

Tejada, M. C. Garcia, J.L. Gonzalez, and M.T. Hernandez. 2006. Use of organic amendment as a strategy for saline soil remediation: Influence on the physical, chemical and biological properties of soil. Soil Biol. Biochem. 38:1413-1421. doi:10.1016/j.soilbio.2005.10.017

Tejada, M. J.L. Gonzalez. 2005. Beet vinasse applied to wheat under dryland conditions affects soil properties and yield. Eur. J. Agron. 23:336-347.

Thies, J. E., P. W. Singleton and B. B. Bohlool. 1991. Influence of the size of indigenous rhizobia populations on establishment and symbiotic performance of introduced rhizobia on field-grown legumes. Appl. Environ. Microbiol. 57:19-28.

Tillman, D. C. Balzer, J. Hill, and B. L. Befort. 2011. Global food demand and the sustainable intensification of agriculture. Proc. Natl. Acad. Sci. U.S.A.. 108:20260-20264. DOI:10.1073/pnas.1116437108.

Unkovich, M.J., D.F. Herridge, M.B. Peoples, R.M. Boddey, G. Cadisch, K.E. Giller, B. Alves, P. Chalk. 2008. Measuring plant-associated nitrogen fixation in agricultural systems. ACIAR Monogr. 136. Commonwealth of Australia, Canberra, Australia.

Unkovich, M.J., J.S. Pate. 2000. An appraisal of recent field measurements of symbiotic N_2 fixation by annual legumes. Field Crops Res. 65:211-228. doi:10.1016/S0378-4290(99)00088-X

USDA [United States Department of Agriculture, Natural Resources Conservation Service]. 2014. Soil health assessment. http://www.nrcs.usda.gov/wps/portal/nrcs/main/soils/health/assessment/. Accessed on 27 October 2014.

van Olphen, H. 1963. An introduction to clay colloid chemistry. John Wiley & Sons. New York, NY, USA.

Vessey, J.K. 1994. Measurement of nitrogenase activity in legume root nodules: in defense of the acetylene reduction assay. Plant Soil. 158:151-162. doi: 10.1007/BF00009490

Vinuesa, P. K. Rohas-Jiménez, B. Contreras-Moreira, S.K. Mahana, B.N. Prasad, H. Moe, S.B. Selvaraju, H. Thiefelder, D. Werner. 2008. Multilocus sequence analysis for assessment of the biogeography and evolutionary genetics of four *Bradyrhizobium* species that nodulate soybeans on the Asiatic continent. Appl. Environ. Microbiol. 74:6987-6996. doi:10.1128/AEM.00875-08

Wallenius, K., H. Rita, S. Simpanen, A. Mikkonen, R.M. Niemi. 2010. Sample storage for soil enzyme activity and bacterial community profiles. J. Microbiol. Methods. 81:48-55. doi:10.1016/j.mimet.2010.01.021

Weaver, R. W. and Frederick, L. R. 1972. A new technique for most-probable-number counts of Rhizobia. Plant and Soil. 36:219-222. doi:10.1007/BF01373474

Wessén, E. and S. Hallin. 2011. Abundance of archaeal and bacterial ammonia oxidizers-possible bioindicator for soil monitoring. Ecol. Indic. 11:1696-1968. doi:10.1016/j.ecolind.2011.04.018

Wessén, E., M. Söderström, M. Stenberg, D. Bru, M. Hellman, A. Welsh, F. Thomsen, L. Klemedtson, L. Philippot, and S. Hallin. 2011. Spatial distribution of ammonia-oxidizing bacteria and archaea across a 44-hectare farm related to ecosystem functioning. ISME J. 5: 1213–1225. doi:10.1038/ismej.2010.206

Wright, W. H. 1925a. The nodule bacteria of soybeans. I. Bacteriology of strains. Soil Sci 20: 95-129.

Wright, W. H. 1925b. The nodule bacteria of soybeans. II. Nitrogen-fixation experiments. Soil Sci 20:131-141.

Xu, M.L., C. Ge, Z. Cui, J. Li, H. Fan. 1995. *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybeans. Int. J. System. Bacteriol. 45:706-711. doi:10.1099/00207713-45-4-706

Yao, Y.Z., F.L. Kan, E.T. Wang, G.H. Wei, X. Chen. 2002. Characterization of rhizobia that nodulate legume species of the genus *Lezpedeza* and description of *Bradyrhizobium yuanmingense* sp. nov. Int. J. Syst. Evol. Microbiol. 52:2219-2230. doi:10.1099/ijs.0.01408-0

Zahran, H.H. 1997. Diversity, adaptation and activity of the bacterial flora in saline environments. Biol. Fertil. Soils. 25:211-223. doi:10.1007/s003740050306

Zapata, F., S.K.A. Danso, G. Hardarson, and M. Fried. 1987. Time course of nitrogen fixation in field-grown soybean using nitrogen-15 methodology. Agron. J. 79:172–176. doi:10.2134/agronj1987.00021962007900010035x

Zhang, L., W. Chen, M. Burger, L. Yang, P. Gong, Z. Wu. 2015. Changes in soil carbon and enzyme activity as a result of different long-term fertilization regimes in a greenhouse field. PLoS ONE. 10:e0118371. doi:10.1371/journal.pone.0118371

Zelles, L. 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterization of microbial communities in soil: A review. Biol. Fertil. Soils. 29**:**111–129. doi:10.1007/s003740050533

PAPER 1: BIOLOGICAL INDICATORS PROVIDE SHORT-TERM SOIL HEALTH ASSESSMENT DURING SODIC SOIL RECLAMATION[1]

Abstract

Sodic soil remediation is an expensive, lengthy process during which producers need tools to demonstrate that sodium (Na^+) remediation practices are improving soil health. The objective of this study was to determine if soil biological indicators can provide a short term assessment of the effectiveness of chemical management strategies used to remediate northern Great Plains Na^+ affected soils. This randomized block research experiment was conducted in a grassland which was converted to annual row crops. The soil at the site was an Exline (fine, smectitic, frigid Leptic Natrudolls). The experiment contained two drainage (tile drained and no-drainage) and four chemical amendments (11.2 Mg ha^{-1} of gypsum, 22.4 Mg ha^{-1} of gypsum, 22.4 Mg ha^{-1} spent sugar beet lime, and a no amendment control). Base-line soil samples for biological assessment were collected in the fall of 2012 after tile drainage was installed. The sodium adsorption ratio (SAR) ranged from 0.4 to 16.7 with a range of electrical conductivity (EC) of 0.4 to 0.8 dS m^{-1}. Gypsum and lime amendments were applied in 2013. Soil samples were collected for assessing soil health before and after application of amendments and throughout the growing season. This study utilizes a novel application of successional vector trajectories to compare shifts in measured soil health parameters associated with land use change and remediation of sodicity. Soil samples were analyzed for percent total soil carbon (C), nitrifier

[1] The material in this chapeter was co-authored by Heather Dose, Ann-Marie Fortuna, Larry Cihacek, Jack Norland, Thomas DeSutter, David Clay and Joel Bell. Heather Dose had primary responsibility for collecting samples in the field and was the primary developer of the conclusions that are advanced here. Heather Dose also drafted and revised all versions of this chapter. Ann-Marie Fortuna served as proofreader and checked the math in the statistical analysis conducted by Heather Dose.

and denitrifier gene copies, soil enzyme assays (nitrate reductase, ammonia monooxegenase, urease, β-glucosidase, alkaline phosphatase, arylsulfatase and fluorescein diacetate hydrolysis), EC, pH, SAR, and soil texture. Gene copies and enzyme activities were successfully used to differentiate between land uses and amendment applications. Ammonia oxidizing bacterial gene copies were higher where cropland was amended with gypsum. Successional vectors verified a significant shift in soil health due to land use change and amendment application. Gypsum applications reduced SAR, but increased soil EC. This work demonstrates that soil enzyme activities and gene copy numbers can be used to detect improvements in soil health.

Introduction

Worldwide, salt-affected soils encompass 10% of Earth's terrestrial surface (Pessarakli and Szabolcs, 1999 and Szabolcs, 1999). Currently, the distribution of sodic soils worldwide is increasing due to poor land management decisions and irrigation with poor quality water (Qadir and Oster, 2004). Increases in sodic soil distribution have occurred in China, central Asia (Cai et al., 2003; Gupta and Abrol, 2000), and Australia where nearly 60% of the agricultural land is sodic (Rengasmy, 2006). In the United States, sodic soils are defined as having a sodium adsorption ratio (SAR) (ratio of Na^+ to Ca^{2+} and Mg^{2+}) of 13 or greater, an exchangeable Na^+ percentage greater than 15, electrical conductivity (EC) of less than 4 dS m^{-1}, and pH of 8.5 or greater (Richards, 1954). Excess Na^+ on the cation exchange sites causes clay particles to disperse or swell, and as a consequence these soils have poor structure, low aggregate stability, and reduced water infiltration (Rengasamy and Olsson, 1991). Overall, sodic soils are a poor rooting medium for plant growth and provide lowered or insufficient nutrients. Sodic soils also have reduced biological activity and function due to the limited availability of C substrates that are likely the result of lowered net primary productivity in these soils (Rao and Pathak, 1996).

Remediating the effects of excess Na^+ in sodic soils can be accomplished with soil amendments and land management. Calcium amendments have been shown to reduce the effects of sodicity. Calcium flocculates clay particles leading to improvements in soil structure (Frenkel et al., 1989). Calcium also replaces Na^+ on soil exchange sites and is frequently correlated with increases in soluble Na^+ (Ilyas et al., 1997). Rates of gypsum application can be calculated by taking into account soil cation exchange capacity, target SAR, and current SAR values (Ashworth et al., 1999). After chemical treatment subsurface tile drainage may be used to remove excess Na^+ from the rooting zone (Pessarakli and Szabolcs, 1999). Subsurface drainage can also prevent salt accumulation due to fluctuations in water table depth, capillary rise, and evaporation (Abrol et al., 1988).

In order to provide advice to growers with respect to whether their management strategies have begun to bring about the changes they anticipated, a tool capable of detecting short-term improvements is needed. Successful remediation of sodicity may take years and can be costly (Qadir and Oster, 2002). Soil health is defined as "the capacity of soil to function within ecosystems and land-use boundaries to sustain biological productivity, maintain environmental quality, and promote plant, animal, and human health" (Doran and Parkin, 1994). Use of biological indicators of soil health as a proxy for shifts in nutrient cycling resulting from land use change, amendment application, and tile drainage installation will aid in the early detection of effective remediation strategies, potentially reducing the cost and environmental impact of remediation (Ritz et al., 2009; Wessén and Hallin, 2011; Wessén et al., 2011; Fortuna et al., 2012). Additionally, identifying soil health indicators and monitoring changes in these soil properties will aid land owners in ensuring the long–term productivity of the land. Currently, biological soil health indicators are not widely used to assess remediation progress.

The objectives of this study were to: (1) derive a set of soil health indicators that include functional gene copy numbers and soil enzyme activities in order to assess the effectiveness of these bioindicators in differentiating among land use change, application of soil amendments, and tile drainage treatments used for Na^+ remediation; and (2) determine the magnitude of shifts in soil health parameters using successional vector trajectories following land use and management changes in a northern Great Plains sodic soil.

Materials and methods

Study site

The study area was located in Richland County, North Dakota (46° 16' 53.843"N, 97° 15' 26.893"W) and has a continental climate with an average temperature of 5.8°C with a 20 year mean annual precipitation of 465 mm (NDAWN, 2014). The cumulative rainfall throughout the study period is shown in Figure 2. The study site was located on a sodic Exline loam, sandy substratum soil (Fine, smectitic, frigid Leptic Natrudolls) (Soil Survey Staff, 2014). This soil series was formed from Glacial Lake Agassiz sediments, is characterized by high water tables and is affected by excess sodium chloride (NaCl), sodium sulfate ($NaSO_4$), calcium sulfate ($CaSO_4$), and magnesium sulfate ($MgSO_4$) salts (Franzen 2003).

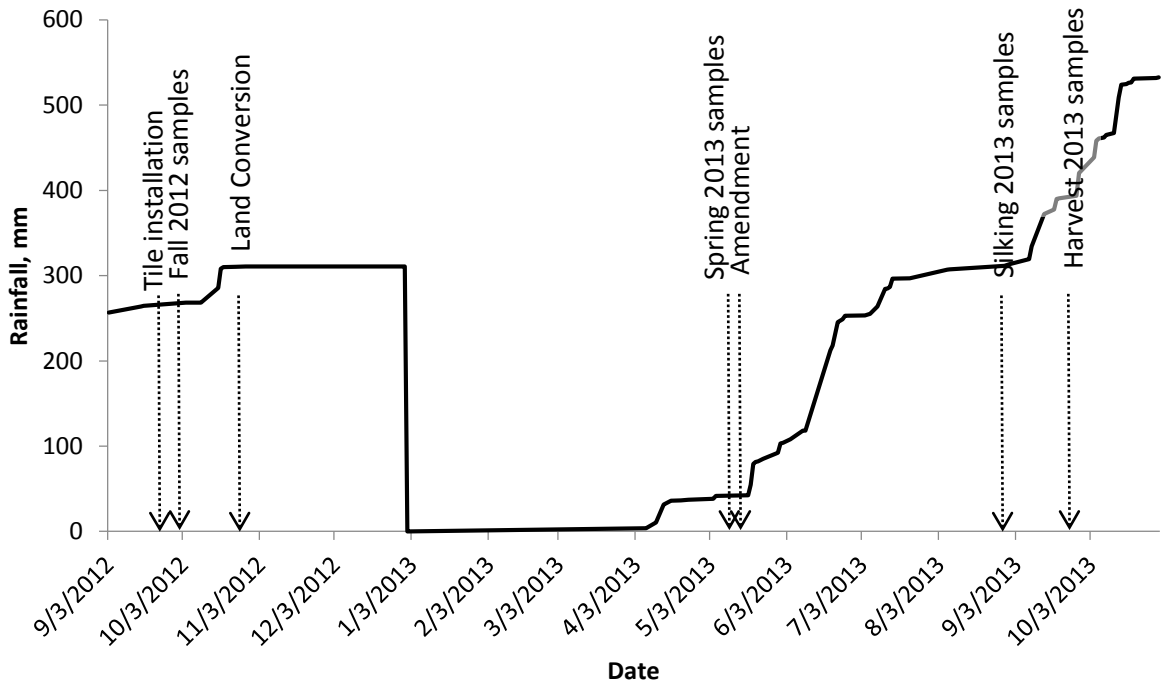

Figure 2. The cumulative rainfall (mm) at the site as measured throughout the study period.

Experimental design

At the onset of the experiment the field was managed as a long–term cool-season perennial hayland. In 2012, tile drainage was installed and the field was plowed, disked, and prepared to be seeded with annual crops. The field experimental design was a randomized complete block split-plot. The experiment contained 3 blocks and each plot was 24 x 24 m. The factorial treatments were 2 drainage treatments and 4 chemical amendments. Whole plot treatments included free drainage and a no drainage control. Tile drainage (10.2 cm diameter, sleeved) was installed to a 1.2 m depth. Four soil amendments were applied on May 14 and 15, 2013. Split plot treatments included soil amendments of 11.2 Mg ha^{-1} of gypsum, 22.4 Mg ha^{-1} of gypsum, 22.4 Mg ha^{-1} spent sugar beet lime, and a no amendment control. Sugar beet lime, which is predominantly calcium carbonate, is a by-product of sugar purification (American Crystal Sugar, 2008) and is readily available locally. The 22.4 Mg ha^{-1} gypsum was spread using

a spinner type broadcast spreader. The 22.4 Mg ha^{-1} lime and 11.2 Mg ha^{-1} gypsum were spread evenly across plots by hand. Fertilizer was applied prior to planting at a rate of 168.0 kg N ha^{-1}, 67.3 kg P ha^{-1}, 16.8 kg K ha^{-1} potassium and 1.9 kg Zn ha^{-1}. Corn (*zea mays* L.) was planted on May 15 and 16 of 2013.

Soil sampling

Soils were sampled from a 1 m^2 geo-referenced area offset by 6 m of the installed tile drainage lines or from the plot center in no drain plots. Three composited soil samples were collected from each plot to a 0 to 30 cm depth using a JMC mud auger (8.3 cm diameter, Clements Associates, Inc., Newton, IA, USA). Soils were stored at -20°C until further analysis. Soil was collected on October 2 (fall 2012), May 10 (spring 2013), August 1, 2013 (corn silking), and September 27 (fall 2013) (Fig. 2).

Chemical and physical analysis of soil

The chemical analysis for pH, EC, SAR, concentration of Mg^{2+}, Ca^{2+}, and Cl^-, total soil carbon (TC), and inorganic soil carbon (IC) was conducted on air-dried ground (<2mm) soil. Both soil pH and EC were measured on a 1:1 soil to water ratio as described by Combs and Nathan (1998) and on saturated paste extracts as described by Rhodes (1996). An atomic adsorption spectrophotometer (Buck Scientific Model 200A, East Norwalk, CT, USA) was used to determine concentrations of Na^+, Mg^{2+}, and Ca^{2+} in saturated paste extracts. The SAR was then calculated as outlined by Richards (1954). An ion specific electrode (Cole-Parmer combination Cl^- electrode, Vernon Hills, IL, USA) was used to measure Cl^- on the saturated paste extract. An Elementar Vario MACRO cube CNHS analyzer was used to test TC (Elementar Americas Inc. Mt. Laurel, NJ, USA). Inorganic C was removed using 1M phosphoric

acid and the difference between TC and IC was considered to be total organic carbon (TOC). Total soil C and IC were measured once and assumed to be constant throughout the study.

Soil texture was determined once on air dried, ground soils sampled in fall 2012. Silt and clay contents were measured using the hydrometer method outlined by Gee and Bauder (1986). Sand contents were determined by sieving. Gravimetric water content was determined for all field samples on each sample date.

DNA extraction and functional gene quantification

DNA was extracted from soils in order to determine if functional gene copy numbers can be used as a sensitive indicator of nutrient cycling and overall soil health. Functional gene copy numbers represent the population of organisms capable of producing an enzyme required for the turnover of a given nutrient such as N, P, or S. Rapid changes in copy numbers can occur as organisms reduce or increase their metabolic response to shifts in nutrient availability. Gene copy numbers can be used as either short–term or long–term indicators of soil health which reflect overall ecosystem health. Gene copies are assessed via quantitative Polymerase Chain Reaction (qPCR) using primers specified for a targeted gene. A standard curve of known copy numbers can then be used to determine the copies in an unknown sample.

Only soil samples collected in the fall of 2012 and 2013 were used for functional gene copy number quantification. These two dates represent the two extremes in management. Using 0.25 g of soil, DNA was extracted using the Power Soil DNA Extraction Kit (MOBIO Laboratories Inc., Carlsbad CA, USA) following manufacturer's instructions. Concentrations of genomic DNA were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All genomic DNA samples were diluted with DNA/RNAse free water to a concentration of 4 ng DNA μl^{-1} that was stored at -80°C until downstream qPCR

application. Real time qPCR was performed in a PikoReal system (Thermo Scientific, Wilmington, DE, USA) using primers designed to amplify genes important in the N cycle as N limits net primary productivity (Vitousek and Howarth, 1991).

All three primer pairs were synthesized by Invitrogen, Carlsbad, CA, USA. The forward primer, *nosZ*1 5'-WCSYTGTTCMTCGACAGCCAG-3' and the reverse primer *nosZ*1R 5'-ATGTCGATCARCTGVKCRTTYTC-3' (Henry et al., 2006) were used to amplify a 259-bp DNA fragment that encodes for the nitrous oxide reductase gene involved in denitrification. The forward primer, *amo*A1F 5'-GGGTTTCTACTGGTGGT-3' and the reverse primer *amo*A2R 5'-CCCCTCKGSAAAGCCTTCTTC-3' (Rotthauwe et al., 1997) were used to amplify a 491-bp DNA fragment of the *amo*A gene in nitrifying bacteria (AOB). The forward primer, Arch-*amo*AF 5'-STAATGGTCTGGCTTAGACG3'-, and the reverse primer Arch-*amo*AR 5'-GCGGCCATCCATCTGTATGT-3' (Francis et al., 2005) were used to amplify a 635-bp DNA fragment of the archaeal *amo*A gene (AOA). Master mixes and thermocycler conditions were held constant for all three primer pairs. Specifically, a 20 µl reaction volume was used that contained 10 µl SYBR Green Master Mix (Applied Biosystems Inc. Foster City, CA, USA), 0.4 µl of 10 µM forward and reverse primer, 2 µl of 4 ng of DNA template, and 7.2µl RNA/DNA free water. The qPCR thermocycler protocol used was 10 min at 95°C followed by 40 cycles of 95°C for 45 s, 56°C for 1 min, and 72°C for 1 min with a data acquisition step, followed by a melt curve analysis. R-squared values were 0.99. Genomic DNA isolated from organismal controls and environmental soil samples were run in triplicate. Separate standard curves were generated for each functional gene using 5 serial dilutions of genomic DNA. Dilutions of the *nosZ* gene isolated from *Pseudomonas aeruginosa* PAO-1 (ATCC 47085, Manassas, VA, USA) ranged from 5.25 ng μl^{-1} to 2.05 fg μl^{-1}. Additional standard curves were generated using five

serial dilutions of *amoA* genes ranging from 400 fg μl^{-1} to 4 ng μl^{-1} isolated from genomic DNA derived from the nitrifying archaea (AOA), *Nitrosopumilus maritimus* SCM1 and the nitrifying bacteria (AOB), *Nitrosomonas europaea* (ATCC 19718, Manassas, VA, USA).

Measurements of soil enzyme activity

All soil enzyme activity levels were determined on a colorimetric basis using a spectrophotomer (Thermo Spectonic 20D+, Thermo Fisher Scientific, Madison, WI, USA). Field moist soils were homogenized and passed through a 2 mm sieve prior to analysis. Soil enzymes critical to C cycling in soil were assayed and included fluorescein diacetate hydrolysis (FDA) (Green et al., 2006) and β-glucosidase (Eivazi and Tabatabai, 1988). FDA estimates the activity of multiple classes of enzymes involved in the breakdown of non-cellulose C compounds and the cleavage of FDA. β-glucosidase activity is related to cellulose decomposition (Deng and Popova, 2011; Prosser et al., 2011). Enzymes involved in the N cycle were measured for activity and include urease (Kandeler and Gerber, 1988), ammonia monooxygenase (Berg and Rosswell, 1985), and nitrate reductase (Abdelmagid and Tabatabai, 1987). The urease enzyme is responsible for the conversion of urea into ammonia. Ammonia monooxygenase regulates the first and rate limiting step in the process of nitrification and is a measure of the conversion of ammonia to hydroxylamine. Nitrate reductase is the second step in denitrification and measures the conversion of nitrate to nitrite. Alkaline phosphatase enzyme, which is responsible for the conversion of organic P to plant available forms, was also assayed following that outlined by Tabatabai and Bremner (1969) and Eivazi and Tabatabai (1977). Finally, arylsulfatase enzyme activity, which is responsible for the conversion of organic sulfur to plant available sulfur forms, was assayed following the method described by Tabatabai and Bremner (1970).

Statistical analysis

To determine which variables can be used as indicators of soil health in a sodic soil, nonmetric multidimensional scaling (NMS) was performed using PC-ORD (McCune & Mefford, 2011) as described by Mather (1976) and Kruskal (1964). Nonmetric multidimensional scaling is an ordination technique that simplifies complex data sets by determining sources of variation displayed graphically as axes. Nonmetric multidimensional scaling is well suited for non-linear data with different scales of measurement and is generally the most effective ordination technique to use in ecological applications (McCune & Grace, 2002). Prior to NMS, data was square root transformed and relativized in order to account for differences in the magnitude among soil health measurements. In PC-ORD, the default "slow and thorough" procedure was chosen with a Sorensen distance measure and a random starting point, resulting in 250 runs with real and randomized data. After NMS analysis, differences in land use, tile drainage treatment and amendment application were assessed using multi-response permutation procedures (MRPP) using the Sorensen distance measure as described in McCune and Grace (2002) and by Stroup and Stubbendieck (1983). The MRPP analysis is a nonparametric test and is used to test differences in groups. Pairwise comparisons using Bonferonni correction were employed to determine differences due to soil amendments.

To determine whether functional gene copy numbers can be used as sensitive indicators of soil health in a sodic soil a number of soil properties were measured that included: SAR, EC, pH, water content, Mg^{2+}, Na^{+}, Ca^{2+}, Cl^{-}, TC, IC, sand, silt, clay, *nos*Z, AOA, AOB, and ratio of AOA to AOB (AOA:AOB) gene copy numbers. Nonmetric multidimensional scaling and MRPP were used as outlined above to determine axes correlations and differences between land use, tile drainage, and soil amendments. Means and standard deviations were calculated for soil health

measurements taken in the fall of 2012 prior to land use conversion, and amendment application, and. during the fall of 2013 in control and amended treatments.

To determine which of the physical, chemical, and enzyme activities could be used as indicators of soil health for a sodic soil, NMS was performed for all four soil sampling time points using soil chemical, physical properties and enzyme activities following the same procedures outlined above. Pairwise comparisons were made between sampling time, drainage, and amendments using MRPP. Means and standard deviations were calculated for soil measures for fall 2012 and fall 2013. Additionally, means and standard deviations for soil measures were calculated for fall 2013 under the various applied soil amendments.

Finally, successional vector overlays in PC-ORD were used to analyze shifts in soil health within the enzyme ordination data. The enzyme assays were chosen because this data set contained all four sampling dates. Successional vectors can be utilized for repeated measures data and to visualize the "trajectory" (McCune and Grace, 2002) of shifts in soil health due to changes in land use and management.

Results and discussion

Functional gene copy numbers as soil health indicators

The use of the ratio of ammonia oxidizing archaea to ammonia oxidizing bacterial (AOA:AOB) gene copies to assess changes in land use, nutrient status, disturbance and climate change is an example in which gene copy numbers were successfully used as a soil health bioindicator (Wessén and Hallin, 2011). The literature indicates that AOA and AOB communities occupy overlapping niches but their growth and activity vary from one another with changes in N concentration, edaphic properties, ecosystem, and changes in land management (Di et al., 2010; Di et al., 2009; Fortuna et al., 2012; Taylor et al., 2010; Wessén et al., 2010).

Overall, soils clustered in distinct patterns based on land use and soil amendment, with less distinct clustering based on tile drainage (Fig. 3). Nonmetric multidimensional scaling ordination resulted in a two dimensional solution. All selected axes for each ordination were significantly different from random (p<0.05) and reduced stress by 5 or more. A final stress of 18.45 with instability of $0.0x10^{-5}$ was reached. Overall, 83.1% of the variation was explained in two axes. The first axis represents 74.0% of the variation. The second axis represents 9.1% of the variation.

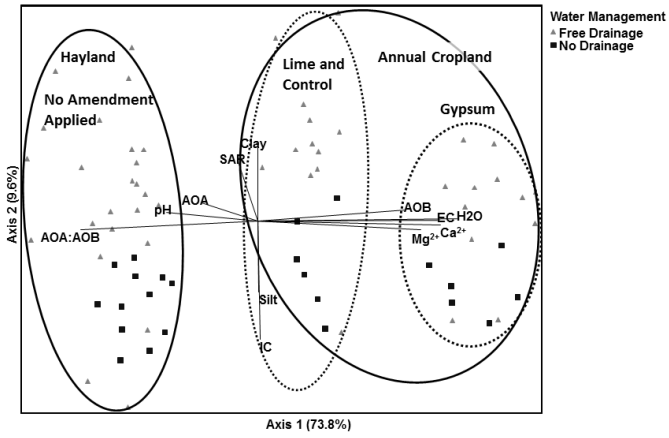

Figure 3. Ordination diagrams using gene copy numbers of *nosZ*, AOA, AOB, and AOA:AOB and soil chemical properties as soil health indicators displayed by water management with land use (solid lines) and amendment application (dashed lines) clusters highlighted.

Soil properties correlated with the two axes are shown in Table 4. Soil properties used as explanatory variables for the structure of the ordination had an absolute r value greater than 0.5. Axis 1 is correlated with EC, water content, Mg^{2+}, Ca^{2+}, AOB, pH, AOA:AOB. Axis 2 represents IC and soil texture (clay, silt). Points that are closer together are more similar, while points that are further apart are more dissimilar. Therefore, axis 1 represents the shift in soil properties resulting from the change in land use (hayfield vs annual crops) and amendments and

application rates (Fig. 3). The two gypsum rates are clustered to the right side of axis 1. The lime and no amendment plots are similar as they are clustered in the middle, and the soils prior to amendment application are clustered separately to the left side of axis 1. Tile drainage treatments are separated by axis 2 with free drainage clustered to the upper half of the ordination diagram and no drainage clustered on the lower half, however this axis represents only 9.6% of the variation within the dataset.

Table 4. The correlation coefficients (r) between soil properties and functional gene copy number ordination analysis for each axis.

	r	
Soil Property	Axis 1	Axis 2
Sodium Adsorption Ratio	-0.278	0.481
Electrical Conductivity	**0.840**[a]	0.042
pH	**-0.601**	0.182
Water Content	**0.885**	0.078
Mg^{+2}	**0.799**	-0.191
Na^{+}	0.321	0.38
Ca^{+2}	**0.847**	-0.137
Cl^{-}	-0.289	-0.115
Total Carbon	0.177	-0.389
Inorganic Carbon	0.076	**-0.68**
Sand Content	-0.069	0.121
Silt Content	0.055	**-0.529**
Clay Content	-0.029	**0.541**
nosZ gene copy numbers	-0.317	-0.034
Ammonia oxidizing archaea *amoA* gene copy numbers	-0.471	0.26
Ammonia oxidizing bacteria *amoA* gene copy numbers	**0.758**	0.202
Ratio of ammonia oxidizing archaea to ammonia oxidizing bacteria *amoA* gene copy numbers	**-0.836**	-0.189

[a] Bold numbers indicate which variables are deemed interpretable for each axis.

Table 5. Pairwise comparisons of multiresponse permutation procedures to compare tile drainage treatment and MPRR to compare soil amendment application differences for soil physical, chemical, functional gene copy numbers and soil enzyme activity levels.

Functional gene copy numbers					
Land Use	vs.	Land Use	T[a]	A[b]	p
Hayland	vs.	Cropland	-37.24	0.23	<0.01
Drainage	vs.	Drainage	T	A	p
No Drain	vs.	Free Drain	-8.43	0.0517	<0.001
Amendment	vs.	Amendment	T	A	p
22.4 Mg Gypsum	vs.	11.2 Mg Gypsum	-2.98	0.05	0.01
22.4 Mg Gypsum	vs.	22.4 Mg Lime	-10.38	0.29	<0.01*
22.4 Mg Gypsum	vs.	Control	-10.67	0.33	<0.01*
11.2 Mg Gypsum	vs.	22.4 Mg Lime	-10.17	0.26	<0.01*
11.2 Mg Gypsum	vs.	Control	.10.46	0.29	<0.01*
22.4 Mg Lime	vs.	Control	-2.91	0.06	0.01
Soil enzyme activity levels					
Land Use	vs.	Land Use	T	A	p
Hayland	vs.	Cropland	-58.38	0.16	<0.01
Tile Drainage	vs.	Tile Drainage	T	A	p
No Drain	vs.	Free Drain	-9.19	0.025	<0.01
Soil Amendment	vs.	Soil Amendment	T	A	p
22.4 Mg Gypsum	vs.	11.2 Mg Gypsum	-1.88	0.01	0.05
22.4 Mg Gypsum	vs.	22.4 Mg Lime	.13.81	0.08	<0.01*
22.4 Mg Gypsum	vs.	Control	-15.61	0.09	<0.01*
11.2 Mg Gypsum	vs.	22.4 Mg Lime	-9.78	0.06	<0.01*
11.2 Mg Gypsum	vs.	Control	-12.77	0.07	<0.01*
22.4 Mg Lime	vs.	Control	-2.45	0.01	0.03

[a] The more negative the T statistic indicates greater differences among groups.
[b] A values represent the chance-corrected within group agreement. When A=0, there is heterogeneity within groups.
* Indicates p values (p<0.05) that are significantly different from each other using Bonferonni correction.

The MRPP analysis resulted in a p value <0.01 for land use, indicating measured soil properties under hayland management were significantly different from conditions after land use conversion to annual cropping and amendment application (Table 5-7). The gypsum amendments raised the Ca^{2+} and Mg^{2+} concentrations over 30 fold and 8 fold, respectively (Table

6). The gypsum applications also reduced the SAR by over 50% from 5.39 in the no amendment plots to 1.5 and 2.54 in the 22.4 and 11.2 Mg gypsum ha^{-1}, respectively (Table 6). However, the soil EC value more than doubled under the gypsum applications (2.31 and 1.57 ds m^{-1} in the 9.1 and 11.2 Mg gypsum ha^{-1}, respectively) compared to the hayland land use (0.34 dS m^{-1}) and lime and no amendment applications (0.52 and 0.11 dS m^{-1}, respectively) (Table 6). The largest change was observed for the AOB copy numbers, where the land use conversion raised the copy numbers from $2.12x10^5$ under hayland to over $1.92x10^6$ in annual row crop (Table 10). Additionally, the AOA copy numbers decreased by nearly 40% from $7.78x10^5$ under hayland to $4.43x10^5$ under cropland (Table 10). The MRPP results for tile drainage were significant ($p<0.01$) (Table 5), but were not correlated with IC content and soil texture. The MRPP analysis also revealed differences among soil amendments ($p<0.01$). Pairwise comparisons, again, using a Bonferonni correction, indicated that the application of 22.4 and 11.2 Mg gypsum ha^{-1} did not result in differences in soil health. However, soil health measurements where gypsum applications were applied did differ relative to the 22.4 Mg lime ha^{-1} and control plots. Application of 22.4 Mg lime ha^{-1} did not result in variations of measured indicators compared to control plots (Table 5).

Table 6. Calculated mean values (±SD) of measured soil chemical properties under hayland management in fall 2012 and annual row crop management in 2013. Additional means for measured soil chemical properties are reported for amendment applications in fall 2013.

Land use	Mg^{2+}, $mmol_{(c)}$ L^{-1}	Na^{+}, $mmol_{(c)}$ L^{-1}	Ca^{2+}, $mmol_{(c)}$ L^{-1}	Cl^{-}, $mmol_{(c)}$ L^{-1}	Sodium Adsorption Ratio	EC, dS m^{-1}	pH	Gravimetric water content, %	Total C
Hayland management	4.15 (±1.39)	7.41 (±6.81)	2.27 (±1.39)	0.95 (±0.37)	4.68 (±4.47)	0.34 (± 0.10)	8.40 (±0.27)	10 (±0.01)	1.41 (±0.19)
Annual crop management	16.85 (±14.41)	11.05 (±7.43)	32.03 (±30.39)	0.78 (±0.38)	3.75 (±4.04)	1.22 (±0.81)	8.13 (±0.44)	20 (±0.01)	1.51 (±0.23)
22.4 Mg ha^{-1} gypsum	32.07 (±7.24)	10.59 (±6.66)	63.62 (±6.00)	0.77 (±0.33)	1.50 (±0.90)	2.31 (±0.26	7.71 (±0.12)	19 (±0.01)	1.49 (±0.17)
11.2 Mg ha^{-1} gypsum	28.53 (±4.85)	16.42 (±5.58)	58.88 (±9.95)	0.72 (±0.18)	2.52 (±0.89)	1.57 (±0.32)	7.84 (±0.16)	20 (±0.02)	1.47 (±0.19)
22.4 Mg ha^{-1} lime	3.55 (±1.09)	9.28 (±9.04)	2.92 (±0.70)	0.78 (±0.51)	5.59 (±5.65)	0.52 (±0.17)	8.53 (±0.29)	19 (±0.02)	1.64 (±0.27)
No amendment	3.25 (±1.31)	7.92 (±6.81)	2.69 (±0.79)	0.83 (±0.51)	5.39 (±5.12)	0.47 (±0.11)	8.45 (±0.36)	19 (±0.01)	1.42 (±0.28)

Table 7. Calculated mean values (±SD) of copy numbers of the nosZ denitrification gene, ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB), and ratio of AOA:AOB under hayland management in fall 2012 and annual row crop management in 2013. Additional means for measured gene copy numbers are reported for amendment applications in fall 2013.

Land use	*nosZ*, gene copy numbers, g soil	Ammonia oxidizing archaea (AOA) gene copy numbers, g soil	Ammonia oxidizing bacteria (AOB) gene copy numbers, g soil	Ratio of AOA:AOB gene copy numbers, g soil
Hayland management	8.52×10^5 ($\pm 2.02 \times 10^5$)	7.78×10^5 ($\pm 6.58 \times 10^5$)	2.12×10^5 ($\pm 1.35 \times 10^5$)	4.24 (±2.62)
Annual crop management	6.69×10^5 ($\pm 2.65 \times 10^5$)	4.43×10^5 ($\pm 3.02 \times 10^5$)	1.92×10^6 ($\pm 1.18 \times 10^6$)	0.30 (±0.30)
22.4 Mg ha^{-1} gypsum	6.96×10^5 ($\pm 3.49 \times 10^5$)	2.36×10^5 ($\pm 7.98 \times 10^4$)	1.37×10^6 ($\pm 7.63 \times 10^5$)	0.25 (±0.19)
11.2 Mg ha^{-1} gypsum	6.89×10^5 ($\pm 3.35 \times 10^5$)	4.51×10^5 ($\pm 2.87 \times 10^5$)	2.54×10^6 ($\pm 1.21 \times 10^6$)	0.19 (±0.10)
22.4 Mg ha^{-1} lime	6.35×10^5 ($\pm 2.30 \times 10^5$)	4.49×10^5 ($\pm 4.11 \times 10^5$)	9.37×10^5 ($\pm 3.84 \times 10^5$)	0.53 (±0.53)
No amendment	7.61×10^5 ($\pm 1.61 \times 10^5$)	6.36×10^5 ($\pm 2.58 \times 10^5$)	2.82×10^6 ($\pm 1.14 \times 10^6$)	0.24 (±0.10)

This study indicates that functional gene copy numbers are sensitive to changes in land use and amendment application (Table 8). Functional gene copy numbers of AOA are correlated with axis 1, which differentiates between land use and amendment applications. However, gene copy numbers were not successful in differentiating between tile drainage treatments. Drainage treatments are differentiated by axis 2, with free drainage clustered toward the positive side of axis 1 and no drainage clustered toward the negative side of axis 2.

Table 8. The sensitivity and selection of functional gene copy numbers and enzyme activity as potential indicators to identify differences in land use, amendment, and water management to be incorporated into a soil health index.

	Functional gene copy numbers	Enzyme Activity Levels	Soil Chemical Measures
Ability to discern land use	AOA:AOB ratio and AOB discern between hayland and cropland	B-glucosidase, alkaline phosphatase, nitrate reductase, and arylsulfatase discern between hayland and crop land	no
Ability to discern soil amendment	yes	no	yes
Ability to discern tile drainage	no	no	no

Gene copy numbers should be considered as metrics for incorporation into soil health indices. Gene copy numbers can be determined more quickly and accurately than enzyme assays. In this study, gene copy numbers of AOB and AOA:AOB were able to differentiate between hayfield and annual crop management and amendment application based on their high correlation with axis 1. Our research supports the findings that fertilized, annual row crop systems select for larger populations of AOB relative to AOA that are favored in unfertilized long-term fallow and grassland managements (Taylor et al., 2012; Taylor et al., 2010). Other

researchers have shown that application of urea fertilizer and manures have no effect or reduce populations of AOA that are favored in oligotrophic environments low in ammonia concentrations (Fortuna et al., 2012; Martens-Habbena et al., 2009; Schleper, 2010; Sims et al., 2012). Nitrogen fertilizer was applied under cropland management, whereas N was not added while the land was under hayland management.

Soil pH has also been shown to influence gene abundance of AOA and AOB. Greater copy numbers of AOB are measured at mid pH ranges while acidic and alkaline conditions have been shown to favor AOA (Bengtson et al., 2012; Prosser and Nicol, 2012). This study supports the concept of a separate niche for AOA in alkaline soil environments. In addition, high Na^{+} concentrations, up to a maximum SAR of 16.7 in this soil prior to amendment applications may have favored archaea. There is evidence that AOB are negatively correlated with increasing salt concentrations in sediments (Li et al., 2011) and that AOA copy numbers are constant across a wide salt gradient in water systems (Erguder et al., 2009). Our research found correlations among nitrifier communities and edaphic processes as did Fortuna et al. (2012) albeit the properties that were correlated varied between the two experiments. In this experiment soil EC, water content, Ca^{+2} and Mg^{+2} concentrations were correlated with AOB gene copy numbers.

Soil enzyme activities as soil health indicators

Soil enzymes have received attention as long-term biological soil health indicators because enzyme activity levels are related to organic matter, soil physical properties, and microbial biomass as well as the low cost and ease associated with these techniques. Enzyme assays have been developed to assess many nutrient transformations including those involved in the C, N, P, and S cycles. Additionally, enzyme activities can detect changes in soil health in as little as one to two years (Bandick and Dick, 1999). Soil enzymes can be intercellular, attached

to outer surfaces of cells, in soil solution, attached to cell debris, or adsorbed to clay minerals and humic colloids (Burns, 1982).

Using enzyme activities as soil health indicators, NMS ordination resulted in a two dimensional solution. All selected axes for each ordination were significantly different from random (p<0.05) and reduced stress by 5 or more. The final stress was 14.98 with instability of $0.1x10^{-5}$. A total of 89.2% of the variation in the data set could be explained by two axes. The first axis represents 51.8% of the variation. The second axis represents 37.3% of the variation (Fig. 4).

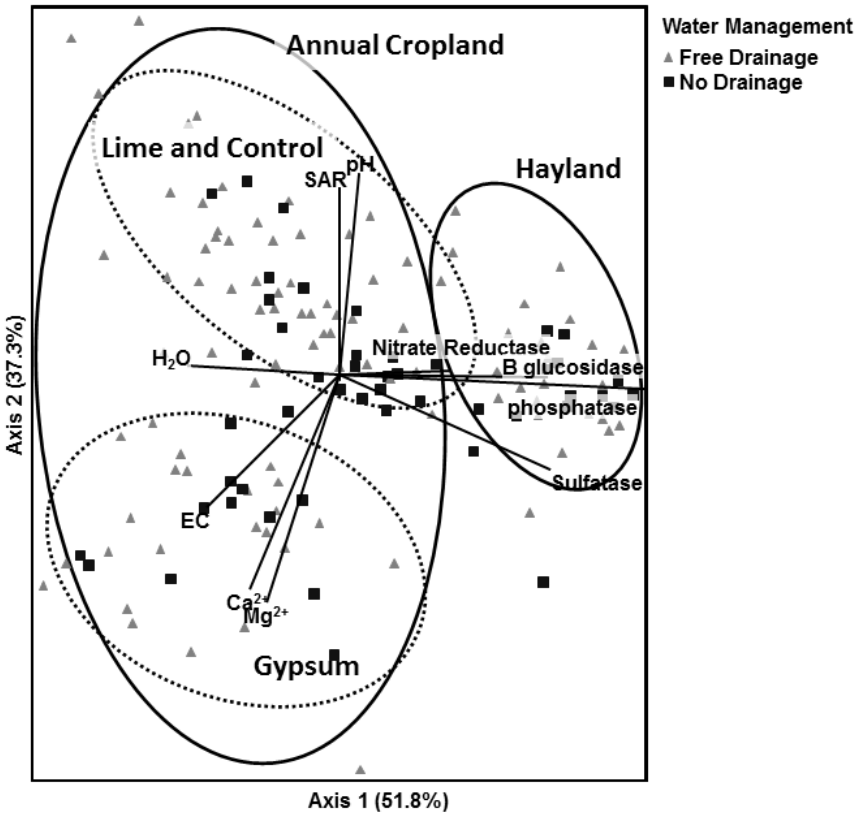

Figure 4. Ordination diagrams using soil enzyme activities and soil chemical properties as soil health indicators displayed by sampling date with land use (solid lines) and soil amendment (dashed lines) clusters highlighted.

Table 9. The correlation coefficients (r) between the individual soil properties with each axis for soil enzyme ordination analysis.

	r	
Soil Property	Axis 1	Axis 2
Sodium Adsorption Ratio	-0.016	**0.708**
Electrical Conductivity	**-0.599**[a]	**-0.596**
pH	0.225	**0.736**
Water Content	**-0.638**	0.166
Mg^{+2}	-0.44	**-0.78**
Na^{+}	-0.402	0.151
Ca^{+2}	-0.493	**-0.757**
Cl^{-}	-0.082	0.354
Nitrate Reductase	**0.541**	0.104
Ammonia monooxygenase	0.175	-0.394
Urease	0.31	-0.411
B-glucosidase	**0.747**	-0.077
Alkaline phosphatase	**0.904**	-0.191
Arylulfatase	**0.752**	**-0.505**
Fluorescein diacetate hydrolysis	0.446	0.088
Total C	0.267	-0.371
Inorganic C	0.126	-0.28
Sand Content	0.04	0.213
Silt Content	0.089	-0.244
Clay Content	-0.115	0.168

[a] Bold numbers indicate which variables are interpretable for each axis.

The two axes that correlated with the measured soil properties are shown in Table 9. Axis 1 represents biological indicators, soil enzyme activities of nitrate reductase, β-glucosidase, alkaline phosphatase, and arylsulfatase. Axis 2 represents primarily the chemical indicators of EC, water content, SAR, pH, Mg^{2+}, and Ca^{2+}. Samples collected from the hayland in fall 2012 clustered separately to the right of the remaining sample time points. Points that are closer together are more similar. Distance between points is relative to their dissimilarities. Therefore

land use had a major influence on soil health indicators (Fig. 4). There was also distinct clustering based on amendment application. The two gypsum rates clustered separately and to the bottom of the diagram as compared to the lime and control amended plots, which cluster toward the top of the diagram. Amendments are more highly correlated with chemical measures of soil health. Again, these parameters clustered separately from the plots that were collected under hayland management. This indicates that amendment applications had differing effects on indicators of soil health.

In addition, MRPP resulted in $p<0.01$ for land use and drainage (Table 5). Although the results of MRPP suggest tile drainage treatments are significantly different ($p<0.01$), there was no distinct clustering of the plots based on tile drainage (Fig. 4). The no drainage treatment clustered more tightly in the middle of the ordination graph as compared to the free drainage treatment points, resulting in a significantly different within cluster distance p value. However, the overlap of the clusters indicated that tile drainage does not have a clear impact on short-term soil health indicators using enzyme activities. The pairwise comparisons using a Bonferroni correction, indicate that the application of 22.4 and 11.2 Mg gypsum do not result in differences in measured soil properties (Table 6 and Table 7). However, the gypsum applications resulted in variable soil properties relative to that of the 22.4 Mg lime and control plots that had no effect on measured soil properties. The MRPP F test resulted in a p value <0.01 for sampling time. Soil properties at each sampling time were significantly different from one another using pairwise comparisons with a Bonferroni correction (Table 5).

Currently, soil enzyme activities as indicators of soil health are favored because assays can be run routinely in soil testing facilities (Dick, 2011). In this study, soil enzyme activities of nitrate reductase (4.81 and 2.67 μg NO_2-N mL^{-1} g soil $5h^{-1}$, respectively), β-glucosidase (184.49

and 91.19 µmol *p*-nitrophenol kg^{-1} soil h^{-1}, respectively), phosphatase (866.15 and 344.02 µmol *p*-nitrophenol kg^{-1} soil h^{-1}, respectively) and arylsulfatase (255.60 and 155.11 µmol *p*-nitrophenol kg^{-1} soil h^{-1}, respectively) were used successfully to differentiate between land uses, and were nearly double those values measured under annual row crops (Table 10). Along with the conversion to cropland, fertilizer applications, such as P likely reduced some enzyme activities (Spiers and McGill, 1979). Greater activity of arylsulfatase has been observed where plant root growth is greater (Castellano and Dick, 1991), such as under grasslands where the volume occupied by living roots tends to be higher than in agricultural systems. Soil enzymes were not effective in determining differences in soil amendment application or tile drainage treatment. However, soil chemical measures were effective at differentiating among amendments and their application rates as pH was reduced by over half a unit and SAR by over 50% in lime and control plots. Electrical conductivity and concentrations of Ca^{2+}, and Mg^{2+} were much higher under both gypsum rates (Table 6).

Table 10. Calculated mean values (±SD) of soil enzyme assays for nitrate reductase, ammonia monooxegenase, urease, β glucosidate, alkaline phosphatase, arylsulfatase and fluorescein diacetate hydrolysis under hayland management in fall 2012 and annual row crop management in 2013. Additional means for measured soil enzymes are reported for amendment applications in fall 2013.

Land Use	nitrate reductase, µg NO_2-N mL^{-1} g soil $24h^{-1}$	Ammonia monooxegenase, µg NO_2-N mL^{-1} g soil $5h^{-1}$	Urease, µg NH_4-N mL^{-1} g soil 2 h^{-1}	β glucosidase, µmol *p*-nitrophenol kg^{-1} soil h^{-1}	Alkaline phosphatase, µg *p*-nitrophenol, g soil h^{-1}	Aryl sulfatase, µg *p*-nitrophenol, g soil h-1	Fluorescein diacetate hydrolysis, mg flourescein released kg^{-1} oven-dried soil $3h^{-1}$
Hayland management	4.81 (±2.16)	1.05 (±0.33)	182.00 (±49.71)	184.49 (±63.07)	866.15 (±151.26)	255.60 (±43.52)	0.36 (±0.04)
Annual crop management	2.67 (±1.23)	1.54 (±0.93)	144.09 (±38.06)	91.19 (±18.11)	344.02 (±69.97)	155.11 (±51.19)	0.22 (±0.02)
22.4 Mg ha^{-1} gypsum	1.98 (±1.11)	1.55 (±0.71)	134.52 (±37.55)	95.92 (±16.08)	356.01 (±47.46)	168.82 (±24.65)	0.21 (±0.03)
11.2 Mg ha^{-1} gypsum	1.95 (±0.88)	1.87 (±1.21)	167.67 (±44.23)	88.59 (±12.27)	368.05 (±52.21)	149.65 (±32.43)	0.22 (±0.03)
22.4 Mg ha^{-1} lime	4.00 (±1.09)	1.67 (±1.18)	137.88 (±29.73)	91.95 (±17.38)	329.68 (±53.50)	152.71 (±78.67)	0.22 (±0.02)
No amendment	2.73 (±0.63)	1.08 (±0.45)	136.29 (±38.97)	88.30 (±27.41)	322.33 (±114.03)	149.27 (±62.44)	0.21 (±0.02)

Successional vector analysis

Successional vector trajectories were analyzed visually for differences in direction and magnitude of shifts based on amendment application (Fig. 5A-D). All plots under hayland vegetation originate to the right of axis 1 and in the middle of axis 2. Amendment applications caused differential shifts in the plots in ordination space. Both gypsum rates had a strong initial shift to the left of axis 1, indicating lowered enzyme activity. The plots also initially shifted to the positive side of axis 2, where the SAR is higher. The gypsum plots then shifted to the negative side of axis 2, indicating reduced SAR, but increased EC. The lime and no amendment plots also initially shifted to the negative side of axis 1 and positive side of axis 2, similar to the gypsum amended plots. Subsequent sampling times resulted in variable shifts for the lime and control plots. However, the lime and control plots successional vectors end in a separate location from the gypsum plots.

Successional vector analysis revealed that changes in land use and management cause shifts in overall soil health. Greater enzyme activity is correlated with increases in soil organic matter and soil health (Dick, 1994). Reductions in enzyme activity under cropland management as observed by a shift in plots to the negative side of axis 1 indicate that soil health is reduced on this sodic soil under cropland management. Additionally, negative shifts due to gypsum application further reduced enzyme activity. A major finding was that microbial communities in the Exline soil were influenced by soil disturbances as observed by the negative changes in soil health after the hayfield was plowed. Questions remain regarding the impact of soil amendments on microbial resilience and enzyme production due to the time required for amendments to leach through the soil profile and react with soil constituents.

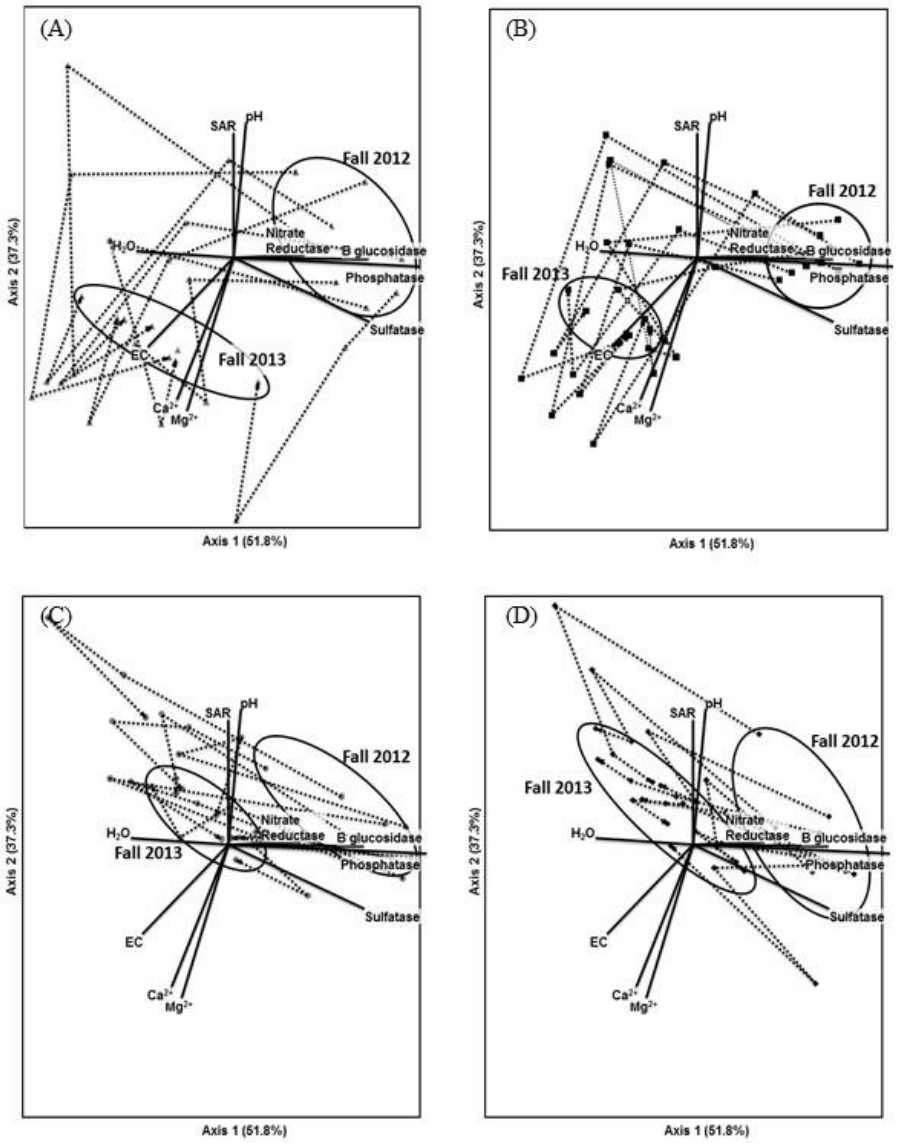

Figure 5. Successional vectors connecting the four sampling dates using the soil enzyme activities as indicators of shifts in soil health for (A) 22.4 Mg gypsum ha^{-1}, (B) 11.2 Mg gypsum ha^{-1}, (C) 22.4 Mg lime ha^{-1}, and (D) control, no amendment plots using the soil enzyme ordination data set. Sampling dates are significantly different for all sampling time points except for the lime amendment and control treatments taken at silking 2013 and in fall 2013.

Effectiveness of gene copy numbers, enzyme activity levels and soil chemical measures in detecting changes in soil management

Table 6 summarizes gene copy numbers, enzyme activity levels and soil chemical measures and their value as measures to assess changes in land use and management. Bacterial

ammonia oxidizers could be used as sensitive measures of land use with bacteria increasing from $2.12x10^5$ copy numbers per gram of soil under hayland management to $1.7x10^6$ copy numbers per gram of soil under cropland management. Also AOB gene copies varied between the 22.4 and 11.2 Mg gypsum ha^{-1} soil amendment applications ($1.37 x10^6$ and $2.54x10^6$, respectively) and the lime and no amendment ($9.37x10^5$ and $2.82x10^6$, respectively). The increase in AOB gene copies measured under cropland management with various amendment applications effectively lowered AOA:AOB ratios as AOB copies were greater (4.24 AOA:AOB under hayland management and reduced to 0.30) under cropland management (Table 7). While the cost of obtaining functional gene copy numbers is high, the average cost per sample is decreasing. Gene copy numbers are also very accurate and more accurately reflect substrate available to soil microorganisms. Enzyme activity levels were able to differentiate between land uses, but were not able to differentiate between soil amendments. However, soil enzyme activity levels are impacted by environmental factors, such as temperature, pH, and water content. Environmental soil factors can change the conformation and effectiveness of stabilized enzymes (Niemi and Vepsalainen, 2005; Daniel et al., 2008). Because of the potential environmental limitations of using soil enzymes, as soil health indicators it has been suggested that incorporating functional gene copy numbers could provide a more meaningful interpretation of soil enzyme activity (Nannipieri et al., 2012). Soil chemical properties are routine measures completed by soil testing laboratories. Chemical properties are easy and inexpensive to measure, and were able to differentiate between soil amendments. Overall, this research highlights the need to incorporate biological indicators into soil health indices used to assess degraded environments, where soil health is more sensitive to land use and management decisions (Freckman and Virginia, 1997).

Conclusions

High predicted input costs and limited arable land worldwide are global problems facing the future of agriculture. Ensuring that current land use and management of sodic soils complies with future needs is important as agricultural practices are intensified and expand to greater marginal acreage to meet the demands of food production. Integrating biological, chemical, and physical soil indicators to monitor changes in soil health in a short time period can aid producers in making sound decisions with respect to land use and management. This work highlights variations in functional gene copy numbers and reductions in soil enzyme activities with change in land use and amendment application. The research findings showed that biological indicators could be used to quantify improvement in soil health. Rapid assessment of changes in soil health can prevent or limit land managers from investing in practices that may not improve soil health, or can serve as a positive indicator of improvement during transition(s) in land use and/or management.

Acknowledgements

This material is based upon work supported by the Natural Resources Conservation Service, U.S. Department of Agriculture; under the Conservation Innovation Grants program. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the U.S. Department of Agriculture. The gypsum amendments were provided by Calcium Products, Ames, IA, USA. Additional support was provided by the (2012-2014) North Dakota Agricultural Experiment Station Fellowship and The North Dakota Water Resources Research Institute Fellowship (2014-2015). The authors would like to thank Nate Derby, Kevin Horsager and Brandon Dahl for their assistance in the field.

References

Abrol, I.P., J.S.P. Yadav, and F.I.Massoud. 1988. Salt-affected soils and their management. Food and Agricultural Organization of the United Nations, Rome, Italy.

Abdelmagid, H.M. and M.A. Tabatabai. 1987. Nitrate reductase activity of soils. Soil Biol. Beochem. 19:421-427. doi:10.1016/0038-0717(87)90033-2

American Crystal Sugar. 2008. The value and benefits of lime. https://www.crystalsugar.com/media/18377/lime.pdf. Accessed on 27 October 2014.

Ashworth, J., D. Keyes, and J-M. Crépin. 1999. A comparison of methods for gypsum requirement of brine-contaminated soils. Can. J. Soil Sci. 79:449-455. doi:10.4141/S99-001

Bandick, A.K. and R.P. Dick. 1999. Field management effects on soil enzyme activities. Soil Biol. Biochem. 31:1471-1479. doi:10.1016/S0038-0717(99)00051-6

Bengtson, P., A. E. Sterngren, and J. Rousk. 2012. Archaeal Abundance across a pH Gradient in an Arable Soil and Its Relationship to Bacterial and Fungal Growth Rates. Appl. Environ. Microbiol.78:5906–5911. doi:10.1128/AEM.01476-12.

Berg, P. and T. Rosswall. 1985. Ammonium oxidizer number, potential and actual oxidation rates in two Swedish arable soils. Biol. Fertil. Soils 1:131-140. doi:10.1007/BF00301780

Burns, R.G. 1982. Enzyme activity in soil: location and possible role in microbial ecology. Soil Biol. Biochem. 14:423-427. doi:10.1016/0038-0717(82)90099-2

Cai, X, D.C. McKinney, and M.W. Rosegrand. 2003. Sustainability analysis for irrigation water management in the Aral Sea region. Sgric. Syst. 76:1043-1066. doi:10.1016/S0308/521X(02)00028-8

Castellano, S.D., and R.P. Dick. 1991. Cropping and sulfur fertilization influence on sulfur transformations in soil. Soil Sci. Soc. Am. J. 54:114-121. doi:10.2136sssaj1991.03615995005500010020x

Combs S.M., M.V. Nathan. 1998. Soil organic matter. In Recommended chemical soil test procedures for the North Central Region, ed. J.R. Brown. Missouri Agricultural Experiment Station SB 1001: University of Missouri.

Daniel, R. 2005. The metagenomics of soil. Nat. Rev. Microbiol. 3:470-478. doi:10.1038/nrmicro1160

Deng, S. and I. Popova. 2011. Carbohydrate Hydrolases. P. 195–198. *In* Methods of soil enzymology, ed. R.P. Dick. Soil Science Society of America Series 9. Madison, WI: Soil Science Society of America. doi:10.2136/sssabookser9.c9

Di, H.J., K.C. Cameron, J.-P. Shen, C.S. Winefield, M. O'Callaghan, S. Bowatte, and J.-Z. He. 2010. Ammonia-oxidizing bacteria and archaea grown under contrasting soil nitrogen conditions. FEMS Microbiol Ecol. 72:386–394. doi:10.111/j.1574-6941.2010.00861.x.

Di, H.J., K.C. Cameron, J.P. Shen, C.S. Winefield, M. O'Callaghan, S. Bowatte, and J.Z. He. 2009. Nitrification driven by bacteria and not archaea in nitrogen rich grassland soils. Nat. Geosci. 2:621–624. doi:10.1038/ngeo613

Dick, R.P. 1994. Soil enzyme activities as indicators of soil quality. *In* Defining soil quality for a sustainable environment, eds. J.W. Doran, D.C. Coleman, D.F. Bezdicek, B.A. Stewart. Madison, WI: Soil Science Society of America. doi:10.2136/sssaspecpub35.c7

Dick, W.A. 2011. Development of a soil enzyme reaction assay. *In* Methods of soil enzymology, ed. R.P. Dick. Soil Science Society of America Series 9. Madison, WI: Soil Science Society of America. dio:10.2136/sssabookser9.c4

Doran J.W. and T.B. Parkin. 1994 Defining and assessing soil quality. *In* Defining soil quality for a sustainable environment, eds. J.W. Doran, D.C. Coleman, D.F. Bezdicek, B.A. Stewart. Madison, WI: Soil Science Society of America. doi:10.2136/sssaspecpub35.c1

Erguder, T.H., N. Boon, L. Wittebolle, M. Marzorati, and W. Verstraete. 2009. Environmental factors shaping the ecological niches of ammonia-oxidizing archaea. FEMS Microbiol Rev. 33:855-869. doi:10.111/j.1574-6976-2009-00179.x.

Eivazi, F. and M.A. Tabatabai. 1977. Phosphatases in soils. Soil Biol. Biochem. 9:197-172. doi:10.1016/0038-0717(77)90070-0

Eivazi, F. and M.A. Tabatabai. 1988. Glucosidases and galactosidases in soil. Soil Biol. Biochem. 20:601-606. doi:10.1016/0038-0717(88)90141-1

Gee, GW and JW Bauder. 1986. Particle-size analysis. p. 383-411. *In* A Klute (ed.) Methods of Soil Analysis, Part 1. Physical and Mineralogical Methods. Agronomy Monograph No. 9 (2ed). American Society of Agronomy/Soil Science Society of America, Madison, WI. doi:10.2136/sssabookser5.1.2ed.c15

Francis, C.A., K.J. Roberts, J.M. Beman, A.E. Santoro and B.B. Oakley. 2005. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. PNAS. 102:14683-14688. doi:10.1073/pnas.0506625102

Franzen, D. 2003. Managing saline soils in North Dakota. North Dakota State University Extension Service SF-1087: North Dakota State University.

Freckman, D.W. and R.A. Virginia. 1997. Low-diveristy Antarctic soil nematode communities: distribution and response to disturbance. Ecology. 78:363-396. doi:10.1890/0012-9658(1997)078[0363:LDASNC]2.0.CO;2

Frenkel, H., Z. Gerstl, and N. Alperovitch. 1989. Exchange-induced dissolution of gypsum and the reclamation of sodic soils. Soil Sci. 40:599–611. doi:10.111/j.1365-2389-1989.tb01301.x

Fortuna, A-M., C.W. Honeycutt, G. Vandemark. T.S. Griffin, R.O. Larkin, Z. He, B.J. Wienhold, K.R. Sistani, S.L. Albrecht, B.L. Woodbury, H.A. Torbert, J.M. Powell, R.K. Hubbard, R.A. Eigenberg, R.J. Wright, J.R. Alldredge, J.B. Harsh. 2012. Links among nitrification, nitrifier communities, and edaphic properties in contrasting soils receiving dairy slurry. J. Environ. Qual. 41:262-272. doi:10.2134/jeq2011.0202

Green, V.S., D.E. Scott, and M. Diack. 2006. Assay for fluorescein diacetate hydrolitic activity:Optimization for soil samples. Soil Biol. Biochem. 38:693-701 doi:10.1016/j.soilbio.2005.06.020

Gupta, P.K., and I.P. Abrol. 2000. Salinity build-up and changes in the rice-wheat system of the Indo-Gangetic Plains. Exp Agr. 36:273-284.

Henry, S., D. Bru, B. Stres, S.Hallet, L. Philippot. 2006. Quantitative detection of the nosZ gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, narG, nirK, and nosZ genes in soils. Appl. Environ. Microbiol. 72:5181–5189. doi:10-1128/AEZM.00231-06

Ilyas, M, R.H. Qureshi, and M.A. Quadir. 1997. Chemical changes in a saline-sodic soil after gypsum application and cropping. Soil Technol. 10:247-260. doi:10.1016/S0933-3630(96)00121-3

Kandeler, E and H. Gerber. 1988. Short-term assay of soil urease activity using determination of ammonium. Biol. Fertil. Soils 6:68-72. doi:10.10074/BF00336437

Kruskal V.J. 1964. Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. Psychometrika. 29:1-27. doi:10.1007/BF02289565

Li, M., Cao, H., Hong, Y., & Gu, J. D. (2011). Spatial distribution and abundances of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) in mangrove sediments. Appl. Microbiol. Biotechnol. 89:1243-1254. doi:10.1007/s00253-010-29259-0

Martens-Habbena, W., P.M. Berube, H. Urakawa, J.R. de la Torre, and D.A. Stahl. 2009. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. Nature. 461:976-9. doi:10.1038/nature08465

Mather, P.M. 1976. *Computational methods of multivariate analysis in physical geography*. London, England : Wiley and Sons, Inc.

McCune B., Grace J.B. 2002. *Analysis of ecological communities*. Gleneden Beach, OR: MjM Software Design.

McCune B., Mefford M.J. 2011. PC-ORD. Multivariate Analysis of Ecological Data. Version 6.15 Gleneden Beach, OR: MjM Software.

NDAWN. 2014. North Dakota Agricultural Weather Network. http://ndawn.ndsu.nodak.edu. Accessed on 10 May 2014.

Nannipieri, P., L. Giagoni, G. Renella, E. Puglisi, B. Ceccanti, G. Masciandaro, F. Fornasier, M.C. Moscatelli, and S. Marinari. 2012. Soil enzymology: classical and molecular approaches. Biol. Fert. Soils. 48:743-762. doi:10.1007/s00374-012-0723-0

Niemi, R.M. and M. Vepsalainen. 2005. Stability of the fluorogenic enzyme substrates and pH optima of enzyme activities in different Finnish soils. J. Microbiol. Methods. 60:195-205. doi:10.1016/j.mimet.2004.09.010

Pessarakli, M. and I. Szabolcs. 1999. Soil salinity and sodicity as particular plant/crop stress factors. In: Handbook of plant and crop stress, second edition, revised and expanded. ed. M. Pessarki. New York, NY: Marcel Dekker.

Prosser, J.I. and G.W. Nicol. 2012. Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. Trends Microbiol. 20(11), 523-531 doi:10.1016/j.tim2012.08.001

Prosser, J.A., T.W. Speir, D.E. Stott. 2011. Soil oxidoreductase and FDA hydrolysis. *In* Methods of soil enzymology, ed. R.P. Dick. Soil Science Society of America Series 9. Madison, WI: Soil Science Society of America. doi:10.2136/sssabookser9.c6

Qadir, M. and J.D. Oster. 2002. Vegetative bioremediation of calcareous sodic soils: history, mechanisms, and evaluation. Irrigation Sci. 21:91-101. doi:10.1007/s00271-001-0055-6

Rao, D.L.N. and H. Pathak, 1996.Ameliorative influence of organic matter on biological activity of salt-affected soils. Arid Soil Res. Rehab. 10:311-319. doi:10.1080/15324989609381446

Rengasamy, P. and K.A. Olsson. 1991. Sodicity and soil structure. Aust. J. Soil Sci. 29:935-952. doi:0004-9573/91/060935$05.00

Rengasamy, P. 2006. World salinization with emphasis on Australia. J. Exp. Bot. 57:1017-1023. doi:10.1093/jxb/erj108

Rhodes JD. 1996. Electrical conductivity and total dissolved solids. *In*: D.L. Sparks, A.L. Page, P.A. Hlmke, R.H. Loeppert, P.N. Soltanpour, M.A. Tabatabai, C.T. Johnston, and M.E. Sumner (eds). Methods of Soil Analysis, Part 3, Chemical Methods, ed. Madison, WI: Soil Science Society of America. doi:10.2136/sssabookser5.3.c14

Richards, L.A. (ed) 1954. Diagnosis and Improvement of Saline and Alkali Soils, U.S. Salinity Laboratory. Handbook 60. United States Department of Agriculture: Washington, D.C.

Ritz, K., H.I.J Black, C.D. Campbell, J.A. Harris, and C.Wood. 2009. Selecting biological indicators for monitoring soils: a framework for balancing scientific and technical opinion to assist policy development. Ecol. Indic. 9:1212–1221. doi:10.1016/j.ecolind.2009.02.009

Rotthauwe, J.H., K.P. Witzel, and W. Liesack. (1997). The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. 63:4704-4712. doi:009-2240/97/$04.00+0

Schleper, C. 2010. Ammonia oxidation: different niches for bacteria and archaea. The ISME Journal. 4:1092-1094. doi:10.1038/ismej.2010.111

Sims A., J. Horton, S. Gajaraj, S. McIntosh, R.J. Miles, R. Mueller, R. Reed and Z. Hu. 2012. Temporal and spatial distributions of ammonia-oxidizing archaea and bacteria and their ratio as an indicator of oligotrophic conditions in natural wetlands. Water Res. 46:4121-4129. doi:10.1371/journal.pone.0113515

Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture. Web Soil Survey. Available online at http://websoilsurvey.nrcs.usda.gov/. Accessed

Spiers, G.A. and W.B. McGill. 1979. Effects of phosphorus addition and energy supply on acid phosphatase production and activity in soils. Soil Biol. Biochem. 11:3-8. doi:10.16/0038-0717(79)90110-X

Stroup, W.W. and J. Stubbendieck. 1983. Multivariate statistical methods to describe changes in botanical composition. J. Range Manage. 36:208-212.

Tabatabai, M.A. and J.M. Bremner. 1969. Use of *p*-nitrophenol phosphate for assay of soil phosphatase activity. Soil Biol. Biochem. 1:301-307. doi:10.1016/0038-0717(69)90012-1

Tabatabai, M.A. and J.M. Bremner. 1970. Arylsulfatase activity of soils. Soil Sci. Soc. Am. Proc. 34:225-229. doi:10.2136/sssaj1970-03615995003400020016x

Taylor AE, L.H. Zeglin, T.A.Wanzek, D.D. Myrold and P.J. Bottomley. 2012. Dynamics of ammonia-oxidizing archaea and bacteria populations and contributions to soil nitrification potentials. ISME J. 6:2024–2032. doi:10.1038/ismej.2012.51

Taylor, A.E., L.H. Zeglin, S. Dooley, D.D. Myrold, and P.J. Bottomley. 2010. Evidence for different contributions of archaea and bacteria to the ammonia-oxidizing potential of diverse Oregon soils. Appl. Environ. Microbiol. 76:7691-7698. doi:10.1128/aem.01324-10

Vitousek, P.M., and R.W. Howarth. 1991. Nitrogen limitation on land and in the sea-How can it occur? Biogeochemistry. 13:87–115. doi:10.1007/BF00002772

Wessén, E. and S. Hallin. 2011. Abundance of archaeal and bacterial ammonia oxidizers-possible bioindicator for soil monitoring. Ecol. Indic. 11:1696-1968. doi:10.1016/j.ecolind.2011.04.018

Wessén, E., M. Söderström, M. Stenberg, D. Bru, M. Hellman, A. Welsh, F. Thomsen, L. Klemedtson, L. Philippot, and S. Hallin. 2011. Spatial distribution of ammonia-oxidizing bacteria and archaea across a 44-hectare farm related to ecosystem functioning. ISME J. 5: 1213–1225. doi:10.1038/ismej.2010.206

PAPER 2: PRIMARY SOIL SALINITY REDUCTION: RESPONSE OF NITRIFYING AND DENITRIFYING ORGANISMS[2]

Abstract

Primary and secondary salinity negatively affects soil organisms involved in N transformations. The objective of this study was to contribute new information on soil biological N transformations by examining the impact of primary salinity reduction has on a) soil enzyme activity levels, b) copy numbers of genes involved in key N cycling processes and c) biological community structure. This randomized complete block experiment was conducted on a Bearden silty clay loam, saline phase (fine-silty, mixed, superactive, frigid Aeric Calciaquolls) soil. The experiment contained two drainage treatments as whole plots (tile-drained and no drainage) that included subplots of a check treatment, three cover crop treatments (low, medium and high diversity), and 4 Mg gypsum ha^{-1}. Soil samples (0-30 cm) were collected in the fall of 2013, 2014, and 2015 and extracted for inorganic N, ammonia monooxygenase and nitrate reductase enzyme activity. Additionally, the quantity and diversity of ammonia oxidizing archaea and bacteria (nitrifiers) and denitrifying bacteria were determined. Treatments were successful in reducing salinity as the electrical conductivity was reduced from an average of 7.0 to 4.5 dS m^{-1} at the end of the study. Soil pH slightly increased but no changes were observed in sodium adsorption ratio. Treatments had minimal effect on ammonia monooxygenase enzyme activity with no effect on nitrate reductase activity. Although treatments reduced soil salinity, no changes

[2] The material in this chapter was co-authored by Heather Dose, Thomas DeSutter, Ann-Marie Fortuna, David Clay, Francis Casey and Robert Brueggeman. Heather Dose had primary responsibility for collecting samples in the field and laboratory analyses. Heather Dose was the primary developer of the conclusions that are advanced here. Heather Dose also drafted and revised all versions of this chapter. Thomas DeSutter served as proofreader and checked the math in the statistical analysis conducted by Heather Dose.

occurred in nitrifiers or denitrifiers in both gene copy numbers and community structure. These results suggest salinity induced community tolerance in soils experiencing primary salinity. Ameliorating naturally-induced saline soils can therefore proceed without special focus on restoring N-cycling organisms.

Introduction

Risk of salinization of agricultural land is a current threat to national and international food security (Rengasamy, 2006). Worldwide, salt-affected soils encompass roughly 10% of Earth's land surface in over 100 countries (Pessarkli and Szabolcs, 1999). In the United States saline soils are classified as having an electrical conductivity (ECe) of the saturation extract of greater than 4 dS m^{-1}, sodium adsorption ratio (SARe) of less than 13, exchangeable Na^{+} percentage (ESP) of less than 15, and a pHe less than 8.5 (Richards, 1954). Soil salinity negatively influences plant growth and reduces yields (Plaut et al., 2013) by affecting osmotic balances that can lead to ion specific toxicities (Bernstein and Hayward, 1958; Sarig and Steinberger, 1994; Zahran, 1997).

Aside from reducing plant growth, salinity affects microbial distribution (Bernhard et al., 2007) and biologically mediated N transformations (McClung and Frankenberger, 1985; Saleem and Ahmed, 1988). Soil salinity limits the biological steps of the N cycle by suppressing amidase and urease enzyme activities (Akhtar et al., 2012; Irshad et al., 2005; Laura, 1977) and reducing nitrification and denitrification (Gandhi and Paliwal, 1976; McClung and Frankenberger, 1985; Irshad et al., 2005; Akhtar et al., 2012). The first step of nitrification (converting NH_4 to NO_2) is performed by ammonia oxidizing bacteria (AOB) and archaea (AOA) and is genetically controlled by the *Amo* gene cluster (Rotthauwe et al., 1997; Wutcher et al., 2006). Denitrifying bacteria that possess the *nos* genes (Henry et al., 2006) regulate the last step of denitrification,

which converts N_2O to N_2. The proposed level whereby nitrification and denitrification is impacted by salinity is at an $EC_{1:5}$ of 1.5 dS m^{-1} (ECe 7.8 dS m^{-1}, He et al., 2013) (Zeng et al., 2006).

Salinity, in addition to reducing microbial N transformations, can affect the diversity and spatial distributions of soil organisms. For example, Canfora et al., (2014) identified several bacterial phyla inhabiting extreme saline soil environments that had not been previously identified as being associated with saline soils (*Nitrospira, Deferribacteres, Cyanobacteria/Chloroplast, Tenericutes and Spirochaete*). The authors also stress the microbial community of saline soil environments is of interest for researchers who may find useful biotechnological applications relating to the restoration of these unique environments or to be studied as a model system for bacterial adaptation to selective environmental conditions. Microbial diversity in saline soil environments is understudied and it is estimated that less than 50% of the archaeal and 25% of bacterial species have been described in these environments (Ma and Gong, 2013). Reducing the effects of primary soil salinity therefore should have an impact on microbial function and structure as the soil solution changes in ion speciation and concentration.

Removing secondary salt accumulation, such as that from irrigation or sea spray, by leaching reduces the negative effects of salinity on N transformations and crop yields (Ayars et al., 2011). However, where primary salinization caused by parent materials and high water tables naturally high in salts is concerned, decreasing the negative effects of salinity may include reducing surface evaporation, reducing capillary rise of salt-affected groundwater, tile drainage installation or additions of organic materials (Hanson et al., 2006; Tejada et al., 2006; Ayars et al., 2011; Provin and Pitt, 2015). Changes in soil microbiological functioning as a result of these

management strategies can give a short-term indication of the success of aboveground land management in decreasing salinity (Dose et al., 2015) as biological indicators of soil change can often be detected prior to any chemical and physical measures (Dick, 1994; Dose et al., 2015). Using measures of nitrification and denitrification during salinity reduction can aid in the early detection of effective management approaches, which can help identify optimum strategies to reduce the cost and environmental impact of salinity reduction (Ritz et al., 2009; Wessén and Hallin, 2011; Wessén et al., 2011). Additionally, evaluating the bacterial community structure as an indicator of successful primary salinity reduction is unknown.

Therefore, to better understand if salinity reduction is occurring, the objectives of this study were to: (1) determine the effect of salinity reduction management strategies on soil nitrification and denitrification enzyme activities, 2) identify changes in functional gene copy numbers for nitrifiers and denitrifiers and 3) assess changes in nitrifier and denitrifier community structure during salinity reduction.

Materials and methods

Study site

The study area was located in Grand Forks County, North Dakota, USA (47°52'36.8688"N, 97°12'27.4788"W). The climate of the area is continental with an average temperature of 4.4 °C and 363 mm of precipitation (NDAWN, 2015). Over the study period the cumulative precipitation collected from a rain gauge placed at the southern edge of the field was 379 mm in 2013, 408 mm in 2014 and 369 mm in 2015. The study site was located on a Bearden silty clay loam, saline phase (fine-silty, mixed, superactive, frigid Aeric Calciaquolls) soil (Soil Survey Staff, 2015). The soil series formed from Glacial Lake Agassiz sediments characterized by high water tables and locally excess salts. Calcium and Mg sulfate salts are dominant in the

area, but mixtures of Ca^{2+}-, Mg^{2+}-, and Na^{+}- Cl^{-} salts are present (National Cooperative Soil Survey Database, 2016).

Experimental design

Prior to the start of the experiment, the soil was managed under conservation tillage, but was managed as no-till during the duration of the study. The soil was mapped as a Bearden silty-clay loam, moderately saline phase (Fine-silty, mixed, superactive, frigid Aeric Calciaquolls) (US Soil Survey Staff, 2015). The experiment was a randomized complete block design with a split-split plot arrangement, established in 2013 with three replicates. Each plot was 24 x 24 m in size. The whole plots consisted of tile-drained and non-tile-drained treatments. Tile (10.2 cm diameter, nylon sleeved) was installed in the fall of 2012 to a depth of 1.2 m. The split plots consisted of a control rotation, which included wheat (*Triticum aestivum*, variety Mayville) in 2013, soybeans (*Glycine max* L., variety Asgrow 00932) in 2014, and wheat (variety Mayville) in 2015 and 3 full season cover crop treatments (low, medium and high diversity). Prior to wheat planting, the plots received an application 22.4 kg-N ha^{-1} as urea, which was applied by hand. In 2013, all plots were planted to wheat and cover crops planted in late August following harvest. In 2014 the entirety of cover crop plots were planted with the respective full season mix. In 2015, the cover crop plots were split into thirds with 2/3 of the plots being planted to cover crops and the remaining 1/3 planted to wheat. Further splitting the plots in this way enables comparison of the effects of control, one year of full season cover crops followed by soybean and two years of full season cover crops on soil properties. The cover crop mixes and seeding rates are listed in Table 11. Cover crops were planted with a cone-seeder no-till drill, in 2013 after wheat harvest and in 2014 and 2015 at the same time as annual cash crop planting. Cover crops were terminated with a roller crimper at the time of cash crop harvest. An additional 4 Mg gypsum ha^{-}

[1] treatment was included in the study to reduce the risk of sodification during salt removal and was applied in 2013 prior to cover crop seeding. Weeds in the cash crops and cover crops were controlled by herbicides and hand-pulling, respectively. The surface soil texture was determined on air dried, ground soils using the hydrometer method (Gee and Bauder, 1986) and was predominantly silty clay loam.

Table 11. Full season cover crop mixes, seeding rates and biomass collected in the study. Different letters following Fisher's least significant different test at $p < 0.05$ denote significant differences. Data are mean values (±SE). Uppercase letters denote significance within cover crop mix across year and lowercase letters denote significance within year across cover crop mix.

	Low Diversity	Medium Diversity	High Diversity
Seeding rate:	37 kg ha^{-1}	71 kg ha^{-1}	40 kg ha^{-1}
Species†:	Sunflower, Oil	Sorghum Sudangrass, Sweet	Triticale, Spring
	Oats, Pinnacle	Barley, Certified Lacey	Oats, Pinnacle
	Pea, 4010 Forage	Radish, Daikon	Sorghum Sudangrass, Sweet
	Turnip, Purple Top	Clover, Crimson Dixie	Millet, Golden German
		Rape Seed, Dwarf Essex	Pea, 4010 Forage
			Clover, Crimson Dixie
			Safflower, MonDak
			Turnip, Purple Top
			Buckwheat, Mancan
	Biomass, Mg ha^{-1}		
2013	3.27(±0.17) ABC	3.47(±0.17) AB	3.69(±0.17) A
2014	2.07 EFG	3.06 ABCD	3.03 ABCD
2015 1 year cover crops	2.72 BCDE	2.47 CDEF	2.24 DEFG
2015 2 year cover crops	1.71 FG	1.46 G	1.93 EFG

†sunflower (*Helianthus annuus*), oats (*Avena sativa*), pea (*Pisum sativum* L.), turnip (*Brassica napa*), sorghum sundangrass (*Sorghum X drummondii*), barley (*Hordeum vulgare*), radish (*Raphanus sativus*), clover (*Trifolium incarnatum*), rape seed (*Brassica napus*), triticale (*Triticale hexaploide* Lart.), millet (*Pennisetum glaucum*), safflower (*Carthamus tinctorius*), buckwheat (*Fagopyrum esculentum*)

Soil sampling

Geo-referenced areas offset by 6 m from the installed tile drainage lines or from the plot center in non-tile-drained plots were used to collect soils. Three soil samples were composited from each plot from a 0-30 cm depth using an AMS Dutch auger (7.6 cm diameter, AMS, Inc. American Falls, ID, USA). After passing through a 2 mm sieve at field moist conditions, soils were stored at -20 °C until further analysis. Soil sampling occurred during the fall, post-harvest, for three years on August 29, 2013, September 25, 2014 and August 7, 2015.

Chemical analysis of soil

In order to determine whether soil salinity reduction managements were successful, soil chemical analyses for pHe, ECe, SARe and total carbon (TC) were performed on air dried, ground (<2 mm) soil. Inorganic N was extracted on field moist soil using a 2M KCl solution as outlined by Bremner (1965) and extracts analyzed for NH_4 and NO_3 using a SEAL Auto Analyzer 3 (SEAL Analytical, Mequon, WI, USA). Extracts from the saturated paste method (Rhodes, 1996) were used to determine soil pHe (Accumet Basic AB15, Fisher Scientific, Pittsburg, PA, USA), ECe (Hach sensION378, Hach, Loveland, CO, USA) and the concentrations of Na^+, Mg^{2+} and Ca^{2+} using an atomic absorption spectrophotometer (Buck Scientific Model 200A, East Norwalk, CT, USA) and SARe calculated as outlined by Richards (1954). The combustion method measured TC via a Skalar (Primatics^{ATC}, Skalar Analytical BV, Netherlands).

Soil enzyme activity

To determine whether N transformations improved with soil salinity reduction, measurement of nitrification and denitrification enzyme activities were performed. The first and rate-limiting step to nitrification is regulated by the enzyme ammonia monooxygenase produced

by ammonia oxidizing bacteria and archaea and its activity was determined as outlined by Berg and Rosswall (1985). Nitrate reductase, the enzyme that converts NO_3^- to NO_2^- and is produced by denitrifying bacteria, activity was used to measure the denitrification potential was determined as outlined by Abdelmagid and Tabatabai (1987). Both enzyme activities were colorimetrically quantified from field moist soil samples using a spectrophotometer (Thermo Scientific 20D+, Thermo Fisher Scientific, Madison, WI, USA).

DNA extraction and functional gene quantification

Functional gene copy numbers represent the population of organisms capable of producing enzymes responsible for N transformations. Rapid changes in copy numbers can occur as organisms react to changes in substrate availability. As such, functional gene copy numbers are useful as long-term and short-term indicators of soil and ecosystem health. Gene copies were assessed using quantitative Polymerase Chain Reaction (qPCR) using primers specified for a target gene.

In order to determine changes in functional gene copy numbers of nitrifying bacteria, archaea, and denitrifying bacteria during salinity reduction qPCR was used. Soil DNA was extracted from 0.25 g of soil using the Power Soil DNA Extraction Kit (MOBIO Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's instructions. The quantity of extracted DNA was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All genomic DNA was then diluted to a concentration of 4 ng μL^{-1} with DNA/RNAse free water and stored at -80 °C until downstream qPCR application. Quantitative PCR was performed using the PikoReal system (Thermo Scientific, Wilmington, DE, USA) using primers designed to amplify genes important to nitrification and denitrification.

The *AmoA* gene encodes for ammonia monooxygenase, the first step in nitrification. The forward primer, amoA1F 5'-GGGTTTCTACTGGTGGT-3' and the reverse primer amoA2R 5'-CCCCTCKGSAAAGCCTTCTTC-3' (Rotthauwe et al., 1997) were used to amplify a 491-bp DNA fragment of the *amoA* gene in nitrifying bacteria (AOB). The forward primer Arch-amoA-for 5'-CTGAYTGGGCYTGGACATC'3' and the reverse primer Arch-amoA-rev 5'TTCTTCTTTGTTGCCCAGTA-3' (Wutcher et al., 2006) were used to amplify a 256-bp DNA fragment of the *AmoA* gene in nitrifying archaea (AOA). To determine the quantity of denitrifiers, the forward primer, nosZ1 5'-WCSYTGTTCMTCGACAGCCAG-3' and the reverse primer nosZ1R 5'-ATGTCGATCARCTGVKCRTTYTC-3' (Henry et al., 2006) were used to amplify a 259-bp DNA fragment that encodes for the nitrous oxide reductase. All primer pairs were synthesized by Invitrogen, (Carlsbad, CA, USA).

A 20 µL reaction volume was used for qPCR that contained 10 µL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 0.3 µL of 10 µM forward and reverse primer, 2 µL of 4 ng μL^{-1} DNA template, and 7.4 µL of DNA/RNAse free water. The qPCR thermocycler protocol used was 5 min at 95 °C followed by 40 cycles of denaturing at 95 °C for 15 s, annealing at temperatures optimized for each primer for 30 s with a data acquisition step, and extension at 72 °C for 30 s followed by a melt curve analysis. The annealing temperature was 55 °C for the AOA primers, 56 °C for the AOB primers and 62 °C for the *nosZ* primers. Separate standard curves were generated for each functional gene using five serial dilutions of genomic DNA ranging from five ng μL^{-1} to 500 fg μL^{-1}. Organismal DNA controls used for the AOA primers were isolated from the nitrifying archaea *Nitrosopumilus maritimus* SCM1, while the DNA controls used for the AOB primers were isolated from the nitrifying bacteria *Nitrosomonas europaea* (DSM 28437, DSMZ, Leibniz-Institut, Braunschweig,

Germany). The organismal DNA control used for the *nosZ* primer was isolated from the denitrifying bacteria *Pseudomonas aeruginosa* PAO-1 (ATTC 47085, Manassas, VA, USA). Standard curve R^2 values were 0.99. Genomic DNA isolated from standard curve organismal controls and soil samples were run in triplicate. The average threshold cycle (Ct) was 23.8 for AOB primers, 26.63 for AOA primers and 31.8 for *nosZ* primers.

Sequencing by synthesis

In order to assess changes in soil nitrifier and denitrifier community structure during salinity reduction, sequencing by synthesis with unique sample barcodes was employed. Extracted soil DNA was amplified in triplicate using the amoA1F/amoA2R, Arch-amoA-for/Arch-amoA-rev and nosZ1/nosZ1R primer pairs. First, a 25 µL reaction with 0.2 µL GoTaq Flexi DNA Polymerase (Promega Corporation, Madison, WI, USA), 5 µL buffer, 2.5 µL dNTPs, 5 µL $MgCl_2$, 1.5 µL forward and reverse primers, 2 µL of 4 ng μL^{-1} DNA template, and the remaining volume RNA/DNA free water. The PCR thermocycler conditions used were 5 min at 95 °C followed by 25 cycles of 94 °C for 30 s, 55 °C for the AOA primer, 56 °C for the AOB primer and 62 °C for the *nodZ* primer for 30 s, and 72 °C for 30 s followed by a final extension at 72 °C for 5 min using the GeneAmp PCR System 9700 (Applied Biosystems, Thermo Fischer, Grand Island, NY, USA). PCR products were verified on a 1% agarose gel to ensure proper amplification of the target base pairs. The triplicate amplicons were pooled and a second step-out PCR amplification was used to apply a unique 8-bp dual indexed barcode and adapter oligos to each sample. A 25 µL reaction with 10.2 µL GoTaq Flexi DNA Polymerase (Promega Corporation, Madison, WI, USA), 5 µL buffer, 2.5 µL dNTPs, 5 µL $MgCl_2$, 0.3 µL of unique forward barcode, 0.3 µL of unique reverse barcode 2.5 µL of 4 ng μL^{-1} amplicon from the first PCR reaction, and the remaining volume was RNA/DNA free water. Five cycles using the above

described thermocycler protocols were used to attach the unique barcodes to each sample. PCR products were again verified on a 1% agarose gel. Phillip San Miguel of the Purdue University Genomics Core Facility provided the unique barcode sequences. The barcoded amplicons were pooled by the initial primer, and samples were cleaned and size selected using a BluePippin (Sage Science, Beverly, MA, USA) with a 1.5% agarose cassette. Amplicon concentration was assessed using Qubit 2.0 Fluorometer (Thermo Fischer Scientific, Waltham, MA, USA). The PCR barcoded amplicons were then pooled in equimolar concentrations. Final amplicon quality was assessed using an Agilent bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and qPCR. Genetic sequence data was obtained using the Illumina MiSeq set to 2×250 reads (Illumina, Inc., San Diego, CA, USA) from the Purdue Genomics Laboratory (West Lafayette, IN, USA). Sequence reads were aligned and mapped to reference sequences provided by the FunGene functional gene pipeline and repository (Fish et al., 2013). The number of reads per library, minimum, maximum and mean read length, coverage mean and standard deviation and mean mapping quality can be found in Table A1.

Statistical analysis

In order to determine the effect of salinity reduction strategies on soil chemical properties and enzyme activities and functional gene copy numbers a repeated measures analysis of variance (ANOVA) was run using Proc Mixed in SAS 9.4 (SAS Inc., Cary, NC, USA). Sampling time was repeated per plot. Replication was considered a random factor while sampling time, drainage, cover crops and gypsum application were considered fixed factors. Main effects and interaction effects were assessed post hoc with estimated mean separation using Tukey's procedure at the $p < 0.05$ level in conjunction with the use of an algorithm for determining letter representation of all pair-wise comparisons based on Saxton (1998). Pearson correlations

between enzyme activity, functional gene copy numbers and soil chemical and texture measurements were assessed using Proc Corr in SAS.

To assess changes in soil nitrifier and denitrifier community structure during salinity reduction non-metric multidimensional scaling (NMS) was performed using PC-ORD (McCune and Mefford, 2011) as described by Mather (1976) and Kruskal (1964). Nonmetric multidimensional scaling is an ordination technique suited for non-normal and non-linear ecological data that simplifies complex datasets by determining sources of variation displayed graphically as axes. The default "slow and thorough" procedure with a Sorensen distance measure and a random starting point resulted in 250 runs with real and randomized data using PC-ORD. After NMS analysis, differences in drainage and treatments were assessed using multi-response permutation procedures (MRPP) with a Sorensen distance measure as described in McCune and Grace (2002) and Stroup and Stubbendieck (1983). Multiresponse permutation procedure analysis is a nonparametric tests used to determine differences in groups. Differences due to management were determined using pairwise comparisons with a Bonferroni correction.

Results and discussion

Salinity reduction effects on soil chemical properties

Reducing the salt content is of utmost importance to improve crop growth on saline soils. The results indicate a successful decline in the salt content during the duration of the study period (Table 12). Throughout the study, the ECe of the soil had a continuous decline from 7.01 dS m^{-1} in 2013 to 4.54 dS m^{-1} in 2015 across all treatments and drainage (Table 13).

Table 12. Analysis of variance results F-value and p-value for inorganic N (NH_4 and NO_3), electrical conductivity (ECe) and pHe of the saturated extract and sodium adsorption ratio (SARe) for the main effects of drainage, treatment and time and their interactions. Bold values indicate significance at the p <0.05 level.

		NH_4		NO_3		ECe		pHe		SARe		Total Carbon	
	df	F value	p-value	F value	p-value	F value	p-value	F value	p-value	F value	p-value	F value	p-value
Repetition	2	7.18	**0.0027**	3.14	0.0571	4.53	**0.0188**	3.37	**0.0474**	12.37	**0.0001**	3.68	**0.0368**
Drainage	1	0.80	0.3790	1.32	0.2589	0.37	0.5466	0.55	0.4625	2.87	0.1001	0.79	0.3821
Treatment	7	0.26	0.9630	2.17	0.0645	1.05	0.4207	3.38	**0.0084**	0.42	0.8822	0.92	0.5051
Time	2	16.18	**<0.0001**	27.16	**<0.0001**	62.22	**<0.0001**	152.41	**<0.0001**	5.08	**0.0091**	10.93	**<0.0001**
Time x Drainage	2	1.26	0.2905	0.49	0.6172	0.70	0.5000	4.22	**0.0193**	0.87	0.4245	3.58	**0.0339**
Time x Treatment	14	0.87	0.5947	2.09	**0.0256**	1.03	0.4356	1.25	0.2650	0.76	0.7111	0.86	0.6045
Drainage x Treatment	7	0.55	0.7892	1.59	0.1748	1.72	0.1414	2.52	**0.0357**	0.64	0.7227	0.46	0.8582
Time x Drainage x Treatment	14	0.80	0.6611	1.10	0.3794	1.36	0.2012	0.45	0.9518	2.66	**0.0044**	0.40	0.9701

Table 12. Analysis of variance results F-value and p-value for inorganic N (NH_4 and NO_3), electrical conductivity (ECe) and pHe of the saturated extract and sodium adsorption ratio (SARe) for the main effects of drainage, treatment and time and their interactions. (continued).

		Nitrate reductase		Ammonia mono-oxygenase		AOA gene copies		AOB gene copies		*nodZ* gene copies	
	df	F value	p value	F value	p value	F value	p value	F value	p value	F value	p value
Repetition	2	0.92	0.4093	1.39	0.2641	0.12	0.8886	2.29	0.1180	15.41	**<0.0001**
Drainage	1	0.09	0.7658	0.05	0.8279	4.70	**0.0380**	0.08	0.7816	0.24	0.6285
Treatment	7	0.56	0.7848	1.34	0.2659	20	0.9841	0.65	0.7127	1.02	0.4360
Time	2	7.12	**0.0017**	5.57	**0.0060**	19.69	**<0.0001**	0.25	0.7792	3.34	**0.0420**
Time x Drainage	2	0.28	0.7596	11.71	**<0.0001**	0.08	0.9195	0.96	0.3879	0.77	0.4684
Time x Treatment	14	1.31	0.2294	0.28	0.9945	068	0.7853	1.65	0.0900	0.63	0.8309
Drainage x Treatment	7	0.28	0.9585	0.41	0.8897	1.07	0.4055	1.22	0.3224	3.45	**0.0076**
Time x Drainage x Treatment	14	0.73	0.7374	1.01	0.4586	0.38	0.9762	0.79	0.6719	0.21	0.9989

Table 13. Calculated mean values across years (±SE) for inorganic N (NH_4 and NO_3), electrical conductivity (ECe), pHe and sodium adsorption ration (SARe) of the saturated extract, total carbon, nitrate reductase enzyme activity, ammonia monooxygenase enzyme activity, ammonia oxidizing archaea (AOA), and *nosZ* denitrifier gene copies for the main effect of time. Different letters within columns are significantly different following Fisher's least significant different test at $p < 0.05$.

	NH_4, mg kg^{-1}	NO_3, mg kg^{-1}	ECe, dS m^{-1}	pHe	SARe	Total Carbon, %	Nitrate reductase, µg NO_2-N mL^{-1} g soil $24h^{-1}$	Ammonia monoox-ygenase, µg NO_2-N mL^{-1} g soil $5h^{-1}$	AOA gene copies	*nodZ* gene copies
2013	1.12 (±0.07)a	11.63 (±0.70)a	7.01 (±0.35)a	7.93 (±0.01)c	3.96 (±0.34)a	3.68 (±0.07)ab	1.57 (±0.19)b	2.77 (±0.10)b	3.17 (±0.04)b	4.62 (±0.08)b
2014	1.10a	0.042b	5.37b	7.96b	3.72a	3.83a	1.08c	3.12a	2.94c	4.86ab
2015	0.57b	0.69b	4.54c	8.20a	2.62b	3.66b	2.08a	2.68b	3.31a	4.96a

Although there was a reduction in salinity, sensitive crops may continue to do poorly as the average soil salinity is over 4 dS m^{-1} and meets salinity criteria according to the USDA-NRCS Handbook 60. While the ECe declined through the study period, the pHe increased from 7.93 in 2013 to 8.20 in 2015. Across years under both tile drainage treatments the pHe increased from 7.92 to 8.21 in undrained soils and from 7.94 to 8.20 in drained soils (Fig. 6).

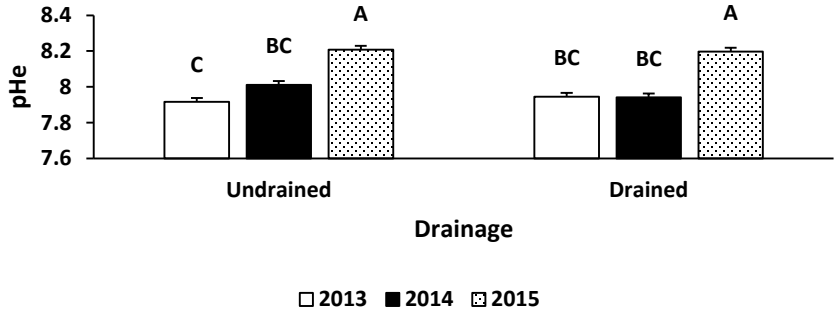

Figure 6. The average soil pHe for the interaction of drainage by sampling year where different letters denote significant difference at $p < 0.05$ following Tukey's procedure. Data are mean values (±SE).

The aboveground treatments also had a significant effect when combined with drainage on the soil pHe. In drained conditions, the pHe of the check treatment increased, while growing one and two years of cover crops on drained conditions reduced the pHe of the soil to 7.89. Finally, the low diversity cover crops had the highest pHe compared to all other treatments in undrained conditions (8.14) (Fig 7). Sunflower (*Helianthus annuus)*, is a high water using crop that was only planted in the low diversity cover crop mix. Although there were no differences in water content in the soil at the time of sampling, perhaps including sunflowers in the mix induced a higher soil pHe. Although not agronomically significant, the increase in soil pHe in combination with the reduction of ECe may be explained by the hydrolysis of water and the replacement of H^+ ions on the cation exchange sites as the salt-derived ions are displaced as

these displaced salts do not react with OH^- in the soil solution (Abrol et al., 1988) which would result in an increase in pHe.

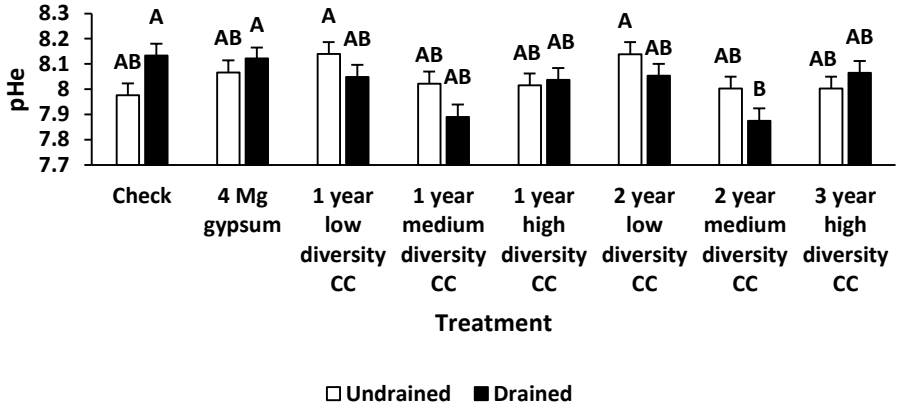

Figure 7. The average soil pHe for the interaction of drainage by treatment where different letters denote significant difference at $p < 0.05$ following Tukey's procedure. Data are mean values (±SE).

Additionally, the leaching of the soluble salts, especially that of the easily exchanged Na^+ ions would reduce SARe, as was observed here as the Na decreased over the study period from 23.1 to 13.4 $mmol_{(c)}\ L^{-1}$, while the sum of Ca^{2+} and Mg^{2+} decreased from 58.9 to 43.4 $mmol_{(c)}\ L^{-1}$. There were initial differences between treatments and tile drainage in 2013 but by 2015 there were no differences in SARe across treatments (Figure 8-9).

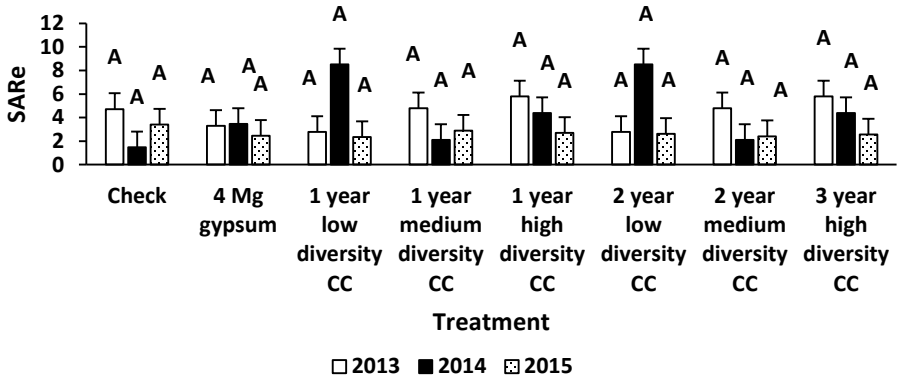

Figure 8. The average soil SARe for the interaction of treatment and sampling year for undrained conditions where different letters denote significant difference at $p < 0.05$ following Tukey's procedure. Data are mean values (±SE).

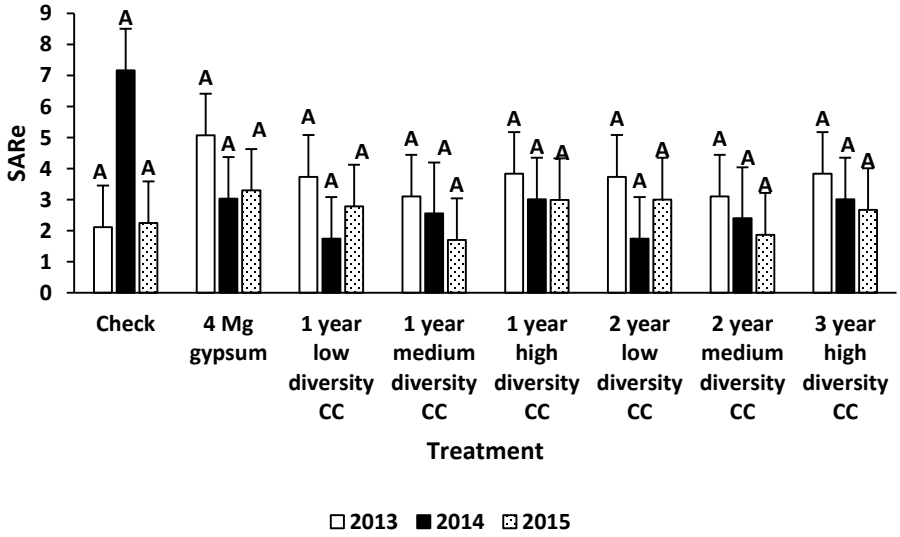

Figure 9. The average soil SARe for the interaction of treatment and sampling year for drained treatments where different letters denote significant difference at $p < 0.05$ following Tukey's procedure. Data are mean values (±SE).

Since the goal of ameliorating soil salinity is reducing soil ions in solution, care must be taken to safeguard against soil sodification which results in clay dispersion and poor physical quality (Arbol et al., 1988). Recent work by He et al. (2013) indicates that soil dispersion can occur at low SARe values (5 or less) when soil ECe is also low (2 dS m^{-1} or less). Although the

SARe of this experiment was reduced across all treatments, the SARe values are lower than 5 which is the recommended SARe for soils in the study region.

The ECe was not affected by treatment, but a steady decline over the study period was observed. During this study, the soil was managed under a no-till system. Residue remaining on the surface under no-till managements can limit the rate of evaporation from the soil surface (van Donk, 2012) thereby reducing the potential to deposit salts at the soil surface. Although biomass collected in the fall of each year (Table 11) was lowest where cover crops were grown for two years, the lack of significance between treatments for measured soil ECe values indicates that a range of 8.7 to 4.2 Mg ha^{-1} biomass is sufficient to reduce water evaporation from the soil surface and limit the accumulation of salts.

Although NO_3 was significantly affected by the interaction of drainage and the year soil samples were collected (Table 12) it is likely a relic of natural soil variability and N fertilizer additions. Higher NO_3 concentrations were observed in 2013 and 2015 when N fertilizer was applied prior to planting wheat. In 2014, no N fertilizer was applied when soybeans were planted hence lower NO_3 values were to be expected. Finally, TC was lowest in 2015 where tile drainage was installed (Table 14) and the statistical significance is likely due to soil variability as the range in TC was small (3.54 to 3.87%).

Table 14. Calculated mean values (±SE) for NO_3, total carbon (TC) and ammonia monooxygenase enzyme activity where different letters denote significant difference at $p < 0.05$ following Tukey's procedure.

	NO_3	TC	Ammonia Monooxygenase
2013 Drained	10.17 (±0.97) A	3.66 (± 0.10) ABC	2.63 (±0.14) B
2013 Undrained	6.39 (±1.19) AB	3.71 (±0.10) CD	2.90 (±0.14) AB
2014 Drained	0.29 (±1.03) C	3.79 (±0.10) AC	3.52 (±0.15) A
2014 Undrained	0.37 (±0.97) C	3.87 (±0.10) AB	2.72 (±0.14) B
2015 Drained	6.19 (±0.97) B	3.54 (±0.10) BD	2.45 (±0.14) B
2015 Undrained	7.61 (±0.97) AB	3.79 (±0.10) ABC	2.90 (±0.14) AB

Salinity reduction effects on soil biological properties

Similar to soil chemical properties, the year soils were sampled had a significant effect on measured soil biological properties (Table 13). The nitrate reductase enzyme activity follows a similar pattern as NO_3 levels in the soil where lowest activity levels of nitrate reductase corresponded to the lowest availability of NO_3 substrate in the soil. There was no relationship between *nosZ* gene copy numbers and soil NO_3 concentrations and additional research has shown *nosZ* genes to be unresponsive to NO_3 levels (Saleh-Lakha et al., 2009). There was a significant interaction effect of drainage and treatment on *nosZ* gene copy numbers (Table 12), but the mean separation following Tukey's procedure did not show statistical differences between the interaction of drainage and treatment (Figure 10).

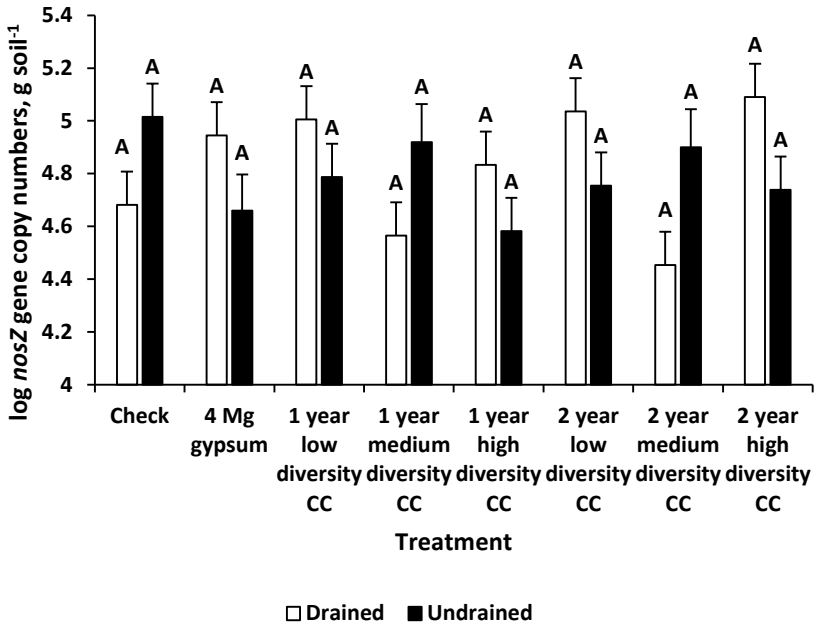

Figure 10. The average soil *nosZ* gene copy numbers for the interaction of drainage and treatments where different letters denote significant difference at $p < 0.05$ following Tukey's procedure. Data are mean values (±SE).

Low levels of NH_4 in the soil corresponded to reduced ammonia monooxygenase activities and AOA increased in number when NH_4 was limiting. Ammonia oxidizing archaea are favored in unfertilized long-term fallow and grassland managements (Taylor et al., 2012; Taylor et al., 2010) characterized by oligotrophic environments low in ammonia concentrations (Martens-Habbena et al., 2009; Schleper, 2010; Sims et al., 2012). Tile drainage had a variable effect on ammonia monooxygenase enzyme activity (Table 14) but no clear pattern emerged.

Salinity reduction effects on soil nitrifier and denitrifier community structure

In total, 352 denitrifing bacterial strains belonging to 19 genus, 23 AOB belonging to 3 three identified genus and over four species and 132 AOA belonging to 2 identified phyla were identified using sequencing by synthesis. Phylogenetic trees using the nearest neighbor method

can be found in Figures 11-13. Multiple sequence alignments were completed using the Geneious software (Kearse et al., 2012) using the MUSCLE alignment option (Figures A1-3).

The denitrifying bacteria found have been described from diverse environments such as the human gut (Ichise et al., 2015), aquatic and terrestrial environments (Yan et al., 2008; Wisniewski-Dye et al., 2011). Many of the identified AOB and AOA sequences were first identified and described in high pH and saline environments including brackish estuaries, methane sea vents and soil (Futamata et al., unpublished; Kim et al., 2008; Jiang et al., 2010; Li et al., 2015).

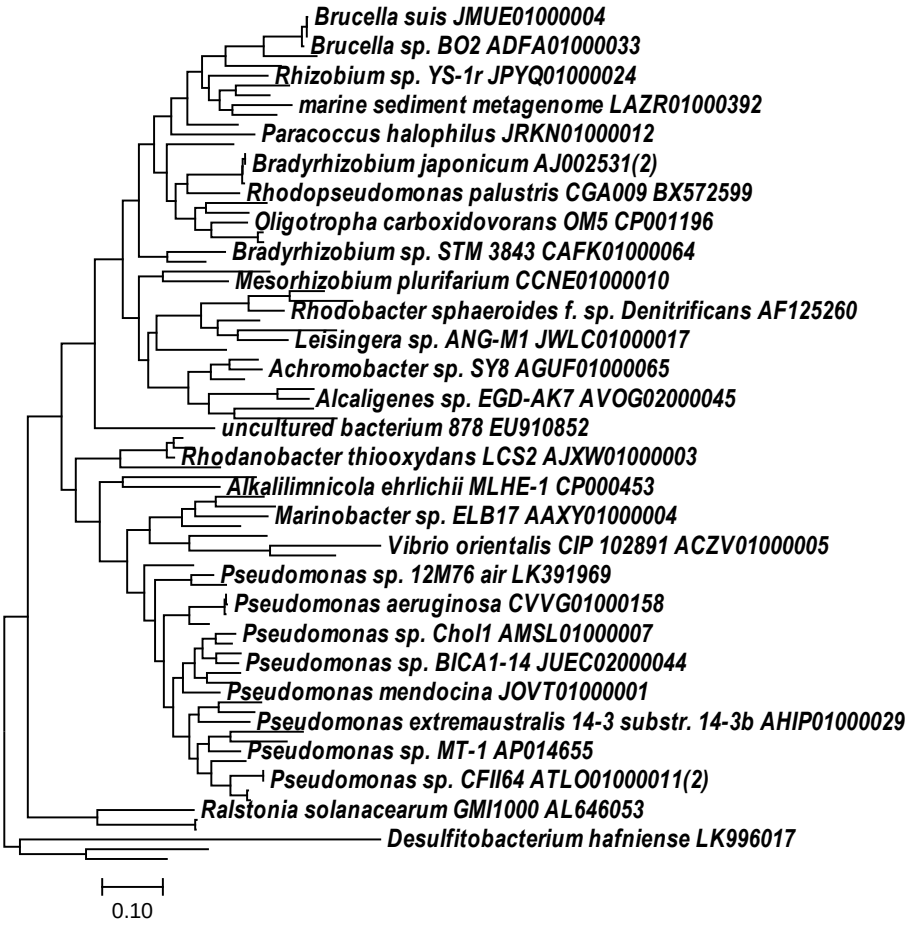

Figure 11. The evolutionary history of *nosZ* genes identified by sequencing by synthesis was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 87 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1545 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2015).

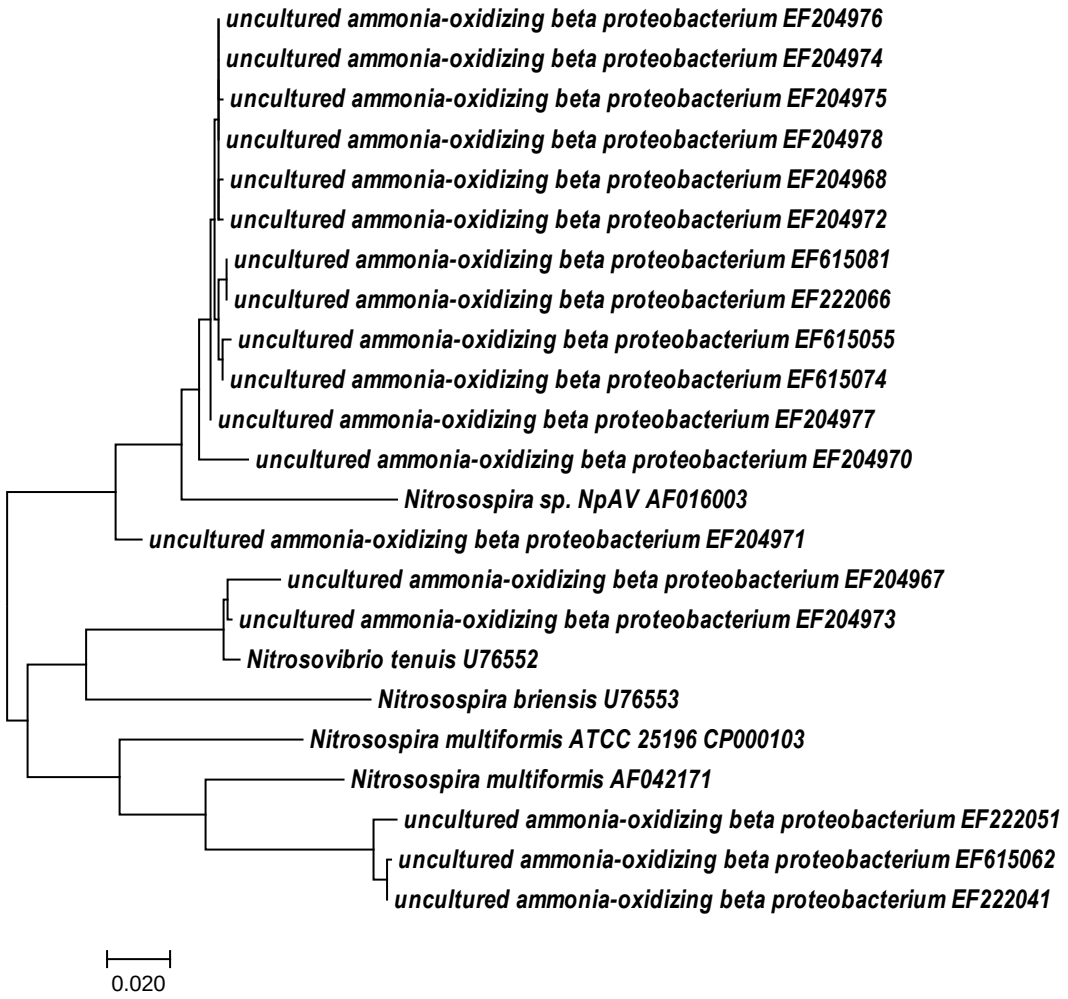

Figure 12. The evolutionary history of ammonia oxidizing bacteria identified by sequencing by synthesis using *amoA* gene primers was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 87 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1545 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2015).

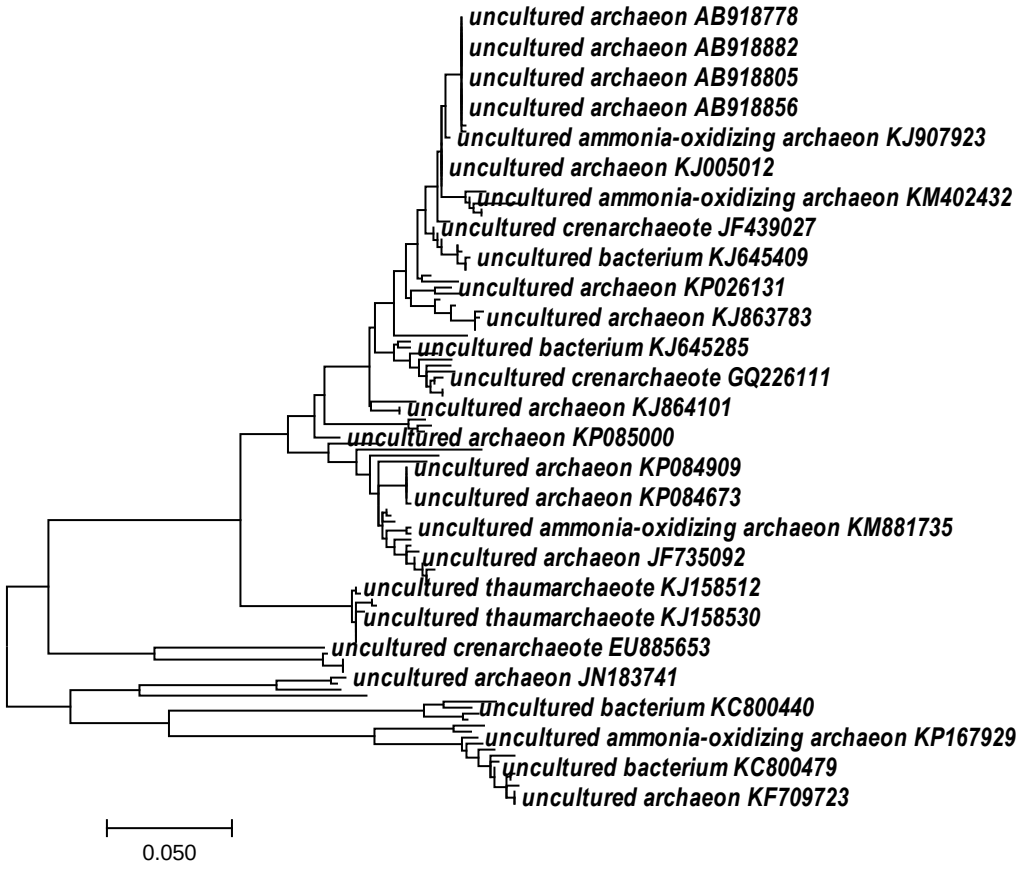

Figure 13. The evolutionary history of ammonia oxidizing archaea identified by sequencing by synthesis using *amoA* gene primers was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 87 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1545 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2015).

Nonmentric multidimensional scaling resulted in nitrifier and denitrifier communities clustering in distinct patterns based on aboveground land management (Fig. 14). Nonmetric multidimensional scaling ordination resulted in a two dimensional solution. All selected axes for each ordination were significantly different from random (p<0.05) and reduced stress by 5 or more. A final stress of 20.08 with instability of 0.0×10^{-5} was reached after 32 iterations. Overall,

78.7% of the variation was explained in two axes. The first axis represents 57.6% of the variation. The second axis represents 21.0% of the variation.

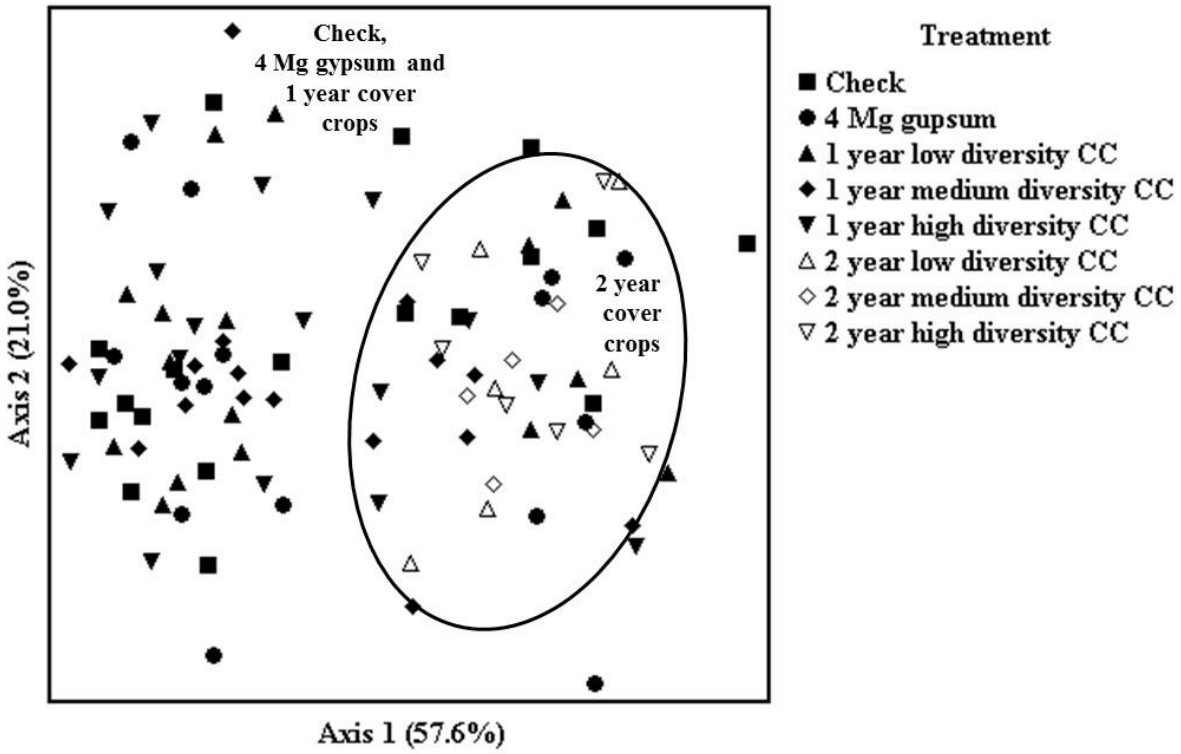

Figure 14. Ordination diagrams of soil chemical and physical properties displayed by treatment with the 2-year cover crop treatments clusters highlighted.

Axis 1 is positively correlated with pHe with an absolute r value greater than 0.5. Points that are closer together are more similar, while points that are further apart are more dissimilar. Therefore, axis 1 represents the shift in nitrifier and denitrifier community structure resulting from the change in pHe when cover crops are grown for two consecutive years as these points cluster to the positive side of axis 1. Nitrifier and denitrifier communities do not cluster based on tile drainage treatments. Several identified denitrifier sequences are strongly correlated to the check, 4 Mg gypsum and 1-year cover crop treatments, where the pHe was lower and include

Achromobacter cycloclastes, Bordetella petrii, Cardiobacterium hominis, Leisingeria sp., *Mesorhizobium plurifarium, Pseudomonas* sp. including *P. stutzeri and P. Tuomuerensis, Ralstonia eutropha, R. solanacearum, Rhodobacter sphaeroides, Streptococcus pneumoniae* and *Thioalkalivibrio thiocyanoxidans. A. cycloclastes* has been shown to have active nitrous oxide reductase enzyme activity over a wide range of pH, however over pH of 8 the activity is lowered due to a reduced availability of H^+ (Fujita and Dooley, 2007) and is likely why *A. cycloclastes* is more abundant at lower soil a pHe. Additionally, other identified strains such as *B. petrii* have been identified and associated with acidic soil pH (Palmer et al., 2010)

The MRPP analysis resulted in a p value <0.001 for treatment. Pairwise comparisons between treatments with a Bonferroni correction indicate that 1-year high diversity cover crops are significantly different from 2 years of high diversity cover crops and 2 years of medium diversity cover crops. All other pairwise comparisons were not significantly different. The composition of the denitrifying bacteria, AOB and AOA from 1-year high diversity cover crop, 2-year medium diversity cover crop, 2-year high diversity cover crop and all other treatments are found in Figure 15-17. The high diversity cover crop mixes harbored fewer *Rhodopseudomonas palustris* and *Afipia felis* than the 2-year medium diversity cover crops and all other treatments.

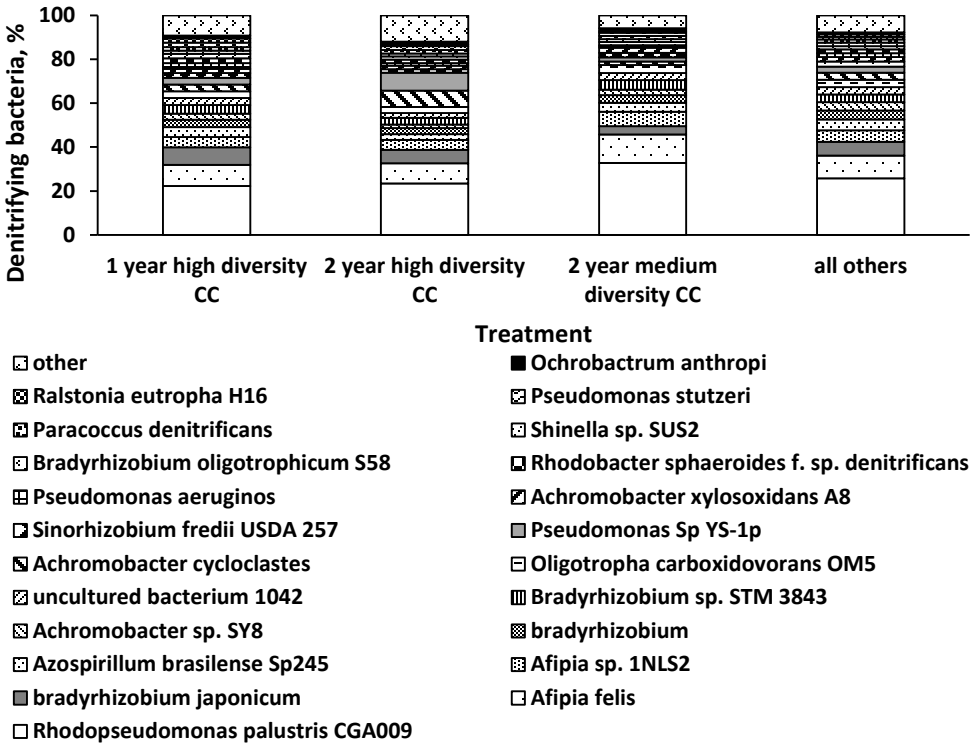

Figure 15. The average composition (%) of identified denitrifying bacteria in the soil under 1- and 2-year high diversity cover crops, 2-year medium diversity cover crops and all other treatments. The community structure of these treatments were identified as being significantly different based on a multiresponse permutation procedure with a Bonferroni correction. Strains were identified by sequencing by synthesis using *nosZ* gene primers.

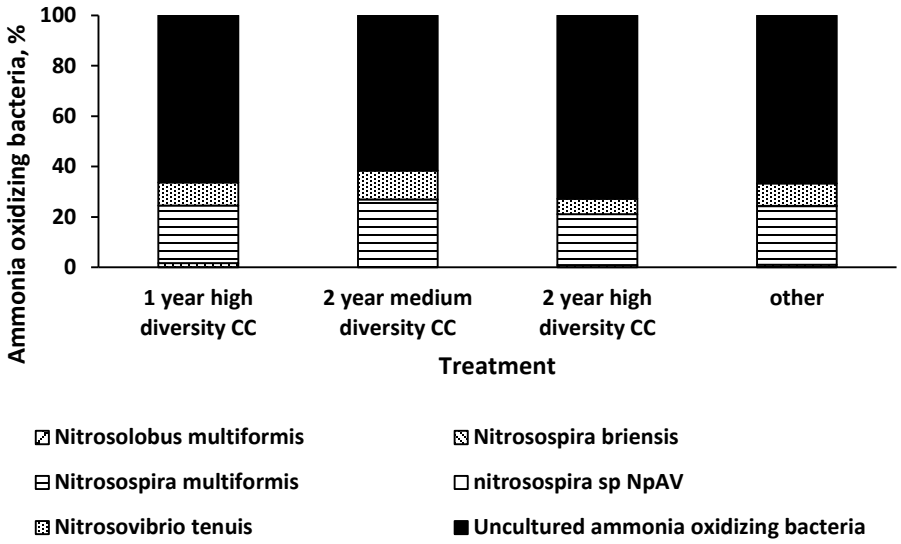

Figure 16. The average composition (%) of identified ammonia oxidizing bacteria in the soil under 1- and 2-year high diversity cover crops, 2-year medium diversity cover crops and all other treatments. The community structure of these treatments were identified as being significantly different based on a multiresponse permutation procedure with a Bonferroni correction. Strains were identified by sequencing by synthesis with ammonia oxidizing bacteria primers.

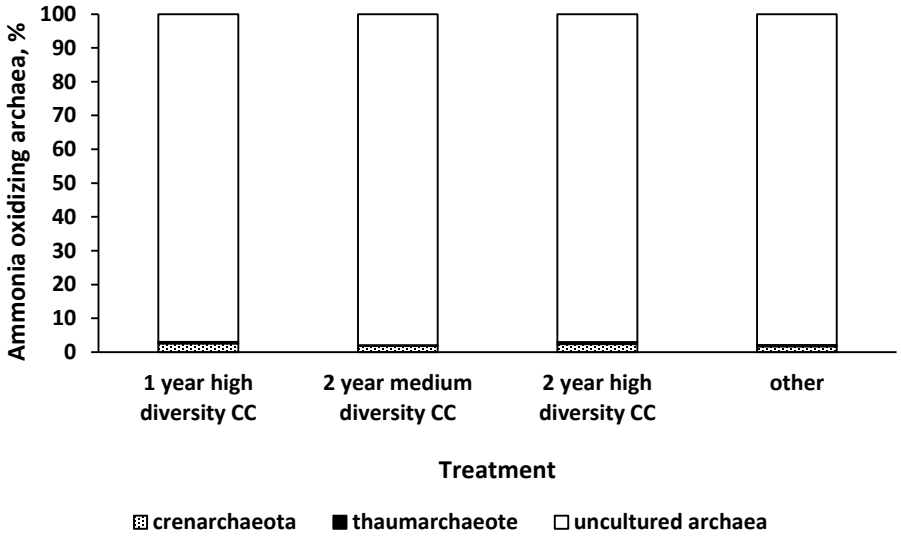

Figure 17. The average composition (%) of identified ammonia oxidizing archaea strains in the soil under 1- and 2-year high diversity cover crops, 2-year medium diversity cover crops and all other treatments. The community structure of these treatments were identified as being significantly different based on a multiresponse permutation procedure with a Bonferroni correction. Strains were identified by sequencing by synthesis with ammonia oxidizing archaea primers.

Both *R. palustris* and *A. felis* are N-fixing bacteria. *R. palustris* is ubiquitous in nature and has the ability to adjust its metabolism based on environmental factors and can be photoautotrophic, photoheterotrophic, chemoautotrophic and chemoheterotrophic (Larimer et al., 2004). *A. felis* is closely related to the slow growing *Bradyrhizobium japonicum* (Saito et al., 1998) and perhaps is less competitive under a diverse cover crop mix as *A. felis* is ologotrophic.

Pearson correlation coefficients between soil chemical and biological properties are presented in Table 15. The community structure of nitrifiers and denitrifiers were influenced by the soil pHe and SARe, which is in agreement with several other authors who attributed soil pH as a main driver in microbial communities in soil (Nicol et al., 2008; Fierer, 2009; Rousk et al., 2010). The lack of strong correlations between soil ECe and measured biological soil properties suggests the nitrifiers and denitrifiers have undergone community induced salinity tolerance (SICT), which is similar to pollution induced community tolerance where the microbial community is exposed to a pollutant overtime. The bacterial community adapts to the presence of the pollutant by selecting for members able to survive the toxic effects of the pollutant (Blanck et al., 1988). Although previous research has shown a negative effect of salinity on microbes and their function (Laura, 1977; McClung and Frankenberger, 1985; Saleem and Ahmed, 1988; Irshad et al., 2005; Akhtar et al., 2012) much of the research was conducted on non-saline soils where solutions of salt concentrations were added. The abrupt change in solution ion concentration by artificially salinizing the soil is likely a shock to the microorganisms and is reflected in the negative response of soil microbial function to increasing salinity gradients. However, SICT, which is a concept that salt will select for tolerant microbial species, has been shown to be induced in non-saline soils in as little as 40 days (Maheshwari, 2015). Microbial communities adapted to higher levels of soil salinity are more tolerant to flucutations in solution

electrical conductivity than microbial communities adapted to non-saline conditions (Rath and Rousk, 2015). For example, bacterial nitrifying cultures adapted to brackishwater showed no alteration in nitrification when grown under non-saline conditions, but nitrifying cultures collected from non-saline conditions were grown under high salinity effluent, the nitrification rate decreased and recovered partially over 54 days (Jonassen, 2013). The concept of SICT of microbial communities in soils is a relatively new field of microbial ecology and its adaptation, to our knowledge, as shown here is the first example SICT occurring in agricultural field soils. Due to these soils exhibiting SICT, management strategies to reduce soil salinity dominated by Ca^{2+} and Mg^{2+} salts may not need to be concerned with microbial functioning and leaching of salts can proceed using best management practices.

Table 15. Pearson correlation coefficient (r) between measured soil inorganic N (NH_4 and NO_3), electrical conductivity (ECe) and pHe on the saturation extract, sodium adsorption ratio (SARe), total carbon, nitrate reductase enzyme activity, ammonia monooxygenase enzyme activity, ammonia oxidizing archaea (AOA) gene copies, ammonia oxidizing bacteria (AOB) gene copies, and denitrifier (*nosZ*) gene copies.

	NH_4	NO_3	ECe	pHe	SARe	Total Carbon	Nitrate Reductase	Ammonia Mono-oxygenase	AOA gene copies	AOB gene copies	*NosZ* gene copies
NH_4	1	-0.08	0.13	-0.31***	0.19*	0.16	-0.06	0.25**	-0.33***	-0.07	0.01
NO_3		1	0.40***	0.01	0.16	0.17*	0.10	-0.27**	0.23**	-0.11	0.01
ECe			1	-0.61***	0.42***	0.49***	-0.05	-0.36***	0.01	0.02	-0.06
pHe				1	-0.14	-0.36***	0.22**	0.03	0.23**	0.06	0.17*
SARe					1	0.24**	-0.05	-0.13	-0.17*	-0.17*	-0.12
Total Carbon						1	-0.05	-0.16	-0.39***	-0.26**	-0.13
Nitrate reductase							1	-0.17*	0.20*	-0.01	0.02
Ammonia mono-oxygenase								1	-0.30**	0.04	-0.17*
AOA gene copies									1	0.52***	0.35***
AOB gene copies										1	0.33***
NodZ gene copies											1

* indicates significance at the p<0.05 level
** indicates significance at the p<0.01 level
*** indicates significance at the p<0.001 level

Conclusions

As the risk of salinization is a global problem facing the future of agriculture, ensuring management strategies reduce soil salinity complies with future needs is important to meet world food demands. This work highlights several salinity reduction strategies such as cover crops and no-till that were successful in reducing soil EC. Findings presented here showed that nitrification, denitrification and the nitrifying and denitrifying communities were not limited or impacted by long-term primary soil salinity and these communities have likely undergone salinity induced community tolerance. The community tolerance is a benefit as salinity is ameliorated and special strategies focusing on the considerations of restoring the microbial community after leaching salts may not be needed.

Acknowledgements

This work was supported by the Natural Resources Conservation Service, U.S. Department of Agriculture; under the Conservation Innovation Grants program. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the U.S. Department of Agriculture. Calcium Products Inc. of Ames, Iowa, USA provided the gypsum amendment. The North Dakota Agricultural Experiment Station Fellowship and the North Dakota Water Resources Research Institute Fellowship provided additional support. The authors would like to thank Joel Bell, Nate Derby, Kevin Horsager, Chandra Hegland and Tom Gross for their assistance in the field and laboratory.

References

Abdelmagid, H.M. and M.A. Tabatabai. 1987. Nitrate reductase activity of soils. Soil Biol. Beochem. 19:421-427. doi:10.1016/0038-0717(87)90033-2

Abrol, I.P., J.S.P. Yadav, and R.I.Massoud. 1988. Salt-affected soils and their management. Soil Resources, Management and Conservation Service, Food and Agricultural Organization of the United Nations. Land and Water Development Division. Rome, Italy.

Akhtar, M., F. Hussain, M.Y. Ashraf, T.M. Qureshi, J. Akhter, and A. Rasool Awan. 2012. Influence of Salinity on Nitrogen Transformations in Soil. Commun. Soil Sci. Plan. 43:12, 1674-1683. doi:10.1080/00103624.2012.681738

Ayars, J., Hoffman, G., and Corwin, D. 2011. Leaching and Rootzone Salinity Control. Agricultural Salinity Assessment and Management: Second Edition, pp. 371-403. doi:10.1061/9780784411698.ch12

Berg, P. and T. Rosswall. 1985. Ammonium oxidizer number, potential and actual oxidation rates in two Swedish arable soils. Biol. Fertil. Soils 1:131-140. doi:10.1007/BF00301780

Bernhard, A. E., J. Tucker, A. E. Giblin, D. A. Stahl. 2007. Fuctionally distinct communities of ammonia-oxidizing bacteria along an estuarine salinity gradient. Environ. Microbiol. 9:1439-1447. doi:10.1111/j.1462-2920.2007.01260.x

Bernstein, L., H.E. Hayward. 1958. Physiology of salt tolerance. Annu. Rev. Plant Phys. 9:25-46. doi:10.1146/annurev.pp.09.060158.000325

Blanck, H., S.A. Wängberg, S. Molander. 1988. Pollution induced community tolerance- a new ecotoxicological tool. *In:* Cairns, J. Jr, J.R. Pratt (eds). Functional testing of aquatic biota for estimating hazards of chemicals. ASTM STP 988.

Bremner, J. M. 1965. Inorganic forms of nitrogen. Pages 1179-1237 in Black, C.A. (Ed) Methods of soil analysis. Part 2. Agronomy No. 9. American Society of Agronomy, Madison, WI. doi:10.2134/agronmonogr9.2.2ed.c33

Canfora, L., G. Bacci, F. Pinzari, G.L. Papa, C. Dazzi, and A. Benedetti. 2014. Salinity and bacterial diversity: to what extent does the concentration of salt affect the bacterial community in a saline soil? PLoS One. 9:e114658. doi:10.1371/journal.pone.0106662

Dick, R. P. 1994. Soil enzyme activities as indicators of soil quality. *In*: Doran, J.W., D.C. Coleman, D.F. Bezdicek, B.A. Stewart (eds.) Defining soil quality for a sustainable environment. Soil Science Society of America special publication. Madison, WI, USA. doi:10.2136/sssaspecpub35.c7

Fierer, N. M.S. Strickland, D. Liptzin, M.A. Bradford, C.C. Cleveland. 2009. Global patterns in belowground communities. Ecol. Lett. 12:1-12. doi:10.1111/j.1461-0248.2009.01360.x

Fish, J. A., B. Chai, Q. Wang, Y. Sun, C. T. Brown, J. M. Tiedje, and J. R. Cole. 2013. FunGene: the Functional Gene Pipeline and Repository. Front. Microbiol. 4:291; doi:10.3389/fmicb.2013.00291

Franzen, D. W. 2013. Managing saline soils in North Dakota. North Dakota State University Extension Service. SF 1087.

Fujita, K., D. M. Dooley. 20007. Insights into the mechanisms of N_2O reduction by reductively activated N_2O reductase from kinetics and spectroscopic studies of pH effects. Inorg. Chem. 46:613-615. doi:10.1021/ic061843f

Gandhi, A. P., and K. V. Paliwal. 2004. Mineralization and gaseous losses of nitrogen from urea and ammonium sulphate in salt-affected soils. Plant Soil. 45:247–255. doi:10.1007/BF00011146

Gee, G.W., Bauder, J.W. 1986. Particle-size Analysis. In Page, L. (ed). Methods of soil analysis, Part 1, Physical and mineralogical methods. 2nd edition. Agronomy Monograph 9, American Society of Agronomy, Madison, WI, USA. doi: doi:10.2136/sssabookser5.1.2ed.c15

Hanson, B.R., S. R. Grattan, A. Fulton. 2006. Agricultural salinity and drainage. Division of Agriculture and Natural Resources Publication 3375. University of California, Davis.

He, Y., T. DeSutter, D. Clay. 2013. Dispersion of pure clay minerals as influenced by Ca to Mg ratios, SAR and EC. Soil Sci. Soc. Am. J. 77:2014-2019. doi:10.2136/sssaj2013.05.0206n

He, Y., T. DeSutter, D. Hopkins, X. Jia, D. A. Wysocki. 2013. Predicting ECe of the saturated paste extract from value of $EC_{1:5}$. Can. J. Soil Sci. 93:585-594. doi:10.4141/CJSS2012-080

Henry, S., D. Bru, B. Stres, S.Hallet, L. Philippot. 2006. Quantitative detection of the nosZ gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, narG, nirK, and nosZ genes in soils. Appl. Environ. Microbiol. 72:5181–5189. doi:10-1128/AEZM.00231-06

Ichise, Y.K., T. Kosuge, M. Uwate, T. Nakae, H. Maseda. 2015. Complete genome sequence of *Pseudomonas aeruginosa* strain 8380, isolated from the human gut. Genome Announc. 3:e00520-15. doi:10.1128/genomeA.00520-15

Irshad, M., T. Honna, S. Yamamoto, A. E. Eneji, and N. Yamasaki. 2005. Nitrogen mineralization under saline conditions. Comm. Soil Sci. Plan. 36:168–189. doi:10.1081/CSS-200059116

Jiang, H., Q. Huang, H. Dong, P. Wang, F. Wang, W. Li, C. Zhang. 2010. RNA-based investigation of ammonia oxidizing archara in hot springs of Yunnan Province, China. Appl. Environ. Microbiol. 76:4538-4541. doi:10.1128/AEM.00143-10

Jonasses, K.R. 2013. Effect of salinity shifts on microbial community composition in different nitrifying biofilms in continuous moving bed biofilm reactors. Norwegian University of Science and Technology. Trondheim, Norway.

Kandeler, E., C. Poll, W. T. Frankenberger Jr., and M. A. Tabatabai. 2011. Nitrogen cycle enzymes. *In*: Dick, R. P. (ed.) Methods of soil enzymology. Soil Science Society of America Series 9, Soil Science Society of America, Madison, WI. doi: doi:10.2136/sssabookser9.c10

Kearse, M., R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, S. Buxton, A. Cooper, S. Markowitz, C. Duran, T. Thierer, B. Shaton, P. Mintjies, A. Drummond. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 28:1647-1649. doi:10.1093/bioinformatics/bts199

Kim, O.S., P. Junier, J.F. Imhoff, K.P. Witzel. 2008. Comparative analysis of ammonia monooxygenase (amoA) genes in the water column and sediment-water interface of two lakes and the Baltic Sea. FEMS Microbiol. Ecol.. 66:367-378. doi:10.1111/j.1574-6941.2008.00565.x.

Kruskal V.J. 1964. Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. Psychometrika. 29:1-27. doi:10.1007/BF02289565

Kumar, S., G. Stecher, K. Tamura. 2015. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. (*Submitted*).

Laura, R.D. 1977. Salinity and nitrogen mineralization in soil. Soil Biol. Biochem. 5:333–336. doi:10.1016/0038-0717(77)90005-0

Li, H., B.S. Weng, F.Y. Huang, J.Q. Su, X.R. Yang. 2015. pH regulates ammonia-oxidizing bacteria and archaea in paddy soils in Southern China. Appl. Microbiol. Biotechnol. 99:6113-6123. doi:10.1007/s00253-015-6488-2

Ma B, Gong J. 2013. A meta- analysis of the publicly available bacterial and archaeal sequence diversity in saline soils. World J. Microb. Biot. 29:2325-2334. doi:10.1007/s11274-013-1399-9

Martens-Habbena, W., P.M. Berube, H. Urakawa, J.R. de la Torre, and D.A. Stahl. 2009. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. Nature. 461:976-9. doi:10.1038/nature08465

Mather, P.M. 1976. Computational methods of multivariate analysis in physical geography. Wiley and Sons, Inc., London, England doi:10.1002/esp.3290020419

Maheshwari. A. 2015. Assessing soil microbial tolerance to salt. MS Thesis. Lund University, Lund, Sweden.

McClung, G. W.T. Frankenberger, Jr. 1985. Soil nitrogen transformations as affected by salinity. Soil Sci. 139:405-411. doi:10.1097/00010694-198505000-00005

McCune B., Grace J.B. 2002. Analysis of ecological communities. Gleneden Beach, OR: MjM Software Design. doi:10.1016/S0022-0981(03)00091-1

McCune, B. and M. J. Mefford. 2011. PC-ORD. Multivariate Analysis of Ecological Data. Version 6. MjM Software, Gleneden Beach, Oregon, U.S.A.

National Cooperative Soil Survey. National Cooperative Soil Survey Characterization Database. http://ncsslabdatamart.sc.egov.usda.gov/ Accessed 2 February 2016.

NDAWN. 2015. North Dakota Agricultural Weather Network. http://ndawn.ndsu.nodak.edu. Accessed on 22 November 2015.

Nicol., G.W., S. Leininger, C. Schleper, J.I Prosser. 2008. The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. Environ. Microbiol. 10:2966-2978. doi:10.1111/j.1462-2920.2008.01701.x

Palmer, K., H.L. Drake, M.A. Horn. 2010. Novel and highly diverse acid-tolerant denitrifiers are associated with N_2O fluxes of an acidic fen. Appl. Environ. Microbiol. 76:1125-1134. doi:10.1128/AEM.02256-09

Pessarakli, M. and I. Szabolcs. 1999. Soil salinity and sodicity as particular plant/crop stress factors. In: Pessarkli, M. (ed) Handbook of plant and crop stress, second edition, revised and expanded. Marcel Dekker, New York, NY, USA.

Plaut, Z., M. Edelstein, M. Ben-Hur. 2013. Overcoming salinity barriers to crop production using traditional methods. Crit. Rev. Plant Sci. 32:250-291. doi:10.1080/07352689.2012.752236

Provin, T., J.L. Pitt. 2015. Managing soil salinity. Texas A&M AgriLife Extension. E-60, 3-12.

Rath, K., J. Rousk. 2015. Using growth-based methods to determine direct effects of salinity on soil microbial communities. EGU General Assembly. 12-17 April 2015. Vienna, Austria.

Rengasamy, P. 2006. World salinization with emphasis on Australia. J. Exp. Bot. 57:1017-1023. doi:10.1093/jxb/erj108

Richards, L.A. (ed) 1954. Diagnosis and improvement of saline and alkali soils, U.S. Salinity Laboratory Staff. Handbook 60. U.S. Dept. Agric., Washington, D.C.

Rhodes, JD. 1996. Electrical conductivity and total dissolved solids. *In*: D.L. Sparks, A.L. Page, P.A. Hlmke, R.H. Loeppert, P.N. Soltanpour, M.A. Tabatabai, C.T. Johnston, and M.E. Sumner (eds). Methods of Soil Analysis, Part 3, Chemical Methods, ed. Madison, WI: Soil Science Society of America. doi:10.2136/sssabookser5.3.c14

Ritz, K., H.I.J. Vlack, C.D. Campbell, J.A. Harris and C. Wood. 2009. Selecting biological indicators for monitoring soils: a framework for balancing scientific and technical opinion to assist policy development. Ecol. Indic. 9:1212-1221. doi:10.1016/j.ecolind.2009.02.009

Rotthauwe, J.H., K.P. Witzel, and W. Liesack. 1997. The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. 63:4704-4712. doi:009-2240/97/$04.00+0

Rousk, J. E. Bååth, P.C. Brookes, C.L. Lauber, C. Lozupone, J.G. Caporaso, R. Knight, N. Fierer. 2010. Soil bacterial and fungal communities across a pH gradient in an arable soil. The ISME Journal. 4:1340-51. doi:10.1038/ismej.2010.58

Saito, A., H. Mitsui, R. Hattori, K. MInamisawa, T. Hattori. 1998. Slow-growing and oligotrophic soil bacteria phylogenetically close to *Bradyrhizobium japonicum*. FEMS Microbiol. Ecol. 25:277-286. doi:10.1111/j.1574-6941.1998.tb00480.x

Saleem, T.M., and N. Ahmed. 1988. Soil fertility research and fertilizer use in Pakistan. *In*: Managing soil resources: Proceedings of the First National Congress on Soil Science, 93–97. Lahore, Pakistan: Soil Science Society of Pakistan.

Saleh-Lakha, S., K.E. Shannon, S.L. Henderson, B.J. Zebarth, D.L. Burton, C. Goyer, J.T. Trevors. 2009. Effect of nitrate and acetylene on *nirS, cnorB,* and *nosZ* expression and denitrification activity in Pseudomonas mandelii. Appl. Environ. Micro. 75:5082-5078. doi:10.1128/AEM.00777-09

Sarig, S. and Y. Steinberger. 1994. Microbial biomass response to seasonal fluctuation in soil salinity under the canopy of desert halophytes. Soil Biol. Biochem. 26:1405-1408. doi:10.1016/0038-0717(94)90224-0

Saxton, A.M. 1998. A macro for converting mean separation output to letter groupings in Proc Mixed. *In*: Proc. 23rd SAS Users Group Intl., SAS Institute, Cary, NC, USA.

Schleper, C. 2010. Ammonia oxidation: different niches for bacteria and archaea. The ISME J. 4:1092-1094. doi:10.1038/ismej.2010.111

Sims A., J. Horton, S. Gajaraj, S. McIntosh, R.J. Miles, R. Mueller, R. Reed and Z. Hu. 2012. Temporal and spatial distributions of ammonia-oxidizing archaea and bacteria and their ratio as an indicator of oligotrophic conditions in natural wetlands. Water Res. 46:4121-4129. doi:10.1371/journal.pone.0113515

Stroup, W.W. and J. Stubbendieck. 1983. Multivariate statistical methods to describe changes in botanical composition. J. Range Manage. 36:208-212.

Soil Survey Staff. 2015. Web Soil Survey. Natural Resources Conservation Service, United States Department of Agriculture. Available online at http://websoilsurvey.nrcs.usda.gov/. Accessed on 15 September 2015.

Tamura, K., M. Nei. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10:512-526. doi:0737-4038/93/1003-0002$02.00

Taylor AE, L.H. Zeglin, T.A.Wanzek, D.D. Myrold and P.J. Bottomley. 2012. Dynamics of ammonia-oxidizing archaea and bacteria populations and contributions to soil nitrification potentials. ISME J. 6:2024–2032. doi:10.1038/ismej.2012.51

Taylor, A.E., L.H. Zeglin, S. Dooley, D.D. Myrold, and P.J. Bottomley. 2010. Evidence for different contributions of archaea and bacteria to the ammonia-oxidizing potential of diverse Oregon soils. Appl. Environ. Microbiol. 76:7691-7698. doi:10.1128/aem.01324-10

Tejada, M., C. Garcia, J.L. Gonzalez, M.T. Hernandez. 2006. Use of organic amendment as a strategy for saline soil remediation: Influence on the physical, chemical and biological properties of soil. Soil Biol. Biochem. 38:1413-1421. doi:10.1016/j.soilbio.2005.10.017

Van Donk, S.J. 2012. Tillage and crop residue removal effects on evaporation, irrigation requirements, and yield. Proceedings of the 24th Annual Central Plains Irrigation Conference. 21-22 February 2012. Colby, KA, USA.

Wisniewski-Dye,F., K. Borziak, G. Khalsa-Moyers, G. Alexandre, L.O. Sukharnikov, K. Wuichet, G.B. Hurst, W.H. McDonald, J.S. Robertson, V. Barbe, A. Calteau, Z. Rouy, S. Mangenot, C. Prigent-Combaret, P. Normand, M. Boyer, P. Siguier, Y. Dessaux, C. Elmerich, G. Condemine, G. Krishnen, I. Kennedy, A.H. Paterson, V. Gonzalez, P. Mavingui, I.B. Zhulin. 2011. Azospirillum genomes reveal transition of bacteria from aquatic to terrestrial environments. PLoS Genetics. 7:e1002430. doi:10.1371/journal.pgen.1002430

Wessén, E. and S. Hallin. 2011. Abundance of archaeal and bacterial ammonia oxidizers-possible bioindicator for soil monitoring. Ecol. Indic. 11:1696-1968. doi:10.1016/j.ecolind.2011.04.018

Wessén, E., M. Söderström, M. Stenberg, D. Bru, M. Hellman, A. Welsh, F. Thomsen, L. Klemedtson, L. Philippot, and S. Hallin. 2011. Spatial distribution of ammonia-oxidizing bacteria and archaea across a 44-hectare farm related to ecosystem functioning. ISME J. 5: 1213–1225. doi:10.1038/ismej.2010.206

Wuchter C, Abbas B, Coolen MJL, Herfort L, van Bleijswijk J, Timmers P. 2006. Archaeal nitrification in the ocean. Proc. Natl. Acad. Sci. U.S.A. 103:12317–12322. doi:10.1073/pnas.0600756103

Yan, Y., J. Uang, Y. Dou, M. Chen, S. Ping, J. Peng, W. Lu, W. Zhang, Z. Uao, H. Li, W. Liu, S. He, L. Geng, X. Zhang, F. Yang, H. Yu, Y. Zhan, D. Li, Z. Lin, Y. Wang, C. Elmerich, M. Lin, Q. Jin. 2008. Nitrogen fixation island and rhizosphere competence traits in the genome of root-associated *Pseudomonas stutzeri* A1501. Proc. Natl. Acad. Sci. U.S.A. 105:7564-7569. doi:10.1073/pnas.0801093105

Zahran, H.H. 1997. Diversity, adaptation and activity of the bacterial flora in saline environments. Biol. Fert. Soils. 25:211-223. doi:10.1007/s003740050306

Zeng, W.Z., C. Xu, J.W. Wu, J.S. Huang, and T. Ma. 2013. Effect of salinity on soil respiration and nitrogen dynamics. Soc. Ecol. Chem. Eng. 20:519-530. doi:10.2478/eces-2013-0039

PAPER 3: TILLAGE, FERTILIZER AND CROP ROTATION EFFECTS ON SOYBEAN (*GLYCINE MAX* L.) RHIZOBIUM PERSISTENCE, INFECTIVENESS AND EFFECTIVENESS[3]

Abstract

Biological N fixation (BNF) occurs through a symbiotic relationship between soybeans (*Glycine max* L.) and *rhizobium* bacteria. The objectives of this study were to determine the effects of 1) tillage, 2) fertilizer application to crops grown prior to soybeans and 3) time since inoculation in a four year rotation on the ability of inoculated and naturalized soybean *rhizobium* to persist in the soil, to form nodules and to fix nitrogen (N). Soil was collected in 2014 from 0-15 and 15-30 cm in no-till and conventional till with different N fertilizer (110 kg-N ha^{-1}, 56 kg-N ha^{-1} as manure, and 0 kg-N ha^{-1}) prior to planting, at the soybean V2-4 and R1 growth stage and after harvest. Soybeans were inoculated at planting with commercial inoculants Persistence of *Bradyrhizobium* in the soil was determined by extracting soil DNA followed by quantitative Polymerase Chain Reaction (qPCR) using the *nodZ* primers targeting the gene regulating soybean nodulation. Soybean plants and their roots were collected at V2-4 and R1 growth stages. Nodules were surface sterilized, counted and weighed. DNA was extracted from the nodules and analyzed via qPCR and sequencing by synthesis to determine *rhizobium* community structure within the nodules. RNA was also extracted and analyzed for *nifH* nitrogenase production genes via qPCR. Plant N obtained from BNF was measured as the ratio of plant ureide-N to ureide-N

[3] The material in this chapter was co-authored by Heather Dose, Ann-Marie Fortuna, Ezra Aberle, R. Jay Goos, Jasper Teboh and Blaine Shatz. Heather Dose had primary responsibility for collecting samples in the field and laboratory analysis. Heather Dose was the primary developer of the conclusions that are advanced here. Heather Dose also drafted and revised all versions of this chapter. Ann-Marie Fortuna and R. Jay Goos served as proofreader and checked the math in the statistical analysis conducted by Heather Dose.

plus nitrate-N. *Bradyrhizobium* populations followed a cyclical trend with highest populations after harvest and decreasing steadily throughout the remainder of the rotation. *B. japonicum* USDA 110 persisted in the soil at greatest numbers (>60%), but populations of *B. japonicum* SEMIA 560, 5045, 511, 512, *B. liaoningense* and *B. diazoefficiens* also persisted. *B. elkanii* was identified in low frequency (<0.1%). The taproot nodules were comprised primarily of the *B. japonicum* USDA 110 inoculant, but the lateral root nodules contained other persistent *Bradyrhizobium*. Where manure was applied, *B. elkanii* was able to form lateral nodules. Although fertilizer source used within the rotation did have an effect on the community structure of soybean *rhizobium* within the nodules, there was no effect on BNF in soybeans.

Introduction

The United States is a major producer and consumer of soybeans (Shiro et al., 2013). Biological N fixation (BNF) can account for 25 to 75% of plant N accumulation in soybean and is derived from *rhizobium* species infecting root nodules in leguminous plants or from free-living N fixing diazotroph populations in the soil. (Zapata et al., 1987) with the remainder of the plant nitrogen in soybeans can come from mineralization of organic matter. Soybean (*Glycene max* L.), a high N demand crop, contributes to 77% of worldwide legume crop N fixed with an estimated contribution of 16.44 Tg N yr^{-1} fixed (Herridge et al., 2008). Therefore, incorporation of high value legumes such as soybeans into crop rotations will contribute to sustainable crop production by reducing synthetic fertilizer needs (Bohlool et al., 1992).

Nodulation begins through a series of biochemical signals between the plant and *rhizobium* (Dénarié et al., 1992). Once the signaling molecules have been detected, the induction of the *nodABC* genes, which are found in all symbiotic N fixing bacteria (Martinez et al., 1990), and soybean specific *nodZ* and *nodVW* initiate root hair curling, infection thread formation and

nodulation in soybeans (Göttfert et al., 1990). In soybeans the nodulation process is very specific and is thought to be limited to *Bradyrhizobium japonicum, B. elkanii, Sinorhizobium fredii, B. liaoningense, S. xinjiangense,* and *Mesorhizobium tianshanense* (Jordan, 1982; Scholla and Elkan, 1984; Chen et al, 1988; Koykendall et al., 1992; Chen et al., 1995; Xu et al., 1995; Tan et al., 1997; Peng et al., 2002; Yao et al., 2002; Vinuesa et al., 2008). BNF in soybeans varies in part due to differences in infectiveness among soybean nodulating *rhizobium* and variations in effectiveness among rhizobium strains. Fast-growing strains like *S. fredii* have been shown to be able to form more nodules (be more infective) when competing with slower growing strains such as *Bradyrhizobium japonicum* (Dowdle and Bohlool, 1987).

Once inside the nodule, the *rhizobium* differentiate into bacteroids and begin fixing N through the production of nitrogenase the production of which is regulated by a series of *nif* genes, particularly *nifH* which are found in all *rhizobium* and diazotrophs. Within individual *rhizobium* species, strain level differences in BNF exist. Therefore, competition amongst inoculated and naturalized strains of rhizobium in the soil can have an impact on subsequent BNF (Johnson et al., 1965; Thies et al., 1991). For example, *B japonicum* strain USDA 110 was found to have greater N fixing effectiveness than other *B. japonicum* strains (Sloger, 1969). Inoculated strains are developed to have greater N fixation abilities (effectiveness) but may be less infective relative to naturalized species that may be highly infective but less effective resulting in lowered BNF in soybeans (Singleton and Tavares, 1986). Increases of up to 45% seed protein have been attributed to BNF by inoculating several strains of *rhizobium* in soils where soybean has not been previously grown (Abel and Erdman, 1964). Moreover, lower BNF in soybeans may be unavoidable as roots explore the soil beyond inoculation depth (McDermott and Graham, 1989) and encounter less effective naturalized strains. Where soybeans have been

historically grown ~ 5 to 20% of nodules on the roots are formed from inoculated strains (Caldwell and Vest, 1970; Kuykendall and Weber, 1978).

Differences in tillage, cropping systems and fertilization can also effect BNF and the soil environment. The effects of tillage can influence *Bradyrhizobium* populations in the soil. Conventional tillage, which consists of annual plowing and disking has been shown to reduce soil organic matter (Ferreira et al., 2000) which can have negative effects on the soil microbes by reducing C substrates. In contrast, no till is a system that does not plow the soil and seeds are planted directly through the previous crop residues. It has been shown that no till systems not only increase soil organic matter, but have corresponding higher soil microbial biomass (Hungria and Vargas, 1996) which can lead to higher *Bradyrhizobium* populations, greater nodulation, and greater N fixing capacity (Hungria and Stacey, 1997). The diversity of *Bradyrhizobium* populations has also been shown to be greater under no till soils with diverse cropping patterns involving soybean relative to plowed and disked soils without soybean in the crop rotation (Ferreira et al., 2000). Soil organic matter is important for carryover of *Bradyrhizobium* populations as they can exist in the soil in a saprophytic stage using organic C as energy substrates. However, the alteration and increase in N mineralization under no-till soils could result in a more continuous supply of plant N that may reduce a legume host's need to invest in nodule formation and BNF (Allos and Bartholomew, 1959).

Fertilizer management that includes long-term applications of manure increases the soil organic C, P content, and other essential nutrients which can affect soil microbial populations including *rhizobium* (Sommerfield et al., 1988; Sharpley et al., 1994; Marschner et al., 2003). Populations of *Bradyrhizobium* are also affected by additional soil properties such as pH and nutrient status of Ca, P and Mo (Sylvia, 2005), which can also be altered by manure additions.

Greater crop biomass and the return of residues within a rotation that optimizes soil fertility and plant nutrient uptake can have an effect on residue decomposition and organic C and N mineralization. Together, these C and N mineralization effects of fertilizer management can impact the persistence of *Bradyrhizobium* in the soil.

Quantifying rhizobium populations in soil is commonly done using the Most Probable Number (MPN) method (Weaver and Frederick, 1972), and rhizobium strains can be identified using serology methods (Means et al., 1964). However, these methods are time consuming and less direct as they do not represent the community *in situ*. Advances in molecular microbiology techniques, such as quantitative Polymerase Chain Reaction (qPCR) and sequencing by synthesis allow for rapid quantification of soybean *rhizobium* populations in both the soil and infected nodules of soybean roots by targeting *nifH* genes required for N fixation and *nodZ* genes required for nodulation (Furseth et al., 2010). By targeting the *nodZ* gene nodule, infectiveness can be quickly assessed for not only the number of bacteriods inhabiting the nodules, but also be used to identify the *rhizobium* strains inhabiting nodules through sequencing. The effectiveness of fixing N within the nodules can be both quantified and sequenced using messenger RNA (mRNA). Messenger RNA is produced by active organisms in the synthesis of proteins and verifies the organism's activity. By targeting the mRNA *nifH* genes from within the nodules, it is possible to estimate the quantity of actively fixing *rhizobium*, which can be achieved faster and for more samples than the ratio of plant ureide-N to plant nitrate. These molecular techniques allow researchers to re-examine the infectiveness and effectiveness of inoculant and naturalized *rhizobium* strains in soil planted to soybean and in soybeans grown across a range of crop rotations, tillage managements and N fertilizer regimes. Utilizing molecular techniques the objectives of this study were to determine the influence of tillage and N fertilizer additions on a)

soybean *rhizobium* persistence within a four year crop rotation, b) the infectiveness of and ability of persistent soybean specific *rhizobium* strains in the soil to form nodules and c) the effectiveness of strains within nodules to fix N.

Materials and methods

Study site

The long-term cropping system experiment, established in 1987 at the Carrington Research Center in North Dakota, USA (47° 30’ 28.407”N, -99° 7’ 2.622”W) was utilized for this study. The field experiment was a split-split plot, randomized complete block with 3 replicates. Whole plots consisted of rotation with tillage and N fertility treatments as split plots. The rotation, tillage, and fertility treatments are replicated in triplicate. All entry points are present in all years and the rotation consists of wheat (*Triticum aestivum* L.)-field pea (*Pisum sativum* L.)-corn (*Zea mays* L.)- soybean. Tillage treatments consisted of conventional disk and chisel plow in the fall and no-till. Fertilizer additions consisted of a no N fertilizer control (no added N at any point in the rotation), 112 kg of N ha^{-1} as urea fertilizer applied in the spring only to wheat and corn, and 56 kg N ha^{-1} as composted feedlot manure applied only during the spring of the wheat phase of the rotation. The soil is mapped as a Heimdal (Coarse-loamy, mixed, superactive, frigid Calcic Hapludolls)-Emrick (Coarse-loamy, mixed, superactive, frigid Pachic Hapludolls) loam complex (Soil Survey Staff, 2014). The average temperature for the area is 4 °C, with an average of 280 mm of precipitation falling mostly in the summer (NDAWN, 2014). Optimize (Novozymes BioAg Inc., Brookfield, WI, USA) liquid inoculant was applied with the soybean seed (variety Dairlyand 0404 at 180,000 pls ac^{-1}) at planting on May 21 according to Novozymes BioAg, contained the rhizobium *Bradyrhizobium japonicum*. TagTeam (Novozymes BioAg Inc., Brookfield, WI, USA) liquid inoculant was applied to peas at the time of planting

and according to Novozymes BioAg, contained the fungus *Penicillium bailaii* and the *rhizobium Rhizobium leguminosarum*. Soybean yield, protein and oil seed content were determined at harvest. Glyphosate was applied prior to seeding and twice during the growing season following best management practices. A map of the plots and treatments can be found in Figure A4.

Soil sampling

Five composite random soil samples were collected from each field plot replicate (wheat, field pea, corn and soybean plots) using a soil probe from 0-15 and 15-30 cm prior to planting in May, 2014. Composite soil samples were collected in the same manner only from the soybean plots during the V2-4 growth stage on June 25, during the R1 growth stage on July 16, and again after harvest on October 29. The soils were transported on ice in a cooler and frozen at -20 °C until the soils could be homogenized and passed through a 2 mm sieve. A subsample was collected and stored at -80 °C for DNA extraction, while the remaining soil was stored at -20 °C. Inorganic N was extracted from each sample using 2M KCl as outlined by Bremner (1965) and analyzed for nitrate and ammonium using a SEAL Auto Analyzer 3 (SEAL Analytical, Mequon, WI, USA).

Determining persistence of soybean specific *Rhizobium* using genomic DNA extracted from the soil

Molecular techniques are preferred to the time consuming MPN method for quantifying *rhizobium* as qPCR is a more direct measure. In order to verify whether qPCR counts of *nodZ* genes can be a suitable substitution for MPN, 12 soils collected from conventionally tilled plots receiving no fertilizer prior to planting and a soil collected from a grassy area that had no history of soybean production were used to determine *rhizobium* populations. The samples to calibrate the MPN method to qPCR *nodZ* genes were brought back to the laboratory in a cooler and stored

at 4 °C overnight and analyzed the next day using MPN method described by Weaver and Frederick (1972). To determine the quantity of soybean *rhizobium*, qPCR was used to assess gene copy numbers of the *nodZ* gene specific to in soil as the primer was designed to target *B. japonicum, B. elkanii, Sinorhizobium fredii* and *Rhizobium eltii* only and does not amplify other rhizobium which do not infect soybeans such as *Sinorhizobium meliloti* 2011, *Mesorhizobium loti* MAFF303099, *Rhizobium leguminosarium* and *Sinorhizobium medicae* (Furseth et al., 2010). *Bradyrhizobium japonicum* USDA 110 has 1 copy of the *nodZ* gene within its genome (Kaneko et al., 2002). Genomic DNA (gDNA) was extracted from 0.25 g of soil using the Power Soil DNA extraction kit (MO BIO Laboratories Inc., Carlsbad CA, USA) following manufacturer's instructions. Concentrations of genomic DNA were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All genomic DNA samples were diluted with DNA/RNAse free molecular grade water (G Biosciences, St. Louis, MO, USA) and were stored at -80 °C until downstream qPCR application. Real time qPCR was performed in a PikoReal system (Thermo Scientific, Wilmington, DE, USA) The *nodZ* forward primer, nodZ-a 5'-GGTTTGGCGACTGTCTGTGGTC-3' and reverse primer, nodZ-a 5'-TTCCACCATGTTGGAAAGAATGGTCC-3' amplified a 228-bp DNA fragment as described by Furseth et al. (2010). The primers were synthesized by Invitrogen (Carlsbad, CA, USA). A 20 µl reaction was a mix of 10 µL FAST SYBR Green Master Mix (Applied Biosystems Inc. Foster City, CA, USA), 0.4 µL forward and reverse 10 µM primer, 2 µL of 4ng μL^{-1} template DNA and 7.2µL RNA/DNA free water. The qPCR thermocycler protocol for amplifying the *nodZ* gene was an initial denaturing for 5 min at 94 °C followed by 40 cycles of 94 °C for 10 s, 58 °C for 20 s, and 72 °C for 30 s with a data acquisition step, followed by a melt curve analysis. Standard curves of known gene copy numbers were generated using a serial dilution of genomic DNA

isolated from *Bradyrhizobium japonicum* (ATTC 49852, Manassas, VA, USA) ranging in concentration from 5 ng μL^{-1} to 5 fg μL^{-1} replicated 3 times each and the average threshold cycle (Ct) for the *nodZ* primer was 32.67.

Determining infectiveness using genomic DNA extracted from nodules

To determine root nodule infectiveness, 25 whole soybean plants with roots from each plot were collected during the V2-4 (June 25, 2014) and the R1 (July 16, 2014) growth stages. The plants and roots were stored overnight in a walk-in cooler at 4 °C until a random subsample of ten plants from each plot were selected to have nodules removed. Prior to counting and removal, the roots and nodules were surface sterilized by placing in 95% ethanol for 30 s followed by submersion in 3% H_2O_2 and rinsed in sterile water, a procedure modified from Vincent (1970). The nodules located on the taproot were collected separately from nodules located on the lateral roots. The nodules were counted, removed, weighed and stored at -80 °C. The plant leaflets and axes (stems and petioles) were separated, dried, and weighed.

Nodule gDNA was extracted using the PowerPlant Pro DNA isolation (MO BIO Laboratories Inc., Carlsbad CA, USA) following manufacturer's instructions with slight modifications for cell lysing. Surface sterilized nodules (50 mg) were placed in the PowerPlant bead tubes. Half of the lysing solution was added and nodules were homogenized using a VWR hard tissue homogenizer (VWR International, Randor, PA, USA) set at level 10 until all nodules were lysed. The remainder of the lysing solution was then added along with the contents of 0.1 mm glass bead tubes from MO BIO Laboratories to ensure lysing of the bacteroid cells within the nodule tissue. Extracted DNA was stored at -80 °C until further downstream qPCR analysis for nodule infectiveness by targeting the *nodZ* gene as described above. The *nodZ* gene copy numbers were corrected for the biomass of nodules collected.

Determining effectiveness using messenger RNA extracted from nodules

In order to quantify the effectiveness of nodules at fixing N, mRNA was extracted from the nodules using the PowerPlant RNA isolation kit (MO BIO Laboratories Inc., Carlsbad CA, USA) following manufacturer's instructions with the lysing modification as described above. Extracted mRNA was then converted to cDNA using the SuperScript VILO cDNA synthesis kit and Master Mix (Life Technologies, Grand Island, NY, USA). *Bradyrhizobium japonicum* USDA 110 has 3 copies of the *nifH* gene on its genome (Kaneko et al., 2002). The *nifH* forward primer PolF 5'- TGCGAYCCSAARGCBGAC TC-3' and the reverse primer PolR 5'-ATSGCCATCATYTCRCCGGA-3' described by Poly et al. (2001) were used to amplify a 362-bp DNA fragment regulating N fixation. The primers were synthesized by Invitrogen (Carlsbad, CA, USA). A 20 µl reaction was a mix of 10 µL FAST SYBR Green Master Mix (Applied Biosystems Inc. Foster City, CA, USA), 0.4 µL forward and reverse 10 µM primer, 2 µL of 4ng μL^{-1} template DNA and 7.2µL RNA/DNA free water. The qPCR thermocycler protocol for *nifH* amplification was initial denaturing for 5 min at 94 °C followed by 40 cycles of 94 °C for 45 s, 60 °C for 1 min, and 72 °C for 1 min with a data acquisition step, followed by a melt curve analysis. Standard curves were generated using DNA isolated from *Ensifer meliloti* (ATTC 4400, Manassas, VA, USA) using five serial dilutions ranging from 5 ng μL^{-1} to 5 fg μL^{-1} replicated 3 times each. *E. meliloti* standard curve controls and cDNA from the nodules were run in triplicate, with an average Ct of 27.8. The *nifH* gene copy numbers were corrected for the biomass of nodules collected.

Sequencing by synthesis

In order to explore the diversity and community structure of soybean *rhizobium* persistence and infectiveness sequencing by synthesis was used. Extracted gDNA from soil

collected prior to planting and during the V2-4 growth stage was amplified in triplicate using the *nodZ* primers. Additionally, gDNA collected from nodule samples during the R1 growth stage and from the liquid soybean and pea inoculant was also amplified in triplicate using the n*odZ* primers.

A 25 µL reaction with 1.25 µL RedTaq Ready Mix PCR Reaction Mix (Sigma Aldrich, Saint Louis, MO, USA) 2.5 µL buffer, 0.5 µL dNTPs, 0.3 µL forward and reverse primers, 2.5 µL of 4 ng μL^{-1} DNA template and 17.65 µL RNA/DNA free water was used to amplify target genes. The PCR thermocycler conditions used was 5 min at 95 °C followed by 25 cycles of denaturing for 30 s at 94 °C, annealing for 30 s at 58 °C and extension for 30 s at 72 °C followed by a final extension at 72 °C for 5 min using the GeneAmp PCR System 9700 (Applied Biosystems, Thermo Fischer, Grand Island, NY, USA) modified and optimized from Mao et al. (2011).

The PCR products were checked on a 1% agarose gel to ensure proper amplification of the target gene. The triplicate amplicons were pooled and a second step-out PCR amplification was used to apply a unique dual indexed barcode and adapter oligos to each sample. A 25 µL reaction with 1.25 µL RedTaq Ready Mix PCR Reaction Mix (Sigma Aldrich, Saint Louis, MO, USA) 2.5 µL buffer, 0.5 µL dNTPs, 0.3 µL of unique forward barcode, 0.3 µL of unique reverse barcode 2.5 µL of 4 ng μL^{-1} amplicon from the first PCR reaction and 17.65 µL RNA/DNA free water was used. The thermocycler conditions were initial denature at 94 °C for 5 min followed by 5 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s followed by a final extension at 72 °C for 5 min. The barcoded PCR products were verified on a 1% agarose gel. The unique barcode sequences were provided by Phillip San Miguel of the Purdue University Genomics Core Facility (West Lafayette, IN, USA).

The barcoded amplicons were then pooled by target gene, and samples were size selected using a BluePippin (Sage Science, Beverly, MA, USA) with a 1.5% agarose cassette. Amplicon concentrations were measured using Qubit 2.0 Fluorometer (Thermo Fischer Scientific, Waltham, MA, USA). The barcoded *nodZ* and *nifH* PCR amplicons were then pooled in equimolar concentrations. Final amplicon quality was assessed using both an Agilent bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and qPCR.

Genetic sequence data was then obtained using the Illumina MiSeq set to 2×250 reads (Illumina, Inc., San Diego, CA, USA). The sequence reads were aligned and mapped with Bowtie2 version 2.2.7 (Langmead and Salzberg, 2012) to reference sequences using references described in Menna and Hungaria (2011) and were obtained from GenBank with the National Center for Biotechnology Information. The number of reads per library, minimum, maximum and mean read length, coverage mean and standard deviation and mean mapping quality can be found in Table A1.

Plant nitrogen obtained from biological fixation

In order to relate nodule infectiveness and effectiveness to plant N obtained from BNF, soybean ureide N concentrations were used. The ratio of water-extractable ureide-N divided by the sum of ureide-N plus nitrate-N in soybean axes (stems plus petioles) approximates the proportion of plant uptake of N coming from BNF at the time of sampling (Herridge, 1982a; Herridge, 1982b; van Berkum et al., 1985). Plant samples were analyzed for nitrate-N and ureide-N by the methods of Cataldo et al. (1975) and Goos, et al. (2015), respectively. In order to determine the effects of mineralizable N on soybean BNF, a 7-day anaerobic potentially mineralizable N (PMN) incubation was performed using frozen soil samples collected prior to

soybean planting from 0-15 and 15-30 cm depths following Moebius-Clune et al. (2016) and analyzed for NH_4^+ using a SEAL Auto Analyzer 3.

Statistical analysis

In order to confirm the relationship between MPN *rhizobium* quantification and qPCR *nodZ* gene copies, a correlation analysis was run using Proc Corr in SAS 9.4 (SAS Inc., Cary, NC, USA). To determine the persistence of *rhizobium*, analysis of variance (ANOVA) using the log *nodZ* gDNA gene copy numbers were analyzed with Proc Mixed in SAS 9.4. Crops in the rotation, fertilizer additions, tillage method and soil depth were considered fixed factors, while repetition was considered a random factor. Main effects and interaction effects were assessed post hoc with estimated mean separation using Tukey's procedure at the $p < 0.05$ level in conjunction with the use of an algorithm for determining letter representation of all pair-wise comparisons based on Saxton (1998).

To assess infectiveness of *rhizobium* strains as related to crop management factors the nodule *nodZ* gDNA gene copies were analyzed with ANOVA using Proc Mixed in SAS 9.4 as described above where nodule location on the roots was considered an additional fixed factor. Furthermore, the community structure of persistent *rhizobium* to nodule infectiveness, nonmetric multidimensional scaling (NMS) in PC-ORD version 6.15 (Gleneden Beach, OR, USA) (McCune & Mefford, 2011). Nonmetric multidimensional scaling is an ordination technique suited for non-normal and non-linear ecological data that simplifies complex datasets by determining sources of variation displayed graphically as axes as described by Mather (1976) and Kruskal (1964). In PC-ORD the default "slow and thorough" procedure was chosen with a Sorensen distance measure and a random starting point, resulting in 250 runs with real and randomized data. The Sorensen distance measure was chosen to downplay the effects of rare

sequences identified. Multiresponse permutation procedure (MRPP) analysis, which is a nonparametric test was used to determine community structural differences due to tillage and fertilizer. Multi-response permutation procedures were run in PC-ORD with a Sorensen distance measure as described in McCune and Grace (2002) and Stroup and Stubbendieck (1983) in conjunction with pairwise comparisons using a Bonferroni correction to determine differences due to management.

Pearson correlation in SAS 9.4 was used to determine the relationship between plant N obtained from BNF and cDNA *nifH* copies. By ordinating the *rhizobium* community within the nodules to other measured variables such as nodule counts and weights, soil PMN, soil inorganic N, *nifH* cDNA copies, seed protein, yield and oil content the effects of different strains on BNF can be determined. In order to accomplish this, nonmetric multidimensional scaling was used. The percentage of each *Bradyrhizobium* sequence identified within the nodules was multiplied by the qPCR gDNA *nodZ* gene copy numbers to give a quantity of each *Bradyrhizobium* found in the nodules. Prior to analysis, the data was power transformed to the fourth in order to reduce error associated with scale of measurement. After NMS analysis, differences in tillage and fertilizer management were assessed using multi-response permutation procedures (MRPP) with a Sorensen distance measure in conjunction with pairwise comparisons using a Bonferroni correction.

Results and discussion

Persistence of soybean specific rhizobium in the soil from a four-year crop rotation

The linear correlation between absolute quantification of *nodZ* gene copies and MPN counts of soybean specific *rhizobium* in the soil resulted in an adjusted r^2 value of 0.72 with the MPN estimates greater than measured *nodZ* gene copy numbers (Fig 18). The MPN technique

estimates *rhizobium* soil populations through serial dilutions and the statistical probability of *rhizobium* forming nodules on soybeans grown in pouches, while the qPCR technique measures genetic information and is a more accurate quantification of *in situ rhizobium* populations. Overall, this correlation is similar to that described by Furseth et al. (2010) (adjusted r^2=0.88) where MPN estimates were correlated to absolute qPCR quantification using the threshold cycle. However, our qPCR results of the *nodZ* gene are lower than the MPN methods, but Furseth et al. (2010) found the MPN technique to underestimate *rhizobium* when compared to the *nodZ* qPCR results.

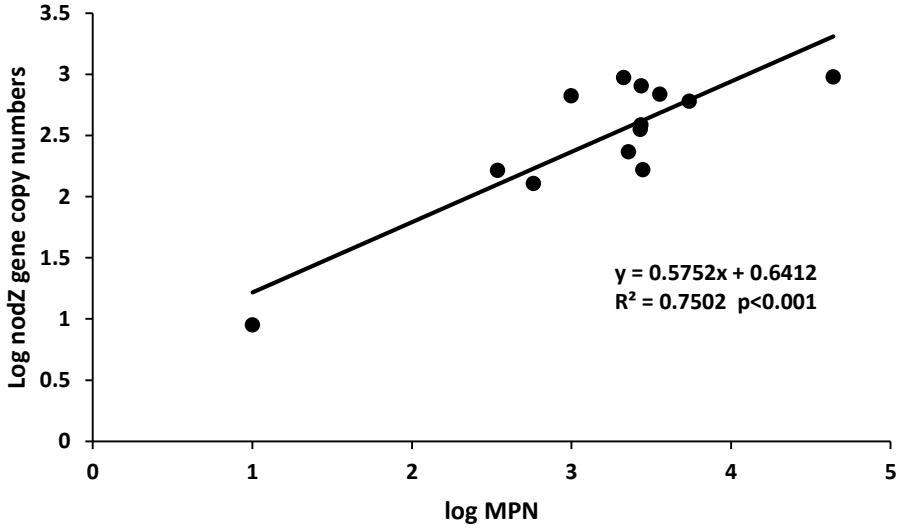

Figure 18. The correlation between enumeration of soybean-associated *rhizobium* by method of quantitative Polymerase Chain Reaction (qPCR) and most probable number (MPN) technique (n=13).

Table 16. Analysis of variance F test and p-value results for soybean specific *rhizobium nodZ* gene copy numbers for the main factors of crop, tillage, fertilizer, and soil depth and their interactions. Bold values are significant at $p < 0.05$.

	Soybean specific *rhizobium nodZ* gene copy numbers	
Effect	F-value	p-value
Crop	9.89	**0.0005**
Tillage	0.20	0.7007
Crop x Tillage	1.18	0.3180
Fertilizer	4.14	0.1061
Crop x Fertilizer	0.73	0.7192
Tillage x Fertilizer	2.09	0.1277
Crop x Tillage x Fertilizer	1.48	0.1382
Soil Depth	239.11	**<0.0001**
Crop x Soil Depth	5.85	**<0.0001**
Tillage x Soil Depth	0.01	0.9317
Crop x Tillage x Soil Depth	1.17	0.3227
Fertilizer x Soil Depth	6.04	**0.0030**
Crop x Fertilizer x Soil Depth	0.58	0.8581
Tillage x Fertilizer x Soil Depth	2.00	0.1391
Crop x Tillage x Fertilizer x Soil Depth	1.53	0.1193

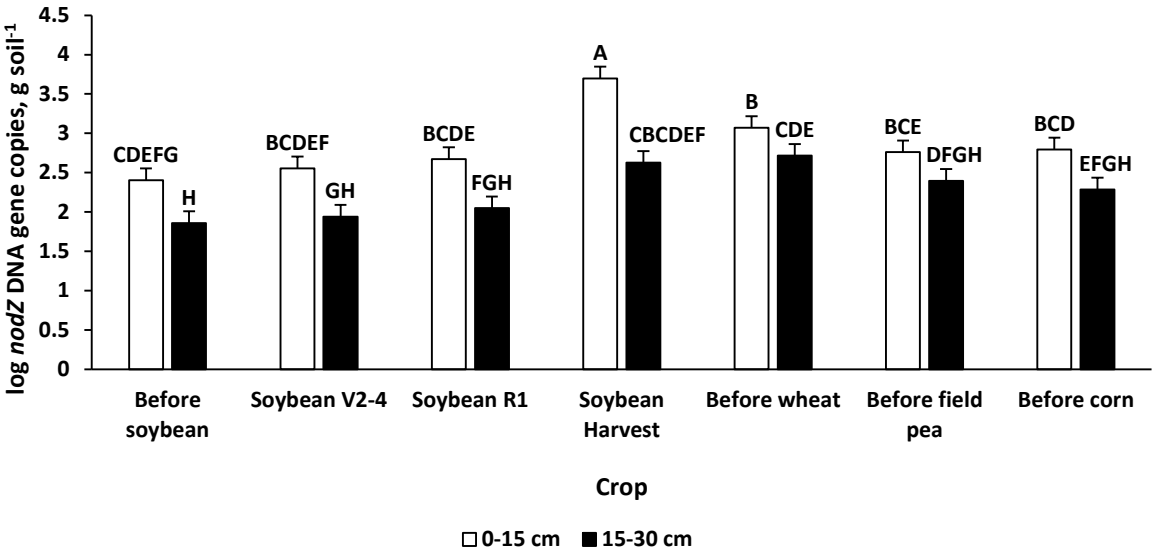

Figure 19. Mean (±SE) of soybean specific *rhizobium* measured by soil *nodZ* gDNA copy numbers through a four year crop rotation at 0-15 and 15-30 cm soil depths. Significant differences are denoted by different letters following Tukey's procedure at $p < 0.05$.

The quantity of soybean specific *rhizobium* in the soil, measured by *nodZ* gDNA gene copy numbers, was affected by the interaction of crop in the rotation, soil depth, and also fertilizer source (Table 16). A cyclical trend was observed for the persistence of soybean specific *rhizobium* (Fig. 19). Overall, the quantity of soybean specific *rhizobium* was greater at the 0-15 cm soil depths than the 15-30 cm depths. At the 0-15 cm soil depths soybean specific *rhizobium* were greatest at soybean harvest (3.70 log *nodZ* gDNA copies) and successively decreased in quantity based on years after inoculation. A similar trend was observed for the 15-30 cm depth except the greatest quantity of soybean specific *rhizobium* was observed prior to wheat planting (2.71 log *nodZ* gDNA copies) and successively decreased in quantity through the remainder of the rotation based on years after inoculation. Fertilizer source did not impact soybean specific *rhizobium* persistence in the soil. However, at the 15-30 cm soil depths the soybean specific inoculant persisted in greater numbers in treatments where manure was applied (2.41 log *nodZ* gDNA copies) and was lowest where 112 kg-N ha^{-1} of UAN was applied (2.14 log *nodZ* gDNA

copies) (Fig. 20). Application of manure has been shown to improve rhizobium persistence in the soil elsewhere (Zengeni et al., 2003).

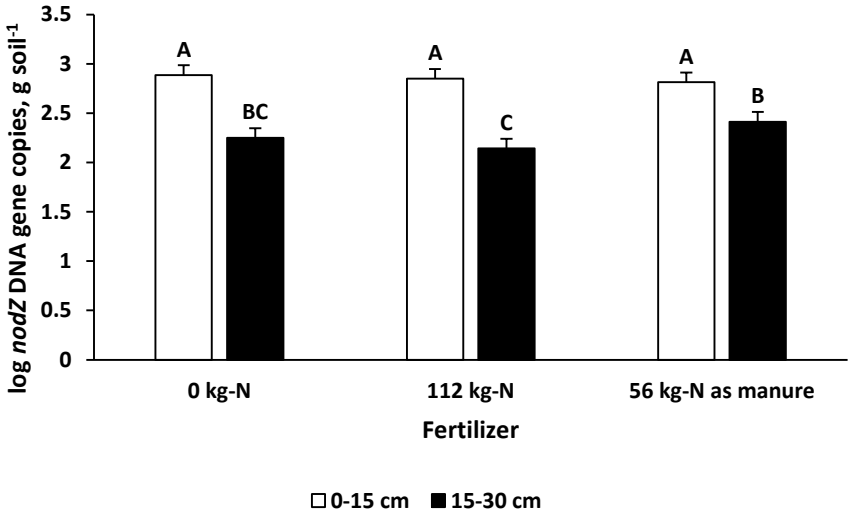

Figure 20. Mean (±SE) of soil *nodZ* gDNA copy numbers receiving N fertility treatments at 0-15 and 15-30 cm soil depths. Significant differences are denoted by different letters following Tukey's procedure at $p < 0.05$.

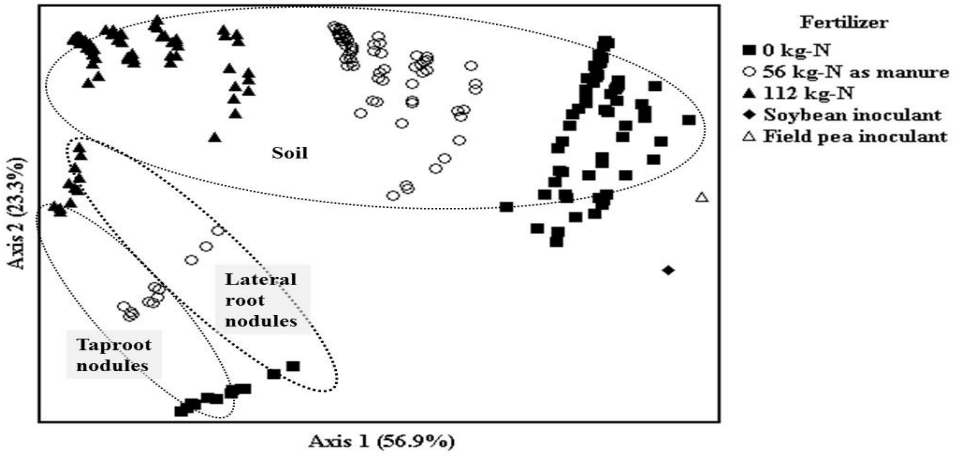

Figure 21. Ordination diagram of identified soybean specific *rhizobium* species as measured by *nodZ* gene copy numbers. Symbols represent samples displayed by fertilizer treatment. Dashed lines highlight clusters of samples collected from the soil, lateral root nodules and taproot nodules.

The persistence of inoculated and naturalized soybean rhizobium is important for maximizing BNF, especially in locations with harsh winters that can limit the ability of

rhizobium strains to overwinter (Bailey, 1989). This study counteracts older literature in which the survivability of *B. japonicum* strains is dependent on climactic conditions. Research by Bailey (1989) in which *B. japonicum* did not overwinter was conducted on a soil that had never been contaminated with *B. japonicum* and although inoculant applied by Bailey contained sufficient cells (between 2.57 and 3.39 x 10^8 *B. japonicum* g^{-1} of peat), perhaps repeated inoculation events would have resulted in a better overwinter population of *B. japonicum* as is the case in the current study where soybean inoculation has been occurring on a four-year basis since 1987. However, this research supports work by Shiro et al. (2013) who suggested that *B. japonicum* is better able to persist in the soil in northern latitudes than *B. elkanii*, and is more successful at inoculating soybean roots at low temperatures (Suzuki et al., 2014). *B. elaknii* has also been shown to dominate in humid climates and acidic soils (Adhikari et al., 2012) and is likely why *B. elkanii* persistence is low in the current study. The presence of *B. elkanii* in the soil and within the lateral root nodules of soils receiving manure additions without being found in the inoculant suggests *B. elkanii* can overwinter in the soil in the northern US Great Plains.

Recommended inoculation rates for soybean suggest a minimum of 10^5 (log 5) *B. japonicum*/seed. The persistence of *B. japonicum* in the soil prior to planting soybean was log 2.4 *nifH* gene copies in the 0-15 cm depths and log 1.86 *nodZ* gene copies in the 15-30 cm soil depths, which is well within the recommended level of inoculant as a range of 10 to 100 individual *Rhizobium* cells results in 63 to 100% nodulation of plants (Perkins, 1925). Although applying inoculant to soybeans where sufficient naturalized populations persist in the soil results in a reduced inoculation efficiency (Boonkerd et al., 1975), the results of this study suggest crop rotations without a host soybean plant can reduce the soybean inoculant populations in the soil. The reductions in inoculant populations due to the absence of a host plant have been

corroborated elsewhere (Ferreira et al., 2000). Therefore, the reduction in inoculated populations in the soil may improve inoculation efficiency for longer rotations with soybeans (greater than 4 year return interval). However, improvements in *Bradyrhizobium* persistence in the soil can occur based on N fertilizer source. Manure is a source of not only organic matter (Sommerfield et al., 1988), but also P and other essential nutrients (Sharpley et al., 1994). The addition of organic matter can aid in harboring soybean *rhizobium* as they are able to persist in a saprophytic life stage. Recent soybean inoculation trials in the northern United States Great Plains found that soybean inoculation increased the 6-year yield average by only 1.7 bushels over non-inoculated soybeans (Franzen, 2013), the lack of response to inoculation emphasizes the importance of persistence *rhizobium* populations.

Nodule infectiveness

Legume inoculant and naturalized soil *rhizobium* populations had an impact on the infectiveness of soybeans. Overall, 12 soybean specific *Bradyrhizobium* species were identified in the soil and within the nodules by sequencing by synthesis. The NMS analysis resulted in a two dimensional solution where the community structure of the soybean specific *rhizobium* in the soil and within the nodules was affected by fertilizer source (Fig 21). All selected axes for each ordination were significantly different from random ($p<0.05$) and reduced stress by 5 or more. A final stress of 8.70 with instability of $0.0x10^{-5}$ was reached after 64 iterations. Overall, 80.3% of the variation was explained in two axes. The first axis represented 56.9% of the variation, while the second axis represented 23.3% of the variation. In the ordination space, points that are closer together are more similar, while points that are further apart are more dissimilar. The samples cluster by fertilizer source along axis 1, while the soil samples cluster to the positive side of axis 2 and the nodules cluster to the negative side of axis 2. Therefore, axis 1

represents the separation between soybean *rhizobium* due to fertilizer sources, while axis 2 represents differences in soybean *rhizobium* community structure found in the soil and found within the nodules. Although the data was transformed prior to NMS, the nodules contained significantly more *Bradyrhizobium* than the soil, which results in separate clusters of the soil and nodule samples. Soil depth ($p=0.06$) and tillage practices ($p=0.61$) did not result in unique soybean specific *rhizobium* community structure either within the soil or within the nodules based on MRPP analysis. However, the results of the MRPP analysis confirm the community structure in the soil is different from the *rhizobium* community found within both the taproot and lateral root nodules ($p<0.001$).

The soil contained a diverse number of *Bradyrhizobium* species. Across all fertilizer treatments, *B. japonicum* USDA 110 comprised the largest portion of soybean *rhizobium* (61%) with an average of 4.9% of the community represented by *B. japonicum* SEMIA 560, 5045, 511, 512 and *B. liaoningense* SEMIA 5025 and *B. diazoefficiens* SEMIA 5080. While the soybean inoculant was dominantly *B. japonicum* USDA 110 (99.7%), *B. japonicum* SEMIA 560, 511, 512, *B. liaoningense* SEMIA 5025 and *B. diazoefficians* SEMIA 5080 were also identified within the soybean inoculant (Fig. 22).

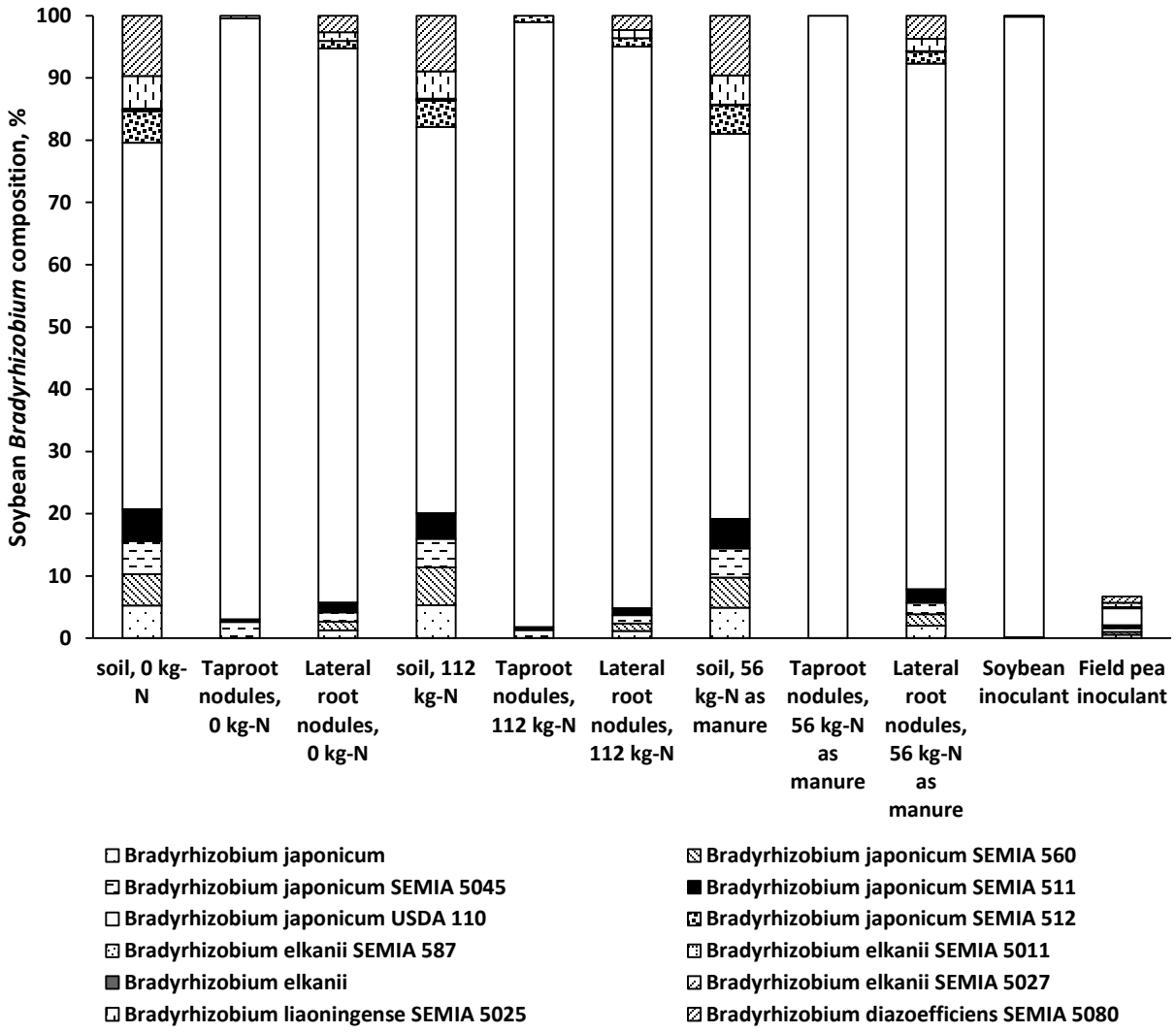

Figure 22. The average composition (%) of identified soybean specific *rhizobium* strains in the soil, taproot and lateral root nodules and inoculant under different fertilizer managements. Strains were identified by sequencing by synthesis and corrected for quantification of *nodZ* gDNA genes within each sample.

Within the taproot nodules the *rhizobium* composition most closely resembled that of the soybean inoculant, where the taproot nodules were predominantly *B. japonicum* USDA 110 (96.6, 97.2 and 100% where 0 kg-N, 112 kg-N and 56 kg-N as manure were applied, respectively). *B. japonicum* SEMIA 5045 was not identified to be within the soybean inoculant but was a minor component inside the nodules where 0 kg-N and 112 kg-N were applied (2.6 and

1.3%, respectively). However, *B. japonicum* SEMIA 511 was found within the soil comprising an average of 4.8% of the identified soybean *rhizobium* community. The diversity of *Bradyrhizobium* strains indentified within the lateral root nodules was more diverse than the taproot nodules or the soybean inoculant. In the lateral root nodules, *B. japonicum* USDA 110 was dominant under all fertilizer regimes (89.1, 90.2 and 84.5% where 0 kg-N, 112 kg-N and 56 kg-N as manure were applied, respectively). Where manure was applied, less than 0.08% of the *rhizobium* within the lateral roots was identified as *B. elkanii* strains 587, 5011 and 5027. A phylogenetic tree illustrating the relationship of the identified *Bradyrhizobium* sequences identified in the soil and within the nodules can be found in Figure 23. The *B. japonicum* strains SEMIA 560, 512, 5045, 511 and USDA 110 are very closely related to *B. liaoningense* SEMIA 5020 and *B. diazoefficens* SEMIA 5080. These strains have roughly 20% difference in the organization of base-pairs. Multiple sequence alignment was completed using Geneious software (Kearse et al., 2012) in conjunction with the MUSCLE alignment and can be found in Figure A5.

Figure 23. Phylogenetic tree of soybean specific *Bradyrhizobium* species and strains found within the nodules identified by sequencing by synthesis of the *nodZ* gene. *Bradyrhizobium* strains marked with an asterisk (*) were found within the soybean inoculant. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-1247.5253) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 363 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2015).

Nodule effectiveness

The amount of N within the soybeans obtained from BNF had a weak positive correlation with the log of *nifH* cDNA copy numbers extracted from the lateral root nodules (r^2=0.11), but the ratio of ureide-N to ureide-N plus nitrate-N within the soybeans does not take into account the rate at which N is fixed. However, these results suggest the quantity of *nifH* cDNA from

within the nodules can be considered further as a research proxy for the activity of N fixation within the roots. Additionally, this is a small dataset (n=36) and the use of a larger datasets covering a larger geographical area and plant N obtained from BNF would be needed to confirm this relationship.

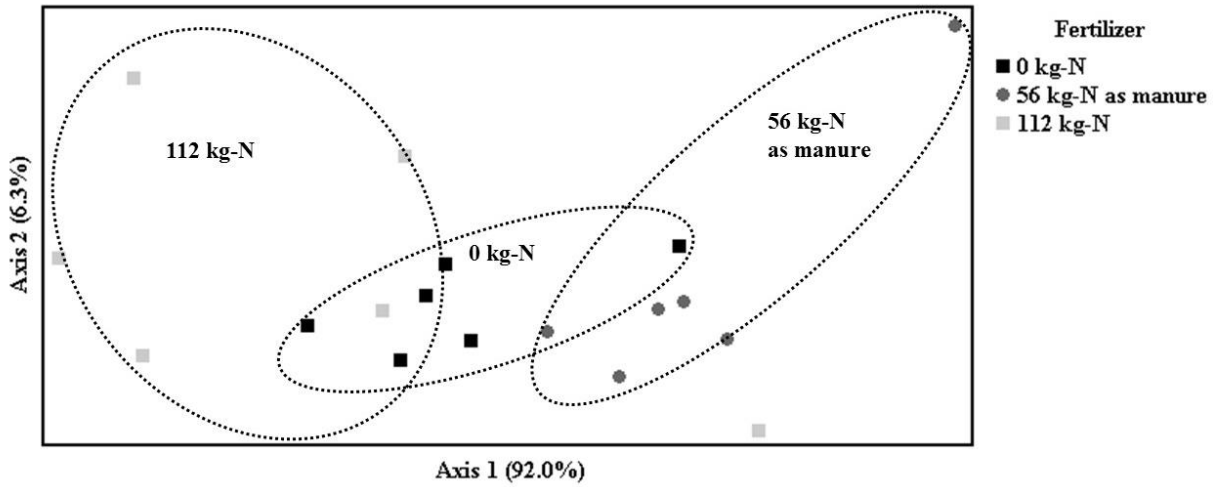

Figure 24. Ordination diagram of soybean *rhizobium* collected from within soybean nodules as related to nodule counts and weights, potentially mineralizable nitrogen, inorganic nitrogen (NO_3^- and NH_4^+), *nifH* cDNA copy numbers within the nodules, seed protein, oil and yield. Dashed line highlight clusters of samples collected from different fertilizer managements.

Nonmetric multidimensional scaling of the *rhizobium* community found within the nodules at the soybean R1 stage along with nodule counts and weights, soil PMN, soil inorganic N and *nifH* cDNA copies from within the nodules and soybean seed protein, oil content and yield resulted in a two dimensional solution. All selected axes for each ordination were significantly different from random ($p<0.05$) and reduced stress by 5 or more. A final stress of 4.54 with instability of 0.0×10^{-5} was reached after 42 iterations. Overall, 98.3% of the variation was explained in two axes. The first axis represented 92.0% of the variation, while the second axis represented 6.3% of the variation (Fig. 24). In the ordination space, points that are closer together are more similar, while points that are further apart are more dissimilar. The samples

cluster by fertilizer management along axis 1 with 56 kg-N as manure clustered to the positive side of axis 1, 0 kg-N fertilizer clustered in the middle of axis 1, and 112 kg-N clustered to the negative side of axis 1. Axis 2 separates the *rhizobium* community structure within the soybean plants. The results of the MRPP analysis confirm fertilizer management ($p<0.01$), but not tillage ($p=0.72$) has an impact on the *rhizobium* community structure found within the nodules.

Bradyrhizobium species, nodule counts and weights, soil PMN, soil inorganic N, *nifH* cDNA copy numbers within the nodules and seed protein, oil content, and yield correlations with the ordination axes is found in Table 17. These measurements are used as explanatory variables for the structure of the orindation where the absolute *r* value is greater than 0.5. Axis 1 positively correlates with taproot and lateral root nodule weights and *B. japonicum* strains SEMIA 560, 5045, 511, 512, USDA 110, *B. liaoningense* SEMIA 5020 and *B. diazoefficens* SEMIA 5080. Axis 2 positively correlates with *B. japonicum* SEMIA 587, 5011, *B. elkanii and B. elkanii* SEMIA 5027. Therefore manure applications in the soil resulted in larger nodules.

Potentially mineralizable N was greatest where 56 kg-N as manure was applied (22.6 mg N L^{-1} mineralized 7 d^{-1}), however the PMN in the manure treatment was not significantly different at the $p<0.05$ level than 112 kg-N (17.9 mg N L^{-1} mineralized $7d^{-1}$) or where 0 kg-N was applied (10.4 mg N L^{-1} $7d^{-1}$). Additionally, there were no correlations with PMN and the *rhizobium* community structure within the nodules. There were also no correlations between the *rhizobium* community structure within the nodules and plant N obtained from BNF. There was a weak, but opposite correlation of PMN and plant N obtained from BNF (Table 17), suggesting where N was mineralized more throughout the growing season, the amount of plant N obtained from BNF decreased.

Table 17. The correlation coefficients (*r*) between soybean nodule counts and weights, soil properties and *Bradyrhizobium* strains.

	r	
	Axis 1	Axis 2
Taproot nodule count	0.134	-0.278
Taproot nodule weight	**0.529**	-0.217
Lateral root nodule count	0.468	0.105
Lateral root nodule count	**0.677**	0.136
Potentially mineralizable N	-0.16	-0.076
Plant N obtained from biological nitrogen fixation	0.169	-0.207
Soil NO_3^-	0.213	0.096
Soil NH_4^+	0.22	0.28
nifH cDNA copies within the nodules	0.471	0.338
Soybean seed protien	-0.09	-0.201
Soybean seed oil content	-0.239	0.066
Soybean yield	0.161	-0.131
Bradyrhizobium japonicum	**0.864**	-0.256
Bradyrhizobium japonicum SEMIA 560	**0.897**	-0.283
Bradyrhizobium japonicum SEMIA 5045	**0.746**	-0.153
Bradyrhizobium japonicum SEMIA 511	**0.88**	-0.391
Bradyrhizobium japonicum USDA 110	**0.693**	0.403
Bradyrhizobium japonicum SEMIA 512	**0.781**	-0.208
Bradyrhizobium elkanii SEMIA 587	0.488	**0.624**
Bradyrhizobium elkanii SEMIA 5011	0.488	**0.624**
Bradyrhizobium elkanii	0.488	**0.624**
Bradyrhizobium elkanii SEMIA 5027	0.488	**0.624**
Bradyrhizobium liaoningense SEMIA 5025	**0.875**	-0.377
Bradyrhizobium diazoefficiens SEMIA 5080	**0.915**	-0.307

The taproot nodules were inhabited predominantly by *B. japonicum* USDA 110 with an observed frequency over 96%. The frequent distribution of *B. japonicum* USDA 110 is not surprising as taproot nodules are frequently formed by the strains used in inoculation as identified within this study and supported by others (Hardarson et al., 1989) and have limited movement in the soil (Lowther and Patrick, 1993). Inoculation efficiency has been shown to be

low in soils with persistent naturalized *rhizobium* as these inoculants generally fail to produce the majority of nodules on soybean roots (McDermott and Graham, 1990). In this study, *B. japonicum* USDA 110 had a frequency of 87% in the lateral root nodules with *B. diazoefficiens* as the next most abundant rhizobium.

Bradyrhizobium liaoningense strains isolated from soybean *rhizobium* roots in China have been shown to be extra-slow growing, but are closely related to *B. japonicum* (Xu et al., 1995). *B. liaoningense* exhibits relatively persistent populations in the soil of this experiment despite its extremely slow growth habit as this *rhizobium* comprises <1% of the field pea inoculant and persists in the soil at an average of over 9%. *B. liaoningense*. *B. diazoefficiens* is also closely related to *B. japonicum* and is commonly used in soybean inoculants in Brazil and South America where it has been shown to have outstanding N fixing capacity (Gomes et al., 2014; Siqueira et al., 2014). Although *B. diazoefficiens* is a very small component of the soybean inoculant *B. diazoefficiens* much like *B. liaoningense* shows potential to persist in the soil in northern climates. The quantity of active *nifH* cDNA within the lateral nodules was related to the plant N obtained from BNF and there is a positive relationship among the active *nifH* cDNA gene copies within the nodules and *B. liaoningense* and *B. diazoefficiens* (Table 17). The increase in effective *nifH* cDNA gene copy numbers in the lateral roots, likely from *B. liaoningense* and *B. diazoefficiens* during later growth stages can be attributed senescence of the taproot nodules. Nodule activity begins to decline on average three to five weeks after formation (Puppo et al., 2004). This work supports the importance of lateral root nodules for late season BNF and is in agreement with McDermott and Graham (1989) and Hardarson et al. (1989). The introduction of *B. liaoningense* and *B. diazoefficiens* in the soil is likely from previous soybean inoculant as inoculation history from the beginning of the experiment (1987) was unavailable.

There results show the potential of including these two species in greater proportions in soybean inoculant in northern climates. Although inoculants are formulated to have high nodule infectiveness and effectiveness, reduced inoculation infectiveness may not be as detrimental to BNF as the community structure of *Bradyrhizobium* within the nodules did not have an effect on plant N obtained from BNF.

Overall, the community structure of *rhizobium* inhabiting the nodules does not correlate with plant uptake of biologically fixed N. Management factors such as fertility and PMN cause differences the ability of strains to persist in the soil, but have little effect on a strains' ability to infect soybean roots and fix N within the nodules. The advances in molecular techniques to measure *nodZ* gDNA nodule infection and *nifH* cDNA gene copies as a proxy for infectiveness and N fixing activity can allow researchers to move beyond measuring nodule sizes or acetylene reduction for N-fixing activity.

Conclusions

The use of qPCR for soybean *rhizobium* enumeration in the soil is correlated to MPN counts and can be used to quickly assess soybean inoculant levels within the soil. Under a four-year crop rotation, soybean *rhizobium* populations exhibited a cyclical trend increasing in number following harvest of the host soybean plant and continually declining through the remainder of the rotation. The population of persistent *Bradyrhizobium* in the soil was, however, lower than recommended inoculation amounts of 10^5 *Bradyrhizobium* per seed. Although soybean inoculant contained 99% *Bradyrhizobium japonicum* USDA 110, several other *B. japonicum* strains and *Bradyrhizobium* species persisted in the soil and the frequency distribution for the other *B. japonicum* strains, *B. liaoningense* and *B. diazoefficiens* which have likely persisted from historically applied inoculants. Manure additions to the soil allowed *B. elkanii* to

persist in the soil and form lateral root nodules. Although previous research has suggested that *B. japonicum* is the only soybean inoculant capable of overwintering in northern climates, these results suggest additional *Bradyrhizobium* are able to overwinter in the harsh US northern Great Plains. The taproot nodules found on the soybeans were predominantly *B. japonicum* USDA 110, presumably from the soybean inoculant, but the lateral root nodules had frequent observations of the persistent *B. liaoningense* and *B. diazoefficiens.* Consequently, inoculated populations are important for early season BNF, but persistent populations of *rhizobium* play an important role in late season BNF, especially in the lateral root nodules. The *nifH* cDNA copy numbers were higher in the lateral root nodules during the soybean R1 phase, especially where manure was applied. However, the *rhizobium* community structure actively fixing N within the nodules was not affected by tillage or fertilizer management and was not related to plant N obtained from BNF. These results suggests that soybean inoculants in the US northern Great Plains should be applied yearly and can be composed of several different *Bradyrhizobium* strains.

Acknowledgements

The North Dakota Soybean Council supported this work. Additionally, the North Dakota Agricultural Experiment Station Fellowship and the North Dakota Water Resources Research Institute Fellowship provided additional support. The authors would like to thank Joel Bell, Brian Johnson and Emma Nygum for their assistance in the field and laboratory.

References

Abel, G.H. L.W. Erdman. 1694. Response of Lee soybeans to different strains of *Rhizobium japonicum*. Agron. J.. 56:423-424.

Adhikari, D., M. Kaneto, K. Itoh, K. Suyama, B.B. Pokharel, Y.K. Gaihre. 2012. Genetic diversity of soybean-nodulating rhizobia in Nepal in relation to climate and soil properties. Plant and Soil. 357:131-145. doi:10.1007/s11104-012-1134-6

Allos, H.F., W.V. Bartholomew. 1959. Replacement of symbiotic fixation by available fixation. Soil Sci. 87:61-66.

Bailey, L.D. 1989. Survival of *Bradyrhizobium japonicum* in Canadian prairie soils. Can. J. Plant Sci. 69:23-30. doi:10.4141/cjps89-004

Barcellos, F.G., P. Menna, J.S. da Silva Batista, M. Hungria. 2007. Evidence of horizontal transfer of symbiotic genes from a *Bradyrhizobium japonicum* inoculant strain to indigenous diazotrophs *Sinorhizobium (Ensifer) fredii* and *Bradyrhizobium elkanii* in a Brazilian savannah soil. Appl. Environ. Microbiol. 73:2635-2643. doi:10.1128/AEM.01823-06

Bohlool, B. B., J. K. Ladha, D. P. Garrity, T. George. 1992. Biological nitrogen fixation for sustainable agriculture: a perspective. Plant Soil. 141:1-11. doi:10.1007/BF00011307

Boonkerd, N., D.F. Weber, D.F. Bezdicek. 1975. Influence of Rhizobium japonicum strains and inoculation methods on soybeans grown in Rhizobia-populated soil. Agron. J. 70:547-549. doi:10.2134/agronj1978.00021962007000040007x

Bremner, J. M. 1965. Inorganic forms of nitrogen. Pages 1179-1237 in Black, C.A. (Ed) Methods of soil analysis. Part 2. Agronomy No. 9. American Society of Agronomy, Madison, WI.

Caldwell, B.E. G. Vest. 1970. Effects of *Rhizobium japonicum* strains on soybean yields. Crop Sci.. 10:19-21. doi:10.2135/cropsci1970.0011183X001000010008x

Calugay, R.J., H. Takeyama, D. Mukoyama, Y. Fukuda, T. Suzuki, K. Kanoh, T. Matsunaga. 2006. Catechol siderophore excretion by magnetotactic bacteriam Magnetospirillum magneticum AMB-1. J. Biosci. Bioeng. 101:445-7. doi:10.1263/jbb.101.445

Chen, W.X., G.H. Yan, L.J. Li. 1988. Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. Int. J. System. Bacteriol. 38:392-397. doi:10.1099/00207713-38-4-392

Chen, W., E. Want, S. Wang, Y. Li, X. Chen, Y. Li. 1995. Characteristics of *Rhizobium tianshanense* sp. nov. a moderately and slowly growing root nodule bacterium isolated from an arid saline environment in Xinjiang, People's Republic of China. Int. J. System. Bacteriol. 45:153-153. doi:0020-7713/95/$04.00 + 0

Crozat, Y., J.C. Cleyet-Marel, A. Corman. 1987. Use of the fluorescent antibody technique to characterize equilibrium survival concentrations of *Bradyrhizobium japonicum* strains in soil. Biol. Fertil. Soils. 4:85-90. doi:10.1007/BF00280356

Dénarié, J., F. Debellé, and C. Rosenberg. 1992. Signaling and host range variation in nodulation. Annu. Rev. Microbiol. 46:497-531. doi:10.1146/annurev.mi.46.100192.002433

Dénarié, J. and P. Roche. 1992. Rhizobium nodulation signals. In: Verma, D.P.S. (ed). 1992. Molecular signals in plant-microbe communication. CRC Press, Boca Raton, LA, USA.

Doran, J.W. 1980. Soil microbial and biochemical changes associated with reduced tillage. Soil Sci. Soc. Am. J. 44:765-771. doi:10.2136/sssaj1980.03615995004400040022x

Dowdle, S.R., B.B> Bohlool. 1987. Intra- and inter-specific competition in *Rhizobium fredii* and *Bradyrhizobium japonicum* as indigenous and introduced organisms. Can. J. Microbiol. 33:900-995. doi:10.1139/m87-174

El-Haris, M.K., V.L. Cochran, L.F. Elliot, D.F. Bezdicek. 1983. Effect of tillage, cropping, and fertilizer management on soil nitrogen mineralization potential. Soil Sci. Soc. Am. J. 47:1157-1161. doi:10.2136/sssaj1983.03615995004700060020x

Ferreira, M. C., D. de S. Andrade, L. M. de O. Chueire, S. M. Takemura, and M. Hungria. 2000. Tillage method and crop rotation effects on the population sizes and diversity of Bradyrhizobia nodulating soybean. Soil Biol. Biochem. 32:627-637. doi:10.1016/S0038-0717(99)00189-3

Fish, J. A., B. Chai, Q. Wang, Y. Sun, C. T. Brown, J. M. Tiedje, and J. R. Cole. 2013. FunGene: the Functional Gene Pipeline and Repository. Front. Microbiol. 4:291; doi:10.3389/fmicb.2013.00291

Fortin, M.-C. 1993. Soil temperature, soil water, and no-till corn development following in-row residue removal. Agron. J.. 85:571-576. doi:10.2134/agronj1993.00021962008500030010x

Franzen, D.W. 2013. Soybean soil fertility. North Dakota State University Extension Service, SF1164.

Furseth, B.L., S.P. Conley, and J.-M. Ané. 2010. Enumeration of soybean-associated rhizobia with quantitative real-time polymerase chain reaction. Crop Sci. 50:2591–2596. doi:10.2135/cropsci2010.03.0141

Gaby J.C., Buckley D.H. 2012. A comprehensive evaluation of PCR primers to amplify the nifH gene of nitrogenase. PLoS ONE 7(7): e42149. doi:10.1371/ journal.pone.0042149

Gomes, D.F., J.S. de Siva Batista, A.A. P. Rolla, L.P. da Silva, C. Block, L.V. Galli-Terasawa, M. Hungria. 2014. Proteomic analysis of free-living *Bradyrhizobium diazoefficiens*: highlighting potential determinants of a successful symbiosis. BMC Genomics. 15:643. doi:10.1186/1471-2164-15-643

Goos, R.J., N. Abdraimova, and B.E. Johnson. 2015. Method for determination of ureides in soybean tissues. Commun. Soil Sci. Plant Anal. 46:242-429.

Göttfert, M., P. Grob, H. Hennecke. 1990. Proposed regulatory pathway encoded by the nodV and nodW genes, determinants of host specificity in Bradyrhizobium japonicum. Proc. Natl. Acad. Sci. U.S.A 87:2680-2684.

Hardarson, G., M. Golbs, S.K.A. Danso. 1989. Nitrogen fixation in soybean (*Glycine max* L. Merrill) as affected by nodulation patterns. Soil Biol. Biochem. 21:783-787. doi:10.1016/0038-0717(89)90171-5

Herridge, D.F. 1982a. Relative abundance of ureides and nitrate in plant tissues of soybean as a quantitative assay of nitrogen fixation. Plant Physiol. 70:1-6. doi:0032-0889/82/70/0001/06/$00.50/0

Herridge, D.F. 1982b. Use of the ureide technique to describe the nitrogen economy of field-grown soybeans. Plant Physiol. 70:7-11. doi:0032-0889/82/70/0007/05/$00.50/0

Herridge, D.F., M.B. Peoples, R.M. Boddey. 2008. Global inputs of biological nitrogen fixation in agricultural systems. Plant and Soil. 311:1-18. doi:10.1007/s11104-008-9668-3

Hungaria, M. and G. Stacey. 1997. Molecular signals exchanged between host plants and rhizobia: basic aspects and potential application in agriculture. Soil Biol. Biochem. 29:819-830.

Hungaria, M. and M.A.T. Vargas. 1996. Exploring the microbial diversity and soil management practices to optimize the contribution of soil microorganisms to plant nutrition. In Stacey, G., B. Mullin, and P.M. Gresshoff (Eds). Biology of Plant-Microbe Interactions. Society of Molecular Plant-Microbe Interactions. St. Paul, MN, USA

Hungria, M., J. Franchini, R. Campo, P. Graham. 2005. The importance of nitrogen fixation to soybean cropping in South America. *In*: Werner, D., W. Newton (Eds). Nitrogen fixation in agriculture, forestry, ecology, and the environment. Springer, Netherlands.

Johnson, H.W., U.M. Means, C. R. Weber. 1965. Competition for nodule sites between strains of Rhizobium japonicum applied as inoculum and strains in the soil. Agron. J. 57:179-185. doi:10.2134/agronj1965.00021962005700020014x

Jordan, D.C. 1982. Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing, root nodule bacteria from leguminous plants. Int. J. System. Bacteriol. 32:136-139. doi:10.1099/00207713-32-1-136

Kaneko, T., Y. Nakamura, S. Sato, K. Minamisawa, T. Uchiumi, S. Sasamoto, A. Watanabe, K. Idesawa, M. Iriguchi, K. Kawashima, M. Kohara, M. Matsumoto, S. Shimpo, H. Tsuruoka, T. Wada, M. Uamada, S. Tabata. 2002. Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA 110. DNA Res. 9:189-197. doi:10.1093/dnares/9.6.189

Kearse, M., R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, S. Buxton, A. Cooper, S. Markowitz, C. Duran, T. Thierer, B. Shaton, P. Mintjies, A. Drummond. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 28:1647-1649. doi:10.1093/bioinformatics/bts199

Kruskal V.J. 1964. Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. Psychometrika. 29:1-27. doi:10.1007/BF02289565

Kumar, S., G. Stecher, K. Tamura. 2015. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. (*Submitted*).

Kuykendall, L.D., B. Saxena, T.E. Cevine, S.E. Udell. 1992. Genetic diversity in *Bradyrhizobium* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. Can. J. Microbiol. 38:501-505. doi:10.1139/m92-082

Kuykendall, L.D., D.F. Weber. 1978. Genetically marked *Rhizobium* identifiable as inoculum strain in nodules of soybean plants grown in fields populated with *Rhizobium japonicum*. Appl. Environ. Microbiol. 36:915-919. doi:0099-2240/78/0036-0915$02.00/0

Langmead, B., S. Salzberg. 2012. Fast gapped-read alignment with Bowtie 2. Nature Methods. 9:357-359. doi:10.1038/nmeth.1923

Larimer, F.W., P. Chain, L. Hauser, J. Lamerdin, S. Malfatti, L. Do, M.L. Land, D.A. Pelletier, J.T. Beatty, A.S. Lang, F.R. Tabita, J.L. Gibson, T.E. Hanson, C. Bobst, J.L. Torres, C. Peres, F.H. Harrison, J. Gibson, C.S. Harwood. 2004. Complete genome sequence of the metabolically versatile photosynthetic bacterium *Rhodopseudomonas palustris*. Nature Biotech. 22:55-61. doi:10.1038/nbt923

Liebig, M.A., D.L. Tanaka, B.J. Wienhold. 2004. Tillage and cropping effects on soil quality indicators in the northern Great Plains. Soil Tillage Res. 78:131-141. doi:10.1016/j.still.2004.02.002

Lowether, W.L., H.N. Patrick. 1993 Spread of *Rhizobium* and *Bradyrhizobium* in soil. Soil Biol. Biochem. 25:607-612. doi:10.1016/0038-0717(93)90200-U

Mao, Y. A.C. Yannarell, R.I. Mackle. 2011. Changes in N-transforming archaea and bacteria in soil during the establishment of bioenergy crops. PLoS ONE. 6:e24750. doi:10.1371/journal.pone.0024750

Marschner P., K. Kandeler, B. Marschner. 2003. Structure and function of the soil microbial community in a long-term fertilizer experiment. Soil Biol. Biochem. 35:453-461. doi:10.1016/S0038-0717(02)00297-3

Martinez, E., D. Romero, and R. Palacios. 1990. The Rhizobium genome. Crit. Rev. Plant Sci. 9:59-93.

Mather, P.M. 1976. Computational methods of multivariate analysis in physical geography. Wiley and Sons, Inc., London, England dio:10.1002/esp.3290020419

Mathew, R.P., Y. Feng, L. Githinji, R. Ankumah, K.S. Balkcom. 2012. Impact of no-tillage and conventional tillage systems on soil microbial communities. Appl. Environ. Soil Sci. 2012:548620. doi:10.1155/2012/548620

McCune B., Grace J.B. 2002. Analysis of ecological communities. Gleneden Beach, OR: MjM Software Design. doi:10.1016/S0022-0981(03)00091-1

McCune, B. and M. J. Mefford. 2011. PC-ORD. Multivariate Analysis of Ecological Data. Version 6. MjM Software, Gleneden Beach, Oregon, U.S.A.

McDermott, T.R. and P.H. Graham. 1989. *Bradyrhizobium japonicum* inoculant mobility, nodule occupancy, and acetylene reduction in the soybean root system. Appl. Environ. Microbiol. 55:2493-2498. doi: 0099-2240/89/102493-06$02.00/0

McDermott, T.R., P.H. Graham. 1990. Competative ability and efficiency in nodule formation of strains of *Bradyrhizobium japonicum*. Appl. Environ. Microbiol. 56:3035-3039. doi:0099-2240/90/103035-05$02.00/0

McDermott, T.R., P.H. Graham, M.L. Ferrey. 1991. Competitiveness of indigenous populations of *Bradyrhizobium japonicum* serocluster 123 as determined using a root-tip marking procedure in growth pouches. Plant and Soil. 135:245-250. doi:0099-2240/89/102493-06$02.00/0

Means, U. M., H. W. Johnson, and R. A. Date. 1964. Quick serological method of classifying strains of rhizobium japonicum in nodules. J. Bacteriol. 87:547-553.

Menna, P., M. Hungria. 2011. Phylogeny of nodulation and nitrogen fixation genes in *Bradyrhizobium*: supporting evidences for the theory of monophyletic origin and spread and maintenance by both horizontal and vertical transfer. Int. J. Syst. Evol. Micr. 61:3052-3067. doi:10.1099/ijs.0.028803-0

Mitchell, R.L., W.J. Russel. 1971. Root development and rooting paterns of soybean (Glycine max (L.) Merrill) evaluated under field conditions. Agron. J. 63:313-316. doi:10.2134/agronj1971.00021962006300020034x

Moebius-Clune, B.N., D.J. Moebius-Clune, B.K. Gugino, O.J. Idowu, R.R. Schindelbeck, A.J. Ristow, H.M. van Es, J.E. Thies, H. A. Shayler, M. B. McBride, D.W. Wolfe, and G.S. Abawi, 2016. Comprehensive Assessment of Soil Health – The Cornell Framework Manual, Edition 3.0, Cornell University, Geneva, NY.

NDAWN. 2014. North Dakota Agricultural Weather Network. http://ndawn.ndsu.nodak.edu. Accessed on 22 December 2014.

Peng, G.X., Z.Y. Tan, E.T. Wang, B. Reinhold-Hurek, W.F. Chen, W.X. Chen. 2002. Identification of isolates from soybean nodules in Xinjiang region as *Sinorhizobium xinjiangense* and a genetic differentiation of *S. xinjiangense* from *Sinorhizobium fredii*. Int. J. Syst. Evol. Microbiol. 52:457-462. doi:10.1099/ijs.0.01921-0

Poly F., L. Ranjard, S. Nazaret, F. Gourbiere, and L. J. Monrozier. 2001. Comparison of *nifH* gene pools in soils and soil microenvironments with contrasting properties. Appl. Environ. Microbiol. 67: 2255–2262. doi:10.1128/AEM.67.5.2255-2262.2001

Puppo, A., K. Groten, F. Bastian, R. Carzaniga, M. Soussi, M.M. Lucas, M.R. de Felipe, J. Harrson, H. Vanacker, C.H. Foyer. 2004. Legume nodule senescence: roles for redox and hormone signaling in the orchestration of the natural aging process. New Phytologist. 165:683-701. doi:10.1111/j.1469-8137.2004.01285.x

Rochette, P., E.G. Gregorich. 1998. Dynamics of soil microbial biomass C, soluble organic C and CO_2 evolution after three years of manure application. Can. J. Soil Sci. 78:283-290. doi:10.4141/S97-066

Saxton, A.M. 1998. A macro for converting mean separation output to letter groupings in Proc Mixed. *In*: Proc. 23rd SAS Users Group Intl., SAS Institute, Cary, NC, USA.

Schlaman, H.R.M., D.A. Phillips, E. Kondorosi. 1998. Genetic organization and transcriptional regulation of rhizobial nodulation genes. *In*: Spaink, H.P., A. Kondorosi, P.J.J. Hooykaas (eds.) The Rhizobiaceae. Dordrecht: Kluwer, The Nederlands.

Scholla, M.H. and H.G. Elkan. 1984. *Rhizobium fredii* sp. nob. A fast-growing species that effectively nodulates soybeans. Int. J. System. Bacteriol. 34:484-486. doi: 10/1984; 34(4). doi:10.1099/00207713-34-4-484

Seefeldt, L.C., M.B. Hoffman, D.R. Dean. 2010. Mechanisms of Mo-dependent nitrogenase. Annu. Rev. Biochem. 78:701-722. doi:10.1146/annurev.biochem.78.070907.103812

Sharpley, A.N., S.C. Chapra, R. Wedepohl, J.T. Sims, T.C. Daniel, K.R. Reddy. 1994. Managing agricultural phosphorus for protection of surface waters: issues and options. J. Environ. Qual. 23:437-451. doi:10.2134/jeq1994.00472425002300030006x

Shiro, S. S. Matsuura, R. Saiki, G.C. Sigua, A. Yamamoto, Y. Umehyara, M. Hayashi, Y. Saiki. 2013. Genetic diversity and geographical distribution of indigenous soybean-nodulating Bradyrhizobia in the United States. Appl. Environ. Microbiol. 79:3610-3618. doi:10.1128/AEM.00236-13

Singleton, P. W. and J. W. Tavares. 1986. Inoculation response of legumes in relation to the number and effectiveness of indigenous Rhizobium populations. Appl. Environ. Microbiol. 51:1013-1018. doi:0099-2240/86/051013-06$02.110/0

Singleton, P.W., H.M. Abdelmagid, J.W. Tavares. 1985. Effect of phosphorus on the effectiveness of strains of *Rhizobium japonicum*. Soil Sci. Soc. Am. J. 49:613-616. doi:10.2136/sssaj1985.03615995004900030016x

Siqueira, A.F., E. Ormeno-Orrillo, R.C. Souza, E.P. Rodriques, L.G. Almeida, F.G. Barcellos, J.S. Batista, A.S. Nakatani, E. Martinez-Romero, A.T. Vasconcelos, M. Hungria. Comparative genomics of Bradyrhizobium japonicum CPAC 15 and Bradyrhizobium diazoefficiens CPAC 7: elite model strains for understanding symbiotic performance with soybean. BMC Genomics. 15:420. doi:10.1186/1471-2164-15-420

Sloger, C. 1969. Symbiotic effectiveness and N_2 fixation in nodulated soybean. Plant Physiol. 44:1666-1668.

Soil Survey Staff. 2014. Web Soil Survey. Natural Resources Conservation Service, United States Department of Agriculture. Available online at http://websoilsurvey.nrcs.usda.gov/. Accessed on 15 September 2014.

Sommerfeld, T.G., C. Chang, T. Entz. 1988. Long-term annual manure applications increase soil organic matter and nitrogen, and decrease carbon to nitrogen ratios. Soil Sci. Soc. Am. J. 52:1688-1672. doi:10.2136/sssaj1988.03615995005200060030x

Stroup, W.W. and J. Stubbendieck. 1983. Multivariate statistical methods to describe changes in botanical composition. J. Range Manage. 36:208-212.

Suzuki, Y., D. Adhikari, K. Itoh, K. Suyama. 2014. Effects of temperature on competition and relative dominance of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* in the process of soybean nodulation. Plant and Soil. 374:915-924. doi:10.1007/s11104-013-1924-5

Tamura, K., M. Nei. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10:512-526. doi:0737-4038/93/1003-0002$02.00

Tan, Z.Y., Z.D. Xu, E.T. Wang, J.L. Gao, E. Martinex-Romero, W.X. Chen. 1997. Phylogenetic and genetic relationships of *Mesorhizobium tianshanense* and related rhizobia. Int. J. System. Bacteriol. 47:874-879. doi:0020-7713/97/$04.00 + 0

Thies, J. E., P. W. Singleton and B. B. Bohlool. 1991. Influence of the size of indigenous rhizobial populations on establishment and symbiotic performance of introduced rhizobia on field-grown legumes. Appl. Environ. Microbiol. 57:19-28. doi:0099-2240/91/010019-10$02.00/0

Trojan, M.D., D.R. Linden. 1998. Macroporosity and hydraulic properties of earthworm-affected soils as influences by tillage and residue management. Soil Sci. Soc. Am. J. 62:1687-1692. doi:10.2136/sssaj1998.03615995006200060029x

Vincent, J.M. 1970. A manual for the practical study of the root-nodule bacteria. IBP Handbook 15. Blackwell Science Publishing, Oxford, UK.

Van Berkum, P., C. Sloger, D.F. Weber, P.B. Cregan, H.H. Keyser. 1985. Relationship between ureide N and N_2 fixation, aboveground N accumulation, acetylene reduction, and nodule mass in greenhouse and field studies with *Glycine max* L. (Merr). Plant Physiol. 77:53-58. doi:0032-0889/85/77/0053/06/$01.00/0

Vinuesa, P. K. Rohas-Jiménez, B. Contreras-Moreira, S.K. Mahana, B.N. Prasad, H. Moe, S.B. Selvaraju, H. Thiefelder, D. Werner. 2008. Multilocus sequence analysis for assessment of the biogeography and evolutionary genetics of four *Bradyrhizobium* species that nodulate soybeans on the Asiatic continent. Appl. Environ. Microbiol. 74:6987-6996. doi:10.1128/AEM.00875-08

Vitousek, P.M., D.N.L. Menge, S.C. Reed, C.C. Cleveland. 2013 Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. Phil. Trans. R. Soc. B. 368: 20130119. doi:10.1098/rstb.2013.0119

Vyn, T.J., G. Opoku, C.J. Swenson. 1996. Residue management and minimum tillage systems for soybean following wheat. Agron. J. 90:131-138. doi:10.2134/agronj1998.00021962009000020002x

Wander, M.M, M.G. Bidart, S. Aref. 1998. Tillage impacts on depth distribution of total and particulate organic matter in three Illinois soils. Soil Sci. Soc. Am. J. 62:1704-1711. doi:10.2136/sssaj1998.03615995006200060031x

Weaver, R.S. and S.K.A. Danso. 1994. Dinitrogen fixation. *In:* Weaver, R.W., J.S. Angle, P.S. Bottomley (eds). Methods of soil analysis. Part 2. American Society of Agronomy. Madison, WI, USA.

Weaver, R.W. and L.R. Frederick. 1972. Effect of inoculum size on nodulation of *Glycine max* L. Merrill, Variety Ford. Agron. J.. 64:597-599. doi:10.2134/agronj1972.00021962006400050014x

Xu, M.L., C. Ge, Z. Cui, J. Li, H. Fan. 1995. *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybeans. Int. J. System. Bacteriol. 45:706-711. doi:10.1099/00207713-45-4-706

Yao, Y.Z., F.L. Kan, E.T. Wang, G.H. Wei, X. Chen. 2002. Characterization of rhizobia that nodulate legume species of the genus *Lezpedeza* and description of *Bradyrhizobium yuanmingense* sp. nov. Int. J. Syst. Evol. Microbiol. 52:2219-2230. doi:10.1099/ijs.0.01408-0

Zapata, F., S.K.A. Danso, G. Hardarson, and M. Fried. 1987. Time course of nitrogen fixation in field-grown soybean using nitrogen-15 methodology. Agron. J. 79:172–176. doi:10.2134/agronj1987.00021962007900010035x

Zengeni, R., S. Mpepereki, K. Giller. 2006. Manure and soil properties effects on rhizobial survival and persistence in smallholder field environments of Zimbabwe. Appl. Soil Ecol. 32:232-242. doi:10.1016/j.apsoil.2005.06.001

GENERAL CONCLUSIONS

Soil microorganisms provide many functions for the environment, agriculture and soil health but have historically been difficult to quantify and measure. Advances in molecular techniques such as quantitative Polymerase Chain Reaction and sequencing by synthesis in conjunction with traditional soil laboratory measurements now allow researchers to improve the understanding of microbial function and diversity. The ability to link the impacts of aboveground land management such as tillage, fertilizer, crop rotations and amendments on belowground microbial functioning such as nutrient cycling and soil health is now feasible. Although this dissertation is but a small step in linking microbial function to land management, the general conclusions of this dissertation are based on the research objectives as follows:

- Research objective 1: Identifying a set of biological soil indicators that can be used to assess changes in soil health due to remediation of soil sodicity.

Several enzymes produced by the soil microbial community can be successfully applied as soil health indicators such as β glucosidase, alkaline phosphatase, nitrate reductase and arylsulfatase. Measured enzyme activity levels decreased due to the conversion of hayland to annual row crop and due to the application of gypsum. Gypsum application increased the salt concentration in the soil which had a negative effect on microbial enzyme activity and ammonia oxidizing archaea. Sugar beet lime had no effect on measured biological soil health indicators. This work identified that soil enzyme activity and the ratio of nitrifying ammonia oxidizing archaea to ammonia oxidizing bacteria are suitable soil health indicators as they were also able to discern soil land uses. Additionally, these nitrifying and also denitrifying soil organisms responded rapidly to soil chemical amendment applications of gypsum and spent sugar beet lime by either increasing or decreasing their populations. As such, the quantity of nitrifying and

denitrifying organisms show potential to be applied as soil health indicators. Finally, a novel application of successional vector trajectories was able to show changes in soil health resulting from alterations in aboveground land management.

- Research objective 2: Determine the impacts of cover crop growth on soil health through the assessment of soil nitrifiers and denitrifiers on a saline soil.

The application of nitrifier and denitrifier soil microorganisms as soil health indicators was applied in a field study to reduce the negative effects of soil salinity on crop growth. Ammonia monooxygenase and nitrate reductase enzyme activity was measured in conjunction with quantifying ammonia oxidizing archaea and bacteria and denitrifying bacteria. Unlike during sodic soil reclamation as identified by research objective 1, the use of nitrifiers and denitrifiers as soil health indicators is not recommended for saline soils. Unlike previous studies where salt was applied to soils to mimic secondary salinity, the salt in this field was from primary soil salinity. As such, the nitrifier and denitrifier communities had evolved under the harsh environmental conditions and exhibited community induced salinity tolerance as enzyme activity and the quantity of organisms was not related to soil salinity. This work is the first to observe salinity induced community tolerance in an agricultural soil. Additionally, many of the nitrifying organisms were previously first described in environments with elevated salinity, where many of the denitrifying bacteria are ubiquitous.

- Research objective 3: Assess biological N fixation and changes in *rhizobium* community in soybeans under different tillage and fertilizer managements.

Symbiotic soil *rhizobium* can infect legume roots and provide N to the plant through biological N fixation. The symbiotic *rhizobium* are applied as an inoculant during seed planting and can persist in the soil. Questions remain regarding the impact of these persistent populations

on biological N fixation and subsequent soil health. The results of this study indicate inoculated soybean (*Glycine max* L.) *rhizobium* populations in the soil decrease in time due to lack of a host legume. Tillage and the application of N fertilizer sources did result in unique populations of *rhizobium* in the soil, but did not affect nodule infectiveness. Fertilizer management, resulted in different *Bradyrhizobium* community structure within the root nodules, larger and more numerous and greater transcription the nitrogenase regulating *nifH* cDNA were found within the nodules where manure was applied. Although there were differences in *rhizobium* structure both in the soil and within the nodules due to fertilizer management, there was no effect on the effectiveness of biological N fixation in soybeans.

While this work has addressed short-term effects of soil aboveground management to belowground microbial processes, questions regarding the long-term effects remain. The long-term effects of increased soil salinity of gypsum application to a sodic soil raises questions as to whether the nitrifying and denitrifying organisms will evolve to tolerate an environment with greater solutes as was exhibited in a long-term saline soil. Effects of sugar beet lime as a reliable amendment to remediate sodicity is also of interest as the solubility of spent sugar beet lime is low. Given more time, how will the microbial community in the soil respond? Finally, if bacteria other than soybean specific *rhizobium* have been identified in soybean nodules, what other organisms could potentially be found within these nodules and do these other organisms increase or decrease biological N fixation? Future studies and continued monitoring are needed to answer these questions.

APPENDIX

Table A1. The sequencing by synthesis samples and primers (soybean specific *nodZ* and nitrogen fixation *nifH*, ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB) and denitrifier *nosZ*) for each library along with reads per library, maximum, minimum and average library read length, coverave mean, standard deviation (SD) and man mapping quality. For the *nodZ* and *nifH* primers the sample nomenclature is as follows: plot, subplot and time of sample collection (preplant, soybean V2-4 or R1 growth stage) and soil depth (0-15 or 15-30 cm) or nodule location (lat for nodules collected from the lateral roots and tap for nodules collected from taproot nodules). For the AOA/AOB/*nosZ* primers, sample nomenclature is as follows: plot number followed by time of sampling. The letter c following the plot number indicates samples take from 2 year cover crop treatments.

nodZ/*nifH* primers	AOA/AOB/*nosZ* primers	reads	minimum read length	maximum read legnth	average read length	coverage mean	coverage SD	mean mapping quality
105-1V6-8lat	111Fall 2013	3861	35	491	104.91	0.09	1.84	0.22
105-3V6-8lat	122Fall 2013	3456	37	492	112.35	0.10	2.16	0.17
105-4V6-8lat	135Fall 2013	2648	36	359	95.00	0.06	1.37	0.16
105-6V6-8lat	143Fall 2013	2104	36	359	105.82	0.06	1.49	0.15
105-10V6-8lat	154Fall 2013	1818	36	359	83.01	0.04	0.84	0.11
105-12V6-8lat	212Fall 2013	2107	36	491	88.57	0.04	1.57	0.12
211-1V6-8lat	223Fall 2013	2086	33	491	118.3	0.07	1.75	0.17
211-2V6-8lat	235Fall 2013	2282	36	491	119.17	0.08	2.12	0.21
211-4V6-8lat	241Fall 2013	2984	36	491	122.54	0.11	3.08	0.18
211-5V6-8lat	254Fall 2013	2889	33	491	105.19	0.08	1.95	0.20
211-10V6-8lat	311Fall 2013	1648	36	491	95.32	0.09	1.96	0.20
211-11V6-8lat	325Fall 2013	3131	33	489	120.77	0.11	2.69	0.18
105-1V2-40-15		729	38	356	214.67	0.07	6.66	0.01
105-3V2-40-15		901	38	230	215.37	0.08	8.34	0.01

Table A1. The sequencing by synthesis samples and primers (soybean specific *nodZ* and nitrogen fixation *nifH*, ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB) and denitrifier *nosZ*) for each library along with reads per library, maximum, minimum and average library read length, coverave mean, standard deviation (SD) and man mapping quality (continued).

nodZ/*nifH* primers	AOA/AOB/*nosZ* primers	reads	minimum read length	maximum read legnth	average read length	coverage mean	coverage SD	mean mapping quality
105-4V2-40-15		1212	35	260	215.10	0.11	11.20	0.01
105-6V2-40-15		212	38	229	181.54	0.02	1.54	0.01
311-2V6-8lat	343Fall 2013	5497	35	491	92.12	0.12	2.78	0.22
311-4V6-8lat	352Fall 2013	5282	35	359	81.68	0.10	1.87	0.13
311-5V6-8lat	514Fall 2013	3170	35	491	81.60	0.06	1.44	0.16
311-10V6-8lat	521Fall 2013	3269	36	491	105.06	0.09	2.32	0.19
311-11V6-8lat	533Fall 2013	3242	30	359	93.44	0.08	1.65	0.18
105-1V6-8tap	545Fall 2013	5069	31	491	163.93	0.31	20.93	0.14
105-3V6-8tap	552Fall 2013	4090	36	491	147.72	0.23	17.65	0.16
105-4V6-8tap	712Fall 2013	6024	36	490	159.60	0.36	24.88	0.18
105-6V6-8tap	721Fall 2013	5735	35	491	151.46	0.31	18.70	0.21
105-10V6-8tap	735Fall 2013	6924	36	491	167.11	0.44	29.13	0.22
105-12V6-8tap	744Fall 2013	6248	36	491	151.07	0.34	21.99	0.21
311-1V2-40-15		186	38	236	185.31	0.01	1.35	0.01
311-2V2-40-15		370	38	281	184.16	0.03	2.69	0.01
311-4V2-40-15		798	38	230	199.35	0.07	6.49	0.01
311-5V2-40-15		591	38	240	163.96	0.04	3.68	0.01
211-1V6-8tap	753Fall 2013	3174	37	354	146.11	0.17	11.89	0.15
211-2V6-8tap	813Fall 2013	3453	37	359	150.23	0.17	14.70	0.12
211-4V6-8tap	821Fall 2013	3381	37	359	147.69	0.18	14.59	0.13
211-5V6-8tap	835Fall 2013	2578	35	491	120.08	0.09	5.18	0.14

Table A1. The sequencing by synthesis samples and primers (soybean specific *nodZ* and nitrogen fixation *nifH*, ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB) and denitrifier *nosZ*) for each library along with reads per library, maximum, minimum and average library read length, coverave mean, standard deviation (SD) and man mapping quality (continued).

nodZ/*nifH* primers	AOA/AOB/*nosZ* primers	reads	minimum read length	maximum read legnth	average read length	coverage mean	coverage SD	mean mapping quality
211-10V6-8tap	842Fall 2013	2669	37	491	136.60	0.12	9.21	0.16
211-11V6-8tap	854Fall 2013	2901	33	490	132.82	0.14	10.53	0.11
311-1V6-8tap	111Fall 2014	3079	37	359	145.44	0.16	12.79	0.13
311-2V6-8tap	122Fall 2014	2768	36	491	132.78	0.12	8.34	0.12
311-4V6-8tap	135Fall 2014	3529	37	359	154.75	0.20	15.20	0.14
311-5V6-8tap	143Fall 2014	3321	36	490	146.81	0.18	13.19	0.14
311-10V6-8tap	154Fall 2014	3272	35	491	144.42	0.17	11.8	0.14
311-11V6-8tap	212Fall 2014	3602	36	491	131.12	0.16	11.32	0.17
105-10V2-40-15		341	38	229	204.30	0.03	2.91	0.01
105-12V2-40-15		272	38	230	171.68	0.02	1.87	0.01
211-1V2-40-15		362	39	229	160.53	0.02	2.23	0.01
211-2V2-40-15		201	43	354	194.98	0.02	1.58	0.01
	223Fall 2014	869	37	491	135.90	0.04	1.61	0.09
	235Fall 2014	1249	37	490	143.20	0.05	1.31	0.16
	241Fall 2014	1639	33	491	153.40	0.07	1.85	0.18
	254Fall 2014	1463	37	491	154.34	0.06	1.40	0.14
	311Fall 2014	1465	35	490	176.92	0.08	1.88	0.17
	325Fall 2014	1649	37	263	156.50	0.08	1.96	0.16
	334Fall 2014	1585	33	491	143.99	0.07	1.59	0.16
	343Fall 2014	1522	35	491	165.44	0.08	2.13	0.17
	352Fall 2014	1826	37	491	156.18	0.09	2.19	0.17

Table A1. The sequencing by synthesis samples and primers (soybean specific *nodZ* and nitrogen fixation *nifH*, ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB) and denitrifier *nosZ*) for each library along with reads per library, maximum, minimum and average library read length, coverave mean, standard deviation (SD) and man mapping quality (continued).

nodZ/*nifH* primers	AOA/AOB/*nosZ* primers	reads	minimum read length	maximum read legnth	average read length	coverage mean	coverage SD	mean mapping quality
	514Fall 2014	1464	36	491	142.38	0.06	1.31	0.15
	521Fall 2014	1452	37	491	139.60	0.06	1.37	0.15
	533Fall 2014	1750	36	491	147.39	0.07	1.67	0.17
311-10V2-40-15		314	44	260	159.26	0.02	1.89	0.01
311-11V2-40-15		351	38	359	166.27	0.02	2.25	0.01
105-1V2-415-30		93	38	332	51.80	0.00	0.02	0.00
105-3V2-415-30		9	47	359	88.40	0.00	0.01	0.00
104-5pre0-15	545Fall 2014	2725	36	355	132.22	0.11	5.41	0.18
104-6pre0-15	552Fall 2014	2207	37	490	154.71	0.12	8.16	0.17
104-8pre0-15	712Fall 2014	2319	36	359	144.91	0.11	7.10	0.13
104-9pre0-15	721Fall 2014	2011	37	491	164.30	0.12	6.32	0.13
104-11pre0-15	735Fall 2014	2526	37	256	158.97	0.13	7.69	0.15
104-12pre0-15	744Fall 2014	1259	31	491	124.40	0.05	1.46	0.12
105-1pre0-15	753Fall 2014	2533	36	491	161.21	0.14	7.92	0.20
105-3pre0-15	813Fall 2014	2387	37	490	137.32	0.11	6.01	0.14
105-4pre0-15	821Fall 2014	1994	38	489	163.89	0.12	7.82	0.12
105-6pre0-15	835Fall 2014	2198	37	491	166.41	0.14	8.47	0.13
105-10pre0-15	842Fall 2014	2387	37	491	155.74	0.14	8.50	0.14
105-12pre0-15	854Fall 2014	2403	37	491	124.92	0.09	4.11	0.16
211-4V2-40-15		357	38	253	198.64	0.03	2.95	0.01
211-5V2-40-15		279	43	229	152.63	0.02	1.54	0.01

Table A1. The sequencing by synthesis samples and primers (soybean specific *nodZ* and nitrogen fixation *nifH*, ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB) and denitrifier *nosZ*) for each library along with reads per library, maximum, minimum and average library read length, coverave mean, standard deviation (SD) and man mapping quality (continued).

nodZ/*nifH* primers	AOA/AOB/*nosZ* primers	reads	minimum read length	maximum read legnth	average read length	coverage mean	coverage SD	mean mapping quality
211-10V2-40-15		626	38	359	203.20	0.05	5.30	0.01
211-11V2-40-15		603	38	229	167.89	0.04	2.93	0.01
109-1pre0-15	111cFall 2015	2132	37	491	141.31	0.11	7.11	0.15
109-3pre0-15	111wFall 2015	2720	37	491	154.47	0.16	11.62	0.13
109-7pre0-15	122wFall 2015	2701	37	490	155.17	0.16	12.12	0.14
109-9pre0-15	135cFall 2015	2187	36	491	140.53	0.12	9.44	0.11
109-10pre0-15	135wFall 2015	1858	37	490	150.47	0.11	7.91	0.13
109-12pre0-15	143wFall 2015	2430	35	491	123.50	0.09	5.51	0.14
111-1pre0-15	154cFall 2015	1912	37	491	134.89	0.09	6.44	0.14
111-2pre0-15	154wFall 2015	1080	37	256	142.69	0.06	5.62	0.03
111-7pre0-15	212wFall 2015	2435	36	491	142.51	0.13	7.86	0.16
111-8pre0-15	223wFall 2015	2426	36	491	130.87	0.11	7.57	0.15
111-10pre0-15	235cFall 2015	2041	33	491	139.25	0.10	5.80	0.15
111-11pre0-15	235wFall 2015	2395	36	260	140.95	0.12	8.11	0.11
105-4V2-415-30		248	38	230	85.93	0.01	0.52	0.01
105-6V2-415-30		56	38	61	53.95	0.00	0.06	0.00
105-10V2-415-30		515	38	229	94.62	0.02	1.32	0.01
105-12V2-415-30		385	38	230	62.47	0.01	0.44	0.01
205-4pre0-15	241cFall 2015	3191	35	491	129.91	0.15	9.94	0.18
205-6pre0-15	241wFall 2015	3213	37	491	141.19	0.17	11.01	0.15
205-7pre0-15	254cFall 2015	4136	37	491	123.27	0.17	12.19	0.18

Table A1. The sequencing by synthesis samples and primers (soybean specific *nodZ* and nitrogen fixation *nifH*, ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB) and denitrifier *nosZ*) for each library along with reads per library, maximum, minimum and average library read length, coverave mean, standard deviation (SD) and man mapping quality (continued).

nodZ/*nifH* primers	AOA/AOB/*nosZ* primers	reads	minimum read length	maximum read legnth	average read length	coverage mean	coverage SD	mean mapping quality
205-9pre0-15	254wFall 2015	1847	36	491	112.94	0.06	2.77	0.16
205-10pre0-15	311cFall 2015	1355	37	249	151.57	0.08	5.85	0.09
205-12pre0-15	311wFall 2015	2507	33	491	132.86	0.11	6.61	0.13
206-5pre0-15	325cFall 2015	3266	36	491	154.73	0.19	14.30	0.12
206-6pre0-15	325wFall 2015	2140	38	359	181.93	0.16	15.84	0.03
206-8pre0-15	334cFall 2015	3331	37	491	116.38	0.13	7.02	0.19
206-9pre0-15	334wFall 2015	2730	36	491	109.49	0.10	5.24	0.15
206-11pre0-15	343wFall 2015	3225	36	491	134.45	0.16	10.46	0.15
206-12pre0-15	352wFall 2015	3734	36	491	172.40	0.25	19.63	0.18
211-1V2-415-30		423	38	229	61.21	0.01	0.48	0.01
211-2V2-415-30		265	38	132	54.82	0.00	0.29	0.00
211-4V2-415-30		512	38	230	58.82	0.01	0.55	0.01
211-5V2-415-30		784	38	281	69.24	0.01	1.06	0.01
208-4pre0-15	514cFall 2015	2243	36	491	139.26	0.11	8.09	0.15
208-6pre0-15	514wFall 2015	2062	37	491	118.67	0.08	5.67	0.12
208-7pre0-15	521cFall 2015	1679	37	491	108.26	0.06	2.48	0.11
208-9pre0-15	521wFall 2015	1437	37	492	138.99	0.07	4.81	0.12
208-10pre0-15	533wFall 2015	1252	36	491	130.63	0.06	3.09	0.11
208-12pre0-15	545cFall 2015	1579	35	491	146.68	0.09	4.90	0.14
211-1pre0-15	545wFall 2015	1631	37	491	128.02	0.07	5.19	0.12
211-2pre0-15	552wFall 2015	1424	37	491	109.97	0.05	2.17	0.16

Table A1. The sequencing by synthesis samples and primers (soybean specific *nodZ* and nitrogen fixation *nifH*, ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB) and denitrifier *nosZ*) for each library along with reads per library, maximum, minimum and average library read length, coverave mean, standard deviation (SD) and man mapping quality (continued).

nodZ/*nifH* primers	AOA/AOB/*nosZ* primers	reads	minimum read length	maximum read legnth	average read length	coverage mean	coverage SD	mean mapping quality
211-4pre0-15	712wFall 2015	1611	37	491	92.74	0.04	1.84	0.11
211-5pre0-15	721cFall 2015	2019	37	256	118.64	0.08	5.41	0.13
211-10pre0-15	721wFall 2015	2368	35	490	107.86	0.08	4.92	0.14
211-11pre0-15	735cFall 2015	2048	37	491	118.66	0.08	4.91	0.13
311-4V2-415-30		154	44	91	54.69	0.00	0.16	0.00
311-5V2-415-30		388	42	229	121.90	0.02	1.56	0.01
311-10V2-415-30		546	43	229	77.60	0.01	0.96	0.01
311-11V2-415-30		568	43	229	69.360	0.01	0.78	0.01
301-4pre0-15	735wFall 2015	2198	37	491	156.51	0.13	9.14	0.12
301-5pre0-15	744cFall 2015	1601	37	491	144.60	0.09	6.26	0.09
301-7pre0-15	744wFall 2015	2322	37	491	138.67	0.12	8.24	0.11
301-8pre0-15	753wFall 2015	1822	37	491	141.50	0.10	6.88	0.15
301-10pre0-15	813wFall 2015	1088	38	491	158.05	0.06	4.41	0.08
301-11pre0-15	821cFall 2015	1490	37	491	109.23	0.05	1.80	0.12
302-5pre0-15	821wFall 2015	2054	37	491	133.28	0.10	6.69	0.11
302-6pre0-15	835cFall 2015	2020	37	490	123.76	0.09	5.94	0.11
302-8pre0-15	835wFall 2015	2409	30	491	136.61	0.12	7.10	0.13
302-9pre0-15	842wFall 2015	2183	37	491	125.42	0.10	6.81	0.13
302-11pre0-15	854cFall 2015	2661	36	491	120.28	0.11	7.49	0.14
302-12pre0-15	854wFall 2015	1933	33	491	145.35	0.10	7.22	0.15
211-10V2-415-30		438	38	229	61.61	0.01	0.42	0.01

Table A1. The sequencing by synthesis samples and primers (soybean specific *nodZ* and nitrogen fixation *nifH*, ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB) and denitrifier *nosZ*) for each library along with reads per library, maximum, minimum and average library read length, coverave mean, standard deviation (SD) and man mapping quality (continued).

nodZ/*nifH* primers	AOA/AOB/*nosZ* primers	reads	minimum read length	maximum read legnth	average read length	coverage mean	coverage SD	mean mapping quality
211-11V2-415-30		261	44	229	58.85	0.00	0.29	0.01
311-1V2-415-30		21	38	175	69.81	0.00	0.01	0.00
311-2V2-415-30		15	38	77	50.13	0.00	0.01	0.00
304-5pre0-15		1831	38	230	201.08	0.16	15.54	0.01
304-6pre0-15		1534	42	229	176.11	0.11	10.90	0.01
304-8pre0-15		1159	43	230	148.80	0.07	6.40	0.01
304-9pre0-15		1236	38	230	191.85	0.1	9.82	0.01
304-11pre0-15		780	38	281	168.96	0.05	5.21	0.01
304-12pre0-15		1256	36	230	142.33	0.07	6.57	0.01
311-1pre0-15		924	37	230	103.85	0.03	2.55	0.01
311-2pre0-15		696	38	230	142.93	0.04	3.64	0.01
311-4pre0-15		592	38	251	111.44	0.02	2.05	0.01
311-5pre0-15		764	38	260	137.21	0.04	3.71	0.01
311-10pre0-15		1216	36	230	134.72	0.06	5.73	0.01
311-11pre0-15		732	38	351	170.23	0.05	4.88	0.01
104-5pre15-30		169	38	281	134.40	0.01	0.78	0.01
104-6pre15-30		358	38	229	203.92	0.03	3.10	0.01
104-8pre15-30		667	38	355	191.06	0.05	5.20	0.01
104-9pre15-30		291	38	229	132.35	0.01	1.32	0.01
104-11pre15-30		245	41	229	165.00	0.02	1.57	0.01
104-12pre15-30		191	38	281	127.66	0.01	0.81	0.01

Table A1. The sequencing by synthesis samples and primers (soybean specific *nodZ* and nitrogen fixation *nifH*, ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB) and denitrifier *nosZ*) for each library along with reads per library, maximum, minimum and average library read length, coverave mean, standard deviation (SD) and man mapping quality (continued).

nodZ/*nifH* primers	AOA/AOB/*nosZ* primers	reads	minimum read length	maximum read legnth	average read length	coverage mean	coverage SD	mean mapping quality
105-1pre15-30		211	43	229	103.90	0.01	0.64	0.01
105-3pre15-30		207	44	229	134.55	0.01	0.98	0.01
105-4pre15-30		161	38	229	65.38	0.00	0.20	0.01
105-6pre15-30		180	38	229	62.50	0.00	0.22	0.01
105-10pre15-30		417	42	286	57.25	0.01	0.44	0.01
105-12pre15-30		277	38	229	58.15	0.00	0.31	0.01
109-1pre15-30		388	38	359	153.22	0.02	2.22	0.01
109-3pre15-30		330	38	229	177.90	0.02	2.31	0.01
109-7pre15-30		1263	38	280	203.51	0.11	10.88	0.01
109-9pre15-30		336	38	229	166.96	0.02	2.21	0.01
109-10pre15-30		248	43	253	185.16	0.02	1.87	0.01
109-12pre15-30		274	38	230	142.74	0.02	1.41	0.01
111-1pre15-30		225	43	229	148.38	0.01	1.24	0.01
111-2pre15-30		206	44	256	177.48	0.02	1.39	0.01
111-7pre15-30		344	38	281	181.42	0.03	2.48	0.01
111-8pre15-30		181	43	281	89.43	0.01	0.40	0.01
111-10pre15-30		483	38	293	62.41	0.01	0.54	0.01
111-11pre15-30		203	38	229	138.37	0.01	0.99	0.01
205-4pre15-30		85	38	229	120.31	0.00	0.33	0.01
205-6pre15-30		185	43	280	181.29	0.01	1.35	0.01
205-7pre15-30		213	43	230	196.76	0.02	1.55	0.01

Table A1. The sequencing by synthesis samples and primers (soybean specific *nodZ* and nitrogen fixation *nifH*, ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB) and denitrifier *nosZ*) for each library along with reads per library, maximum, minimum and average library read length, coverave mean, standard deviation (SD) and man mapping quality (continued).

nodZ/*nifH* primers	AOA/AOB/*nosZ* primers	reads	minimum read length	maximum read legnth	average read length	coverage mean	coverage SD	mean mapping quality
205-9pre15-30		151	44	229	107.97	0.01	0.49	0.01
205-10pre15-30		100	44	281	161.92	0.01	0.54	0.01
205-12pre15-30		185	38	276	160.96	0.01	1.13	0.01
206-5pre15-30		1094	38	260	218.30	0.01	10.35	0.01
206-6pre15-30		301	38	260	202.79	0.03	2.56	0.01
206-8pre15-30		377	38	281	179.31	0.02	2.70	0.01
206-9pre15-30		286	38	229	118.10	0.01	1.02	0.01
206-11pre15-30		591	44	281	179.80	0.04	4.28	0.01
206-12pre15-30		243	44	231	161.76	0.02	1.49	0.01
208-4pre15-30		265	43	281	189.95	0.02	2.07	0.01
208-6pre15-30		254	38	229	198.94	0.02	2.09	0.01
208-7pre15-30		214	44	229	129.99	0.01	0.94	0.01
208-9pre15-30		209	38	231	133.27	0.01	0.96	0.01
208-10pre15-30		195	38	260	123.48	0.01	0.78	0.01
208-12pre15-30		158	44	359	119.85	0.01	0.57	0.01
211-1pre15-30		154	38	229	90.08	0.00	0.35	0.01
211-2pre15-30		109	43	229	79.34	0.00	0.19	0.01
211-4pre15-30		148	38	229	120.58	0.01	0.57	0.01
211-5pre15-30		260	38	280	144.08	0.01	1.34	0.01
211-10pre15-30		256	38	229	79.40	0.01	0.44	0.01
211-11pre15-30		141	43	229	118.90	0.01	0.55	0.01

Table A1. The sequencing by synthesis samples and primers (soybean specific *nodZ* and nitrogen fixation *nifH*, ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB) and denitrifier *nosZ*) for each library along with reads per library, maximum, minimum and average library read length, coverave mean, standard deviation (SD) and man mapping quality (continued).

nodZ/*nifH* primers	AOA/AOB/*nosZ* primers	reads	minimum read length	maximum read legnth	average read length	coverage mean	coverage SD	mean mapping quality
301-4pre15-30		93	38	229	86.73	0.00	0.21	0.01
301-5pre15-30		370	38	230	208.56	0.03	3.28	0.01
301-7pre15-30		227	44	281	160.53	0.01	1.38	0.01
301-8pre15-30		156	44	281	164.76	0.01	0.96	0.01
301-10pre15-30		150	38	230	135.65	0.01	0.64	0.01
301-11pre15-30		128	38	281	136.16	0.01	0.60	0.01
302-5pre15-30		197	38	358	184.63	0.01	1.43	0.01
302-6pre15-30		113	44	229	141.55	0.01	0.57	0.01
302-8pre15-30		148	38	229	138.04	0.01	0.70	0.01
302-9pre15-30		323	38	240	178.89	0.02	2.26	0.01
302-11pre15-30		234	38	229	161.53	0.02	1.44	0.01
302-12pre15-30		237	37	281	155.04	0.01	1.33	0.01
304-5pre15-30		103	43	229	66.49	0.00	0.14	0.01
304-6pre15-30		79	38	229	133.92	0.00	0.37	0.01
304-8pre15-30		95	43	229	130.39	0.00	0.40	0.01
304-9pre15-30		161	38	229	152.59	0.01	0.92	0.01
304-11pre15-30		179	43	229	143.61	0.01	0.84	0.01
304-12pre15-30		199	38	229	158.52	0.01	1.18	0.01
311-1pre15-30		120	38	229	90.76	0.00	0.27	0.01
311-2pre15-30		101	44	229	74.88	0.00	0.16	0.01
311-4pre15-30		106	43	229	71.60	0.00	0.17	0.01

Table A1. The sequencing by synthesis samples and primers (soybean specific *nodZ* and nitrogen fixation *nifH*, ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB) and denitrifier *nosZ*) for each library along with reads per library, maximum, minimum and average library read length, coverave mean, standard deviation (SD) and man mapping quality (continued).

nodZ/*nifH* primers	AOA/AOB/*nosZ* primers	reads	minimum read length	maximum read legnth	average read length	coverage mean	coverage SD	mean mapping quality
311-5pre15-30		61	38	229	69.56	0.00	0.09	0.01
311-10pre15-30		146	38	229	55.92	0.00	0.16	0.00
311-11pre15-30		103	43	288	71.51	0.00	0.13	0.01
soybean inoculant		2585	38	359	234.56	0.27	24.46	0.01
pea inoculant		108	38	359	124.31	0.00	0.31	0.01

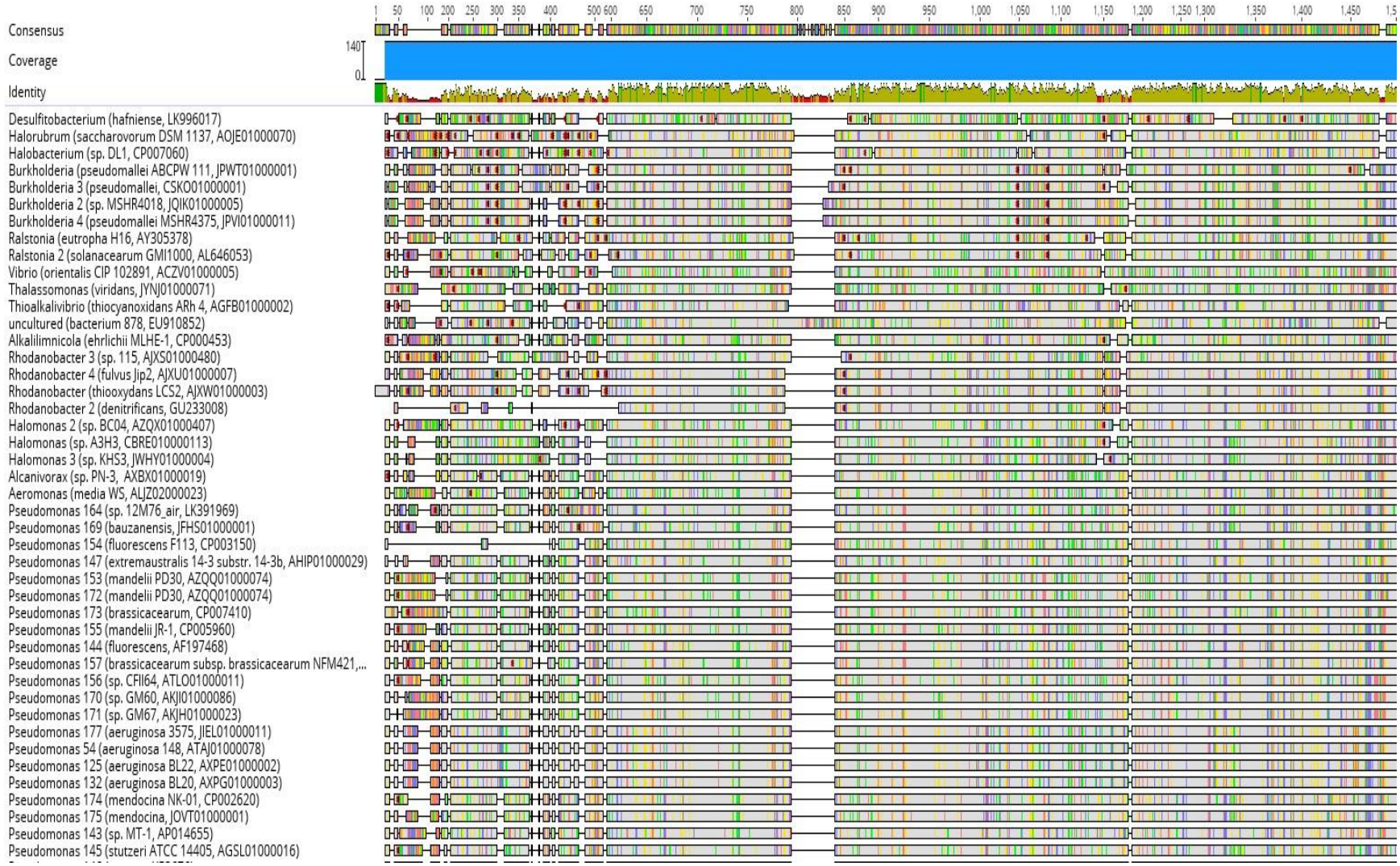

Figure A1. Multiple alignment of sequences identified for *nosZ* gene from sequencing by synthesis.

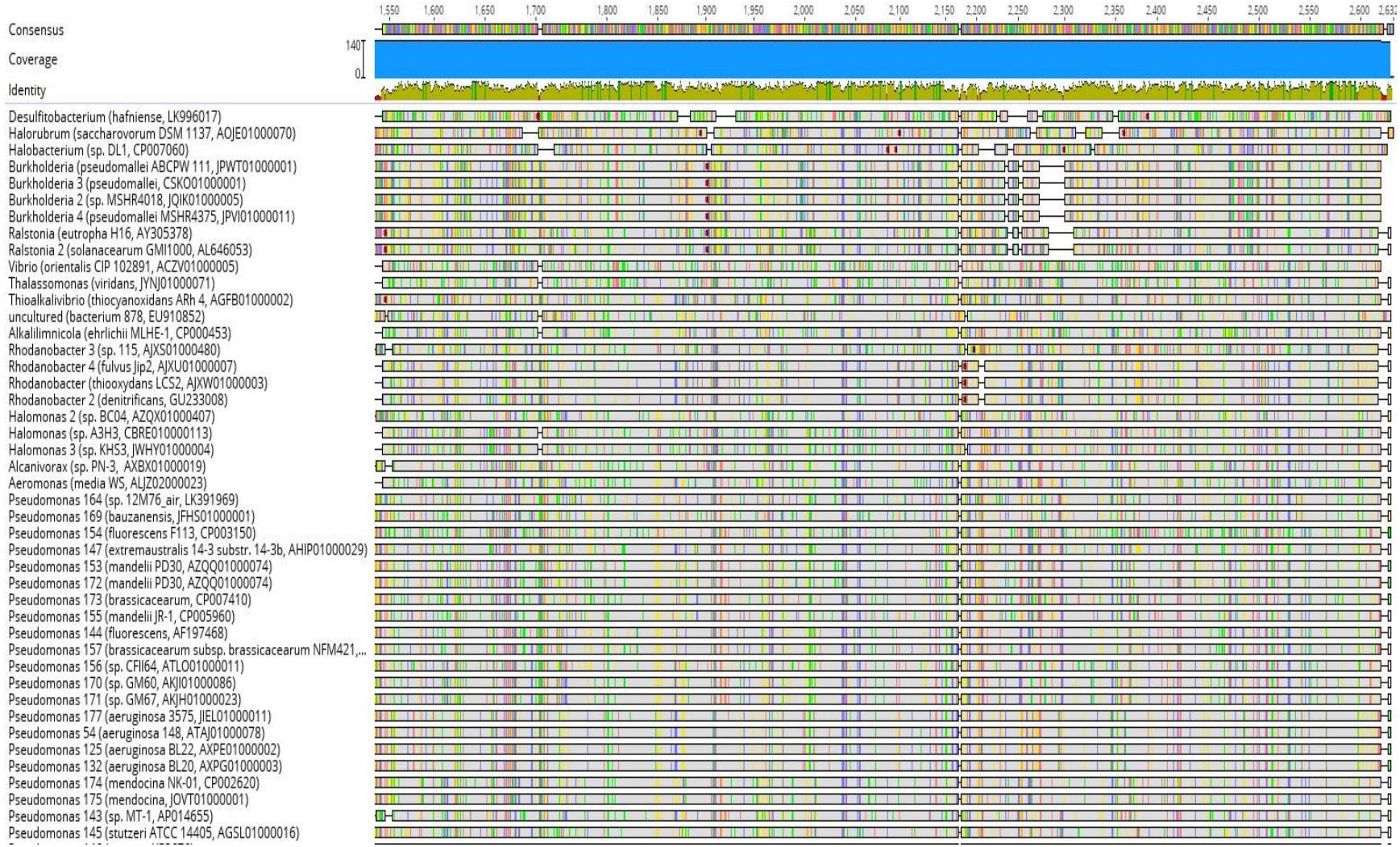

Figure A1. Multiple alignment of sequences identified for *nosZ* gene from sequencing by synthesis (continued).

Figure A1. Multiple alignment of sequences identified for *nosZ* gene from sequencing by synthesis (continued).

Figure A1. Multiple alignment of sequences identified for *nosZ* gene from sequencing by synthesis (continued).

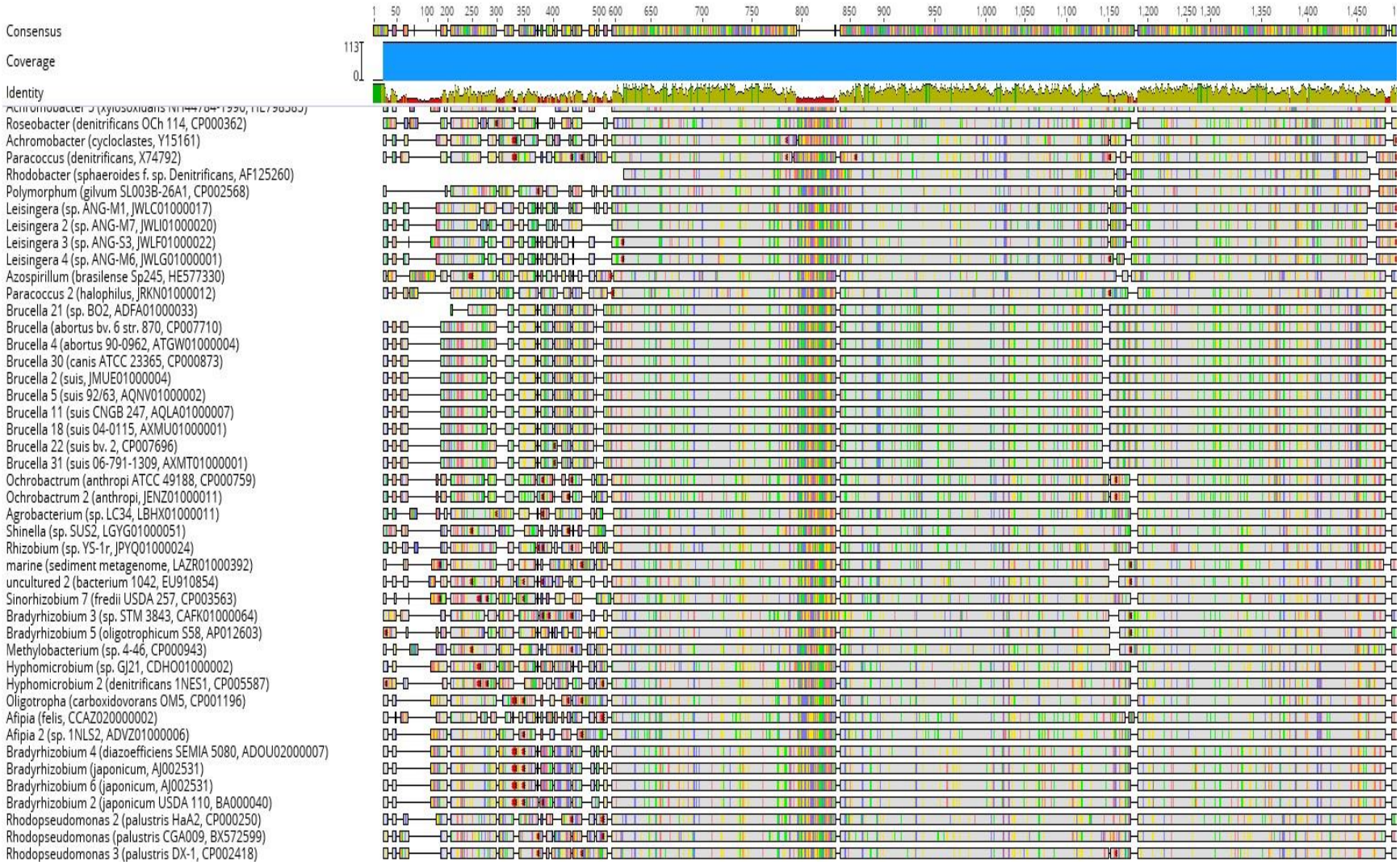

Figure A1. Multiple alignment of sequences identified for *nosZ* gene from sequencing by synthesis (continued).

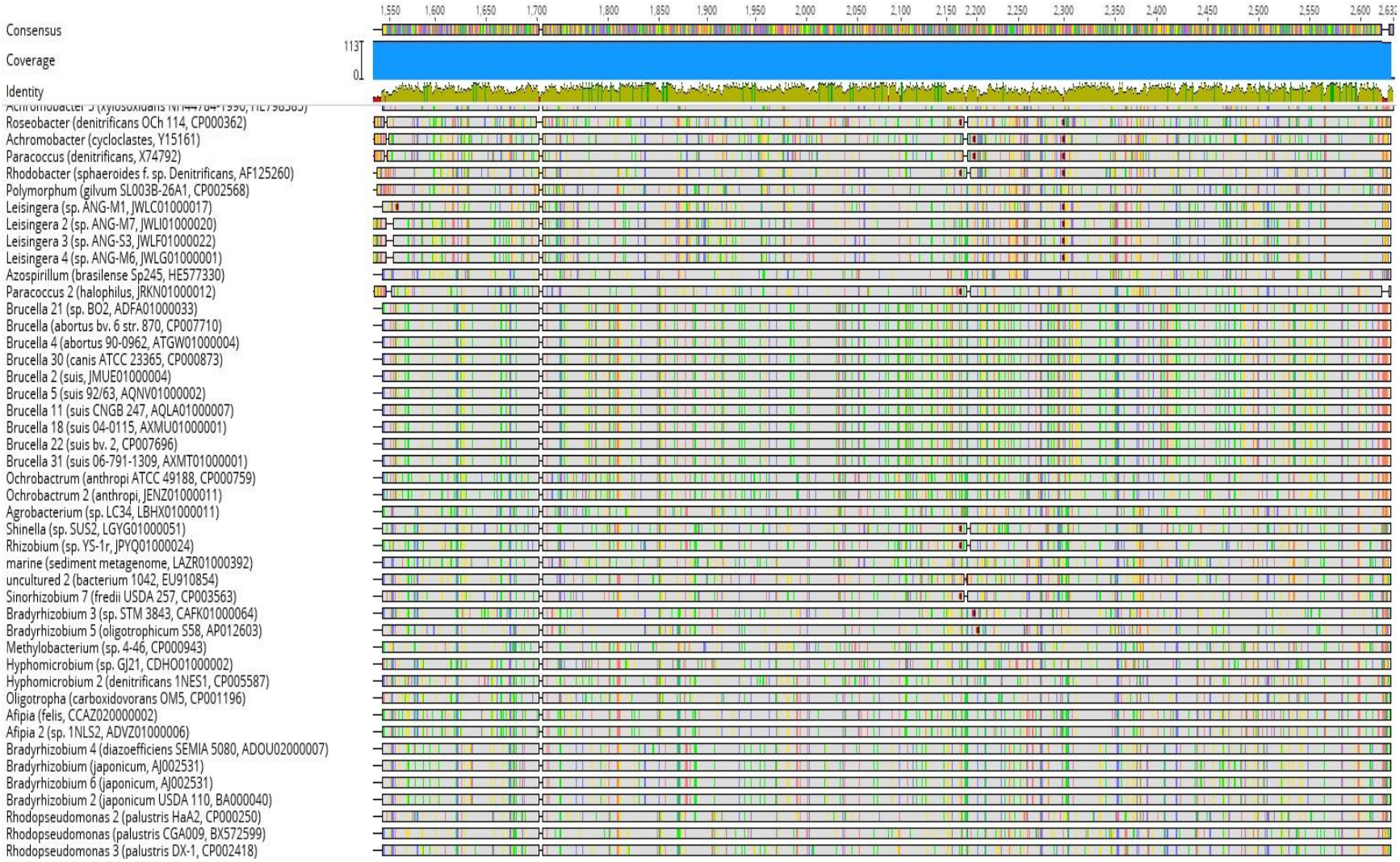

Figure A1. Multiple alignment of sequences identified for *nosZ* gene from sequencing by synthesis (continued).

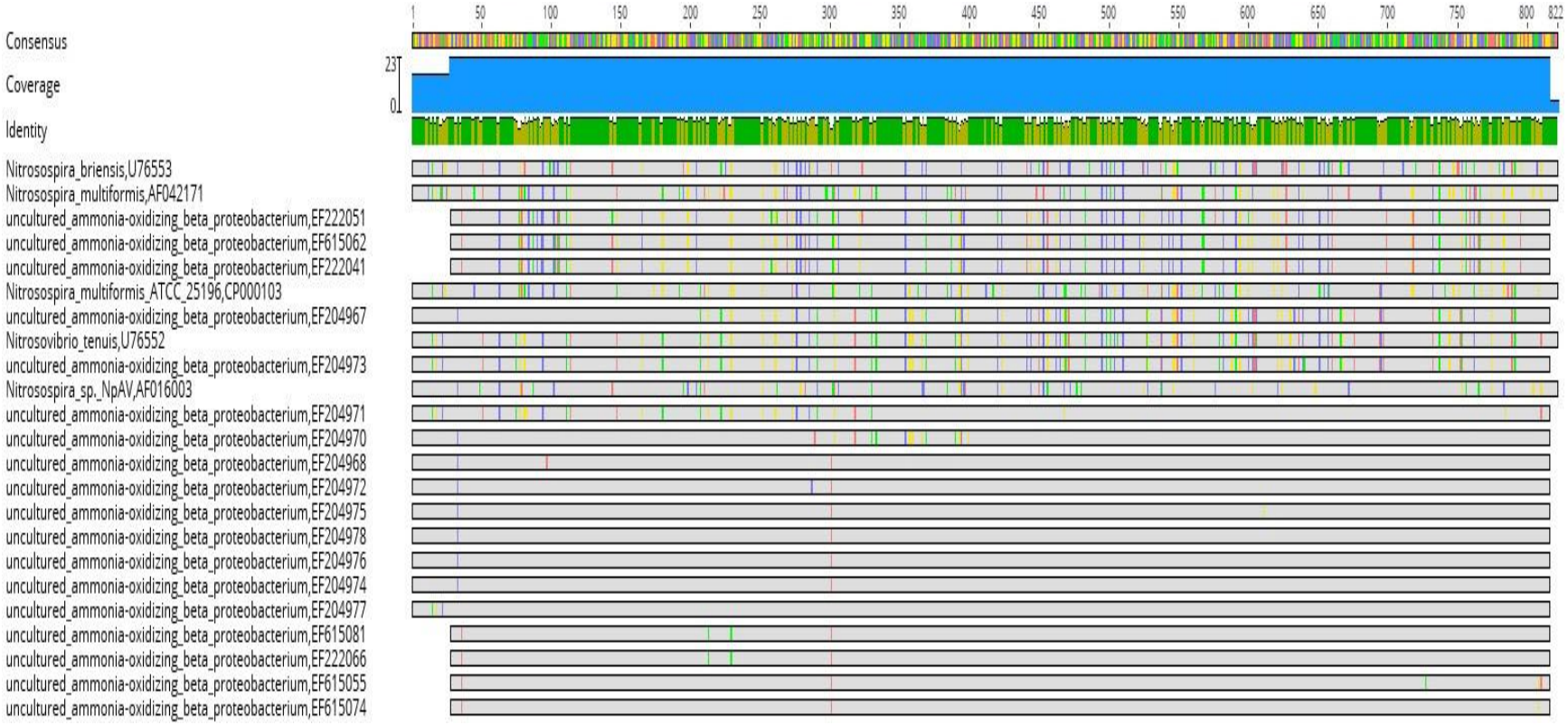

Figure A2. Multiple alignment of sequences identified for ammonia oxidizing bacteria from sequencing by synthesis.

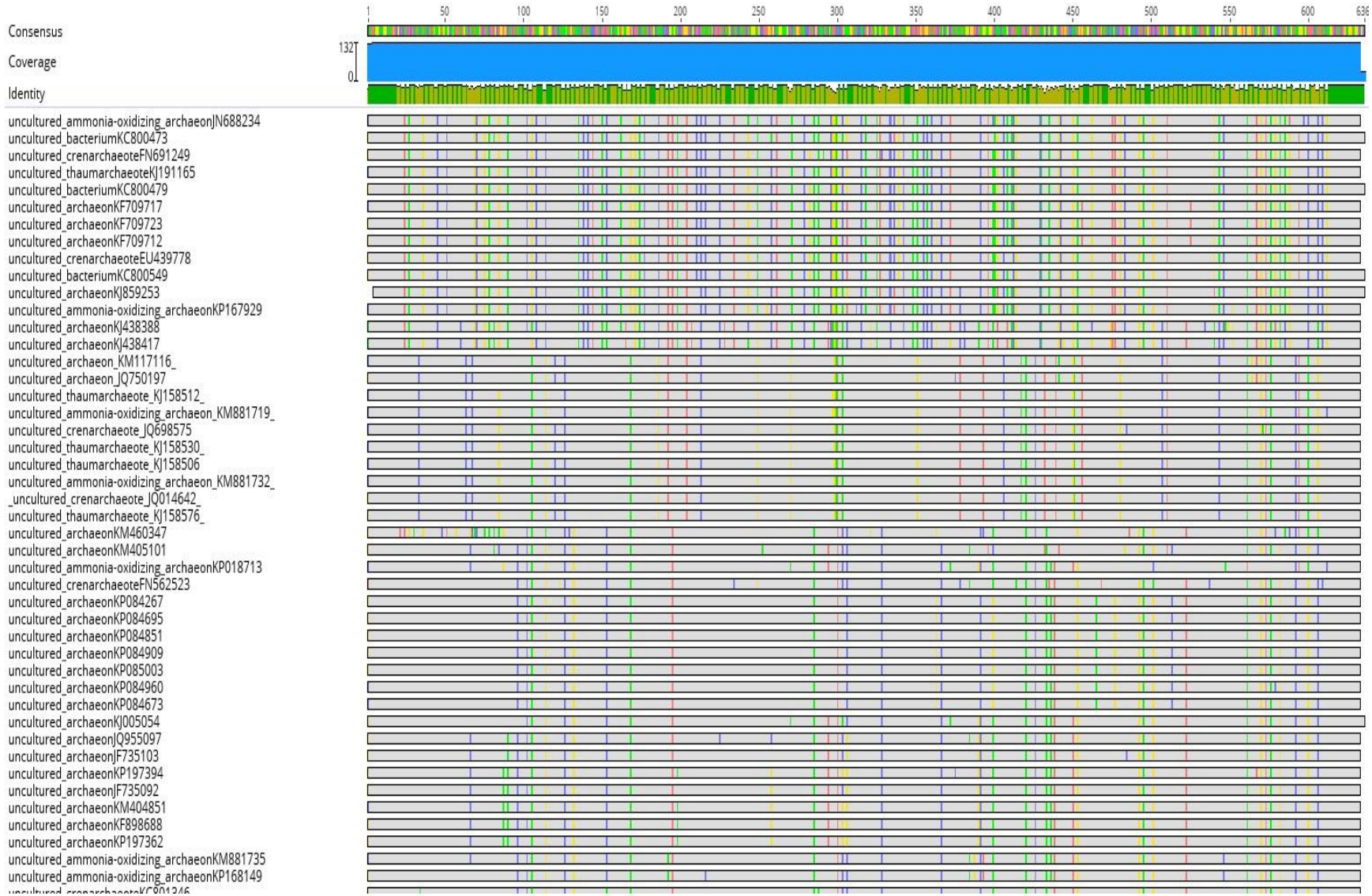

Figure A3. Multiple alignment of sequences identified for ammonia oxidizing archaea from sequencing by synthesis.

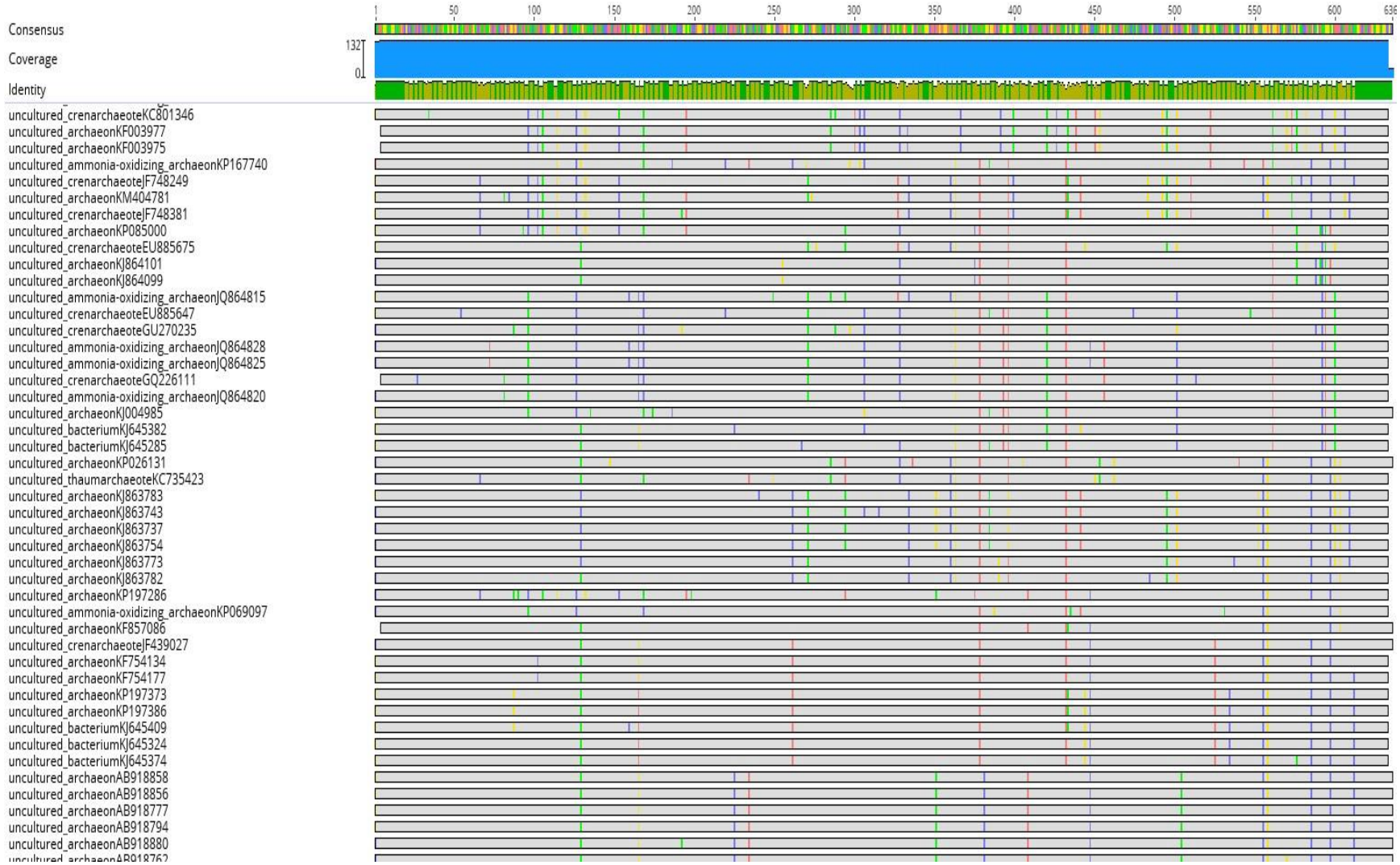

Figure A3. Multiple alignment of sequences identified for ammonia oxidizing archaea from sequencing by synthesis (continued).

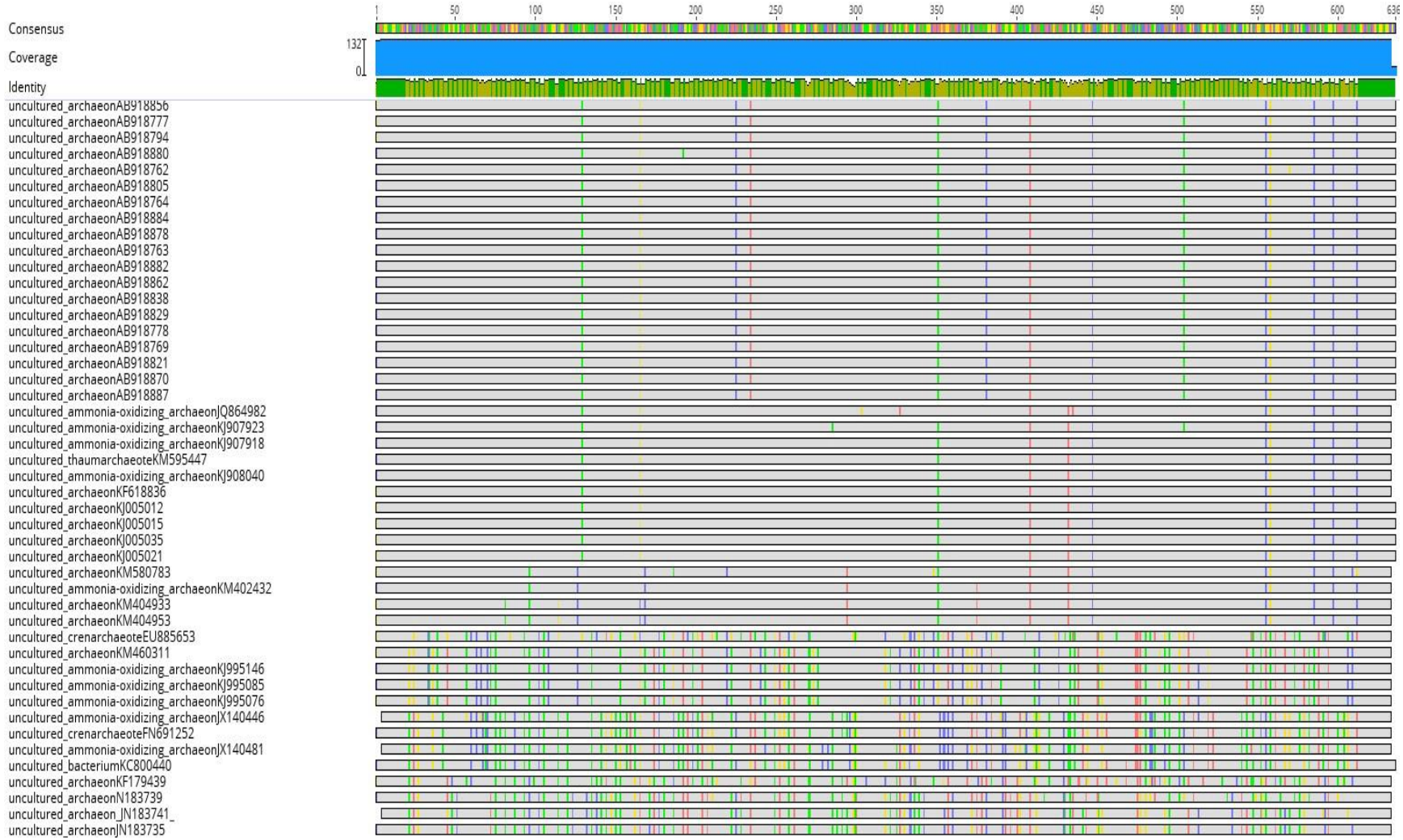

Figure A3. Multiple alignment of sequences identified for ammonia oxidizing archaea from sequencing by synthesis (continued).

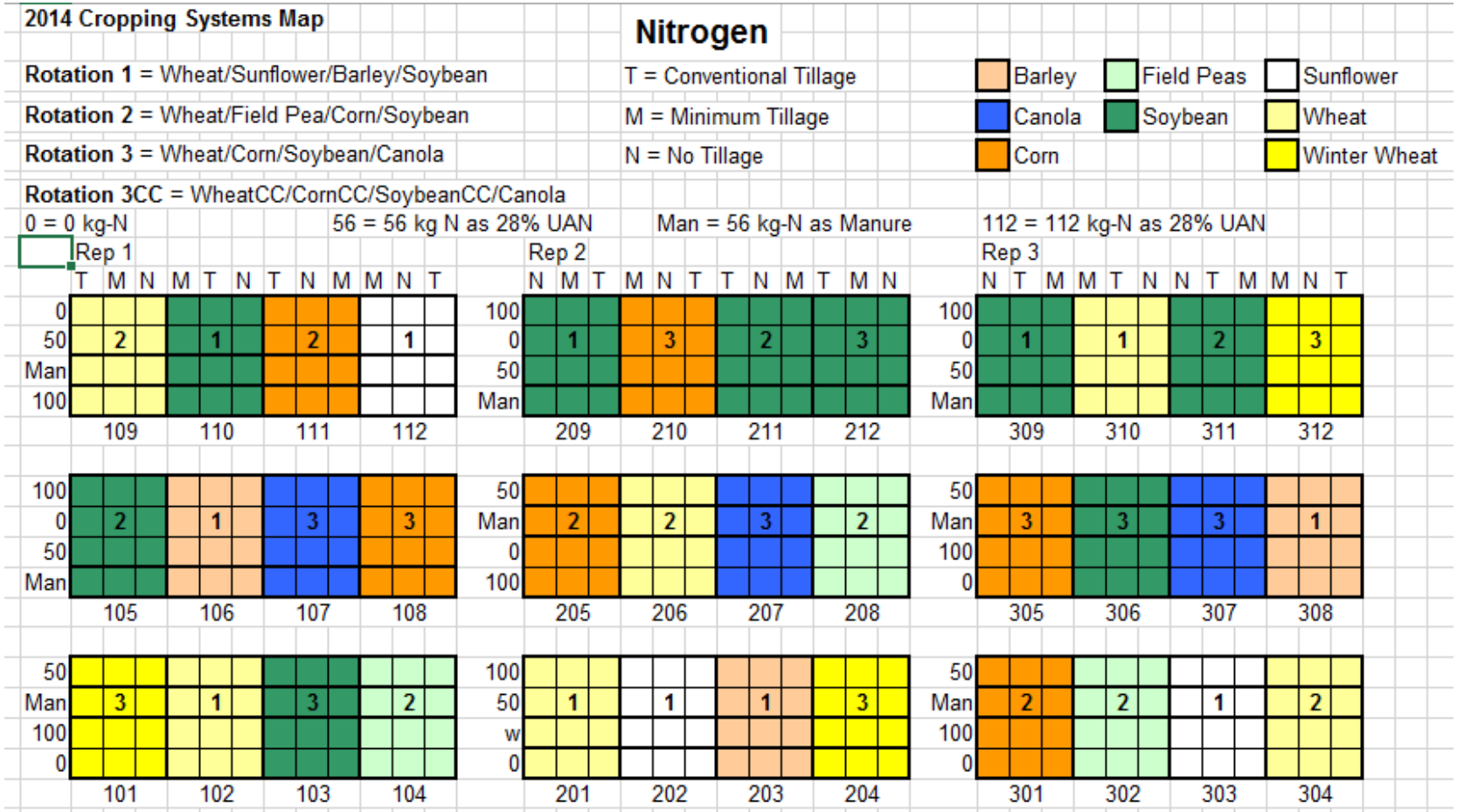

Figure A4. Map of the plots sampled in Carrington, ND. Soil was sampled from 0-15 and 15-30 cm from rotation 2 receiving the following nitrogen fertilizer rates as kg-N ha^{-1}: 0, 112 and 56 manure and under conventional and no-till.

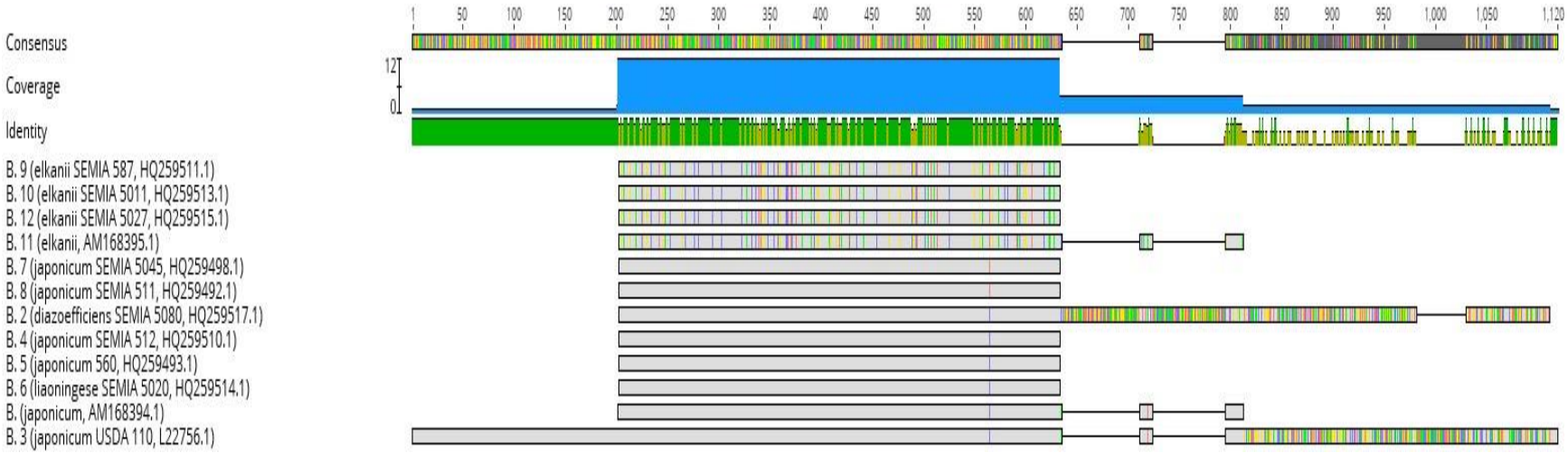

Figure A5. Multiple alignment of sequences identified for soybean specific *Bradyrhizobium* using *nodZ* gene from sequencing by synthesis.