SPATIO-TEMPORAL VARIATION OF DENITRIFICATION DRIVERS

By

LIA SHREWSBURY

A thesis submitted in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE IN SOIL SCIENCE

WASHINGTON STATE UNIVERSITY
Department of Crop and Soil Science

MAY 2014

To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of LIA SHREWSBURY find it satisfactory and recommend that it be accepted.

(Jeff Smith, Ph.D., Honorary Chair)

_____

Dave Huggins, Ph.D., Chair

_____

Kate Reardon, Ph.D.

_____

Lynne Carpenter-Boggs, Ph.D.

ACKNOWLEDGEMENT

I would like to acknowledge the Honorary Chair of my committee, Dr. Jeff Smith, who passed away before completion of this thesis. He was instrumental in the planning, design and implementation of my research. He was an exceptional mentor and I feel blessed to have had his expertise and insight. I would also like to recognize the rest of my committee for their guidance as well as their willingness to step forward in involvement after Dr. Smith's passing. In addition, I would like to acknowledge that funding for this study was provided by a USDA/NIFA grant for the Site-Specific Climate Friendly Farming project.

SPATIO-TEMPORAL VARIATION OF DENITRIFICATION DRIVERS

Abstract

By Lia Shrewsbury, M.S.
Washington State University
May 2014

Chair: Dave Huggins

Nitrous oxide is a potent greenhouse gas. Approximately 80% of current U.S. emissions of nitrous oxide come from soil and agriculture, in particular from the soil microbial processes of denitrification and nitrification. A thorough understanding of denitrification drivers is necessary to accurately predict nitrous oxide emissions from a particular agroecological landscape. However, current predictive models of denitrification do not consider spatio-temporal variation of drivers, nor the size and structure of the denitrifier microbial population. This study identifies the environmental and biological drivers of denitrification at different topographical positions (summit, backslope and footslope) and seasons (autumn, winter, spring and summer) within an agricultural field. In addition, the spatio-temporal variation of denitrifier and nitrifier microorganism abundance in soil was evaluated. Soil environmental measurements of soil water content, nitrate (NO_3-N) concentration, ammonium (NH_4-N) concentration, soluble total nitrogen, soluble non-purgeable organic carbon, pH, electrical conductivity, total carbon and nitrogen, mineral fraction carbon and nitrogen, and particulate organic matter carbon and nitrogen were measured. The abundance of nitrate reductase (*nirK*) and bacterial and archaeal ammonia monooxygenase (*amoA*) gene copies in soil were determined by quantitative PCR and community structure of the same populations was determined by T-RFLP. A short-term assessment of potential denitrification and basal denitrification in soils were performed using the acetylene inhibition method. A multivariate stepwise regression analysis of potential and basal

denitrification was performed using the soil environmental and biological measurements as possible explanatory variables. The predictive power of both potential and basal denitrification models was at times improved when spatio-temporal variation was considered. For potential denitrification, the overall model R-square was 0.69, while the model R-square for separate topographical positions and seasons ranged from 0.26 to 0.84. The predictive power of the potential denitrification model was improved further at times when *nirK* abundance was considered. In addition, it was found that the abundance of denitrifier and nitrifier microorganisms varies across the landscape over time. This study demonstrates the need to account for spatio-temporal variation when modeling and predicting denitrification rates in order to improve estimates of denitrification rates and thus more accurately prediction soil nitrous oxide emissions.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iii

ABSTRACT iv

LIST OF TABLES ix

LIST OF FIGURES x

SPATIO-TEMPORAL VARIATION OF SOIL DENITRIFICATION AND ITS ENVIRONMENTAL DRIVERS

ABSTRACT 1

INTRODUCTION 2

Importance of nitrous oxide and its sources 2

Predicting and modeling denitrification 3

METHODS 5

Description of study site 5

Soil sampling 6

Soil analyses 7

Short-term denitrification incubations 8

Statistical analysis 9

RESULTS 11

Spatial and temporal variation of soil characteristics 11

Spatial and temporal variation of denitrification 15

Identifying drivers of denitrification incubations 18

DISCUSSION 22

SPATIO-TEMPORAL VARIATION OF DENITRIFIER AND NITRIFIER MICROBIAL COMMUNITIES IN SOIL, AND INFLUENCE ON DENITRIFICATION RATE

ABSTRACT 26

INTRODUCTION 27

Sources of nitrous oxide 27

The study of denitrifier and nitrifier communities..... 28

Relationship of denitrifier population size and structure to denitrification 30

Purpose of current study 33

METHODS 34

Study site and soil sampling 34

Soil characteristics and denitrification 34

Molecular analyses of denitrifiers and nitrifiers 35

a. DNA extraction 35

b. Quantitative PCR 35

c. PCR and terminal restriction fragment length polymorphism analysis 37

Statistical analysis 39

RESULTS 40

Spatial and temporal variation of denitrifier and nitrifier abundance 40

nirK abundance and community structure as predictors of denitrification 43

Potential drivers of denitrifier and nitrifier population size and structure 45

Denitrifier and nitrifier community structure 50

DISCUSSION 54

REFERENCES 58

LIST OF TABLES

1. Soil environmental measurements 13

2. Soil carbon and nitrogen measurements 14

3. Stepwise multivariate regression models of potential denitrification 20

4. Stepwise multivariate regression models of basal denitrification 21

5. Stepwise multivariate regression models of potential denitrification with *nirK* abundance added 44

6. Stepwise multivariate regression models of *nirK* abundance 47

7. Stepwise multivariate regression models of bacterial *amoA* abundance 48

8. Stepwise multivariate regression models of archaeal *amoA* abundance 49

9. Community structure of denitrifier and nitrifier populations 52

LIST OF FIGURES

1. Figure 1; Digital elevation map of study site 7

2. Figure 2; Spatio-temporal variation of denitrification 17

3. Figure 3; Spatio-temporal variation of denitrifier and nitrifier abundance 42

4. Figure 4; Moving window analysis of *nirK*, bacterial *amoA* and archaeal *amoA* percent change in community structure 53

Dedication

This thesis is dedicated in memory of Dr. Jeff Smith

SPATIO-TEMPORAL VARIATION OF SOIL DENITRIFICATION AND ITS ENVIRONMENTAL DRIVERS

ABSTRACT

Nitrous oxide is a gaseous emission from the soil microbial processes of denitrification and nitrification that contributes significantly to climate change. A thorough understanding of denitrification drivers is necessary to accurately predict and estimate nitrous oxide emissions from a landscape. However, current models that have been developed to predict denitrification do not consider both spatial and temporal variation of drivers. This study identifies denitrification drivers at different topographical positions (summit, backslope and footslope) and seasons (autumn, winter, spring and summer) within a field to ascertain whether spatio-temporal variation of denitrification and its drivers exists and to describe this variation in rates and drivers for each topographical position and season. Soil environmental measurements of soil water content, nitrate concentration, ammonium concentration, soluble total nitrogen, soluble non-purgeable organic carbon, pH, electrical conductivity, total carbon and nitrogen, mineral fraction carbon and nitrogen, and particulate organic matter carbon and nitrogen were measured as possible drivers of denitrification. A short-term potential denitrification (denitrification enzyme activity) incubation and a short-term basal denitrification incubation were performed using the acetylene inhibition method. A stepwise multivariate regression analysis of potential and basal denitrification was performed using measured soil characteristics as possible explanatory variables. Soil environmental drivers of both potential and basal denitrification differed among the topographical positions and seasons. The predictive power of the model was at times

improved when spatio-temporal variation was considered, with seasons and topographical positions analyzed separately. For potential denitrification, the model R-square for samples across all seasons and topographical positions was 0.69, but the model R-square for separate topographical positions and seasons ranged from 0.26 to 0.84. For basal denitrification, the model R-square for samples across all seasons and topographical positions was 0.54, but the model R-square for separate topographical positions and seasons ranged from 0.29 to 0.95. This study demonstrates the need to consider spatio-temporal variation when modeling and predicting denitrification rates, as it can improve estimates of denitrification and thus contribute to better predictions of soil nitrous oxide emissions.

INTRODUCTION

Importance of nitrous oxide and its sources

Nitrous oxide (N_2O) is a long-lived, potent greenhouse gas with a global warming potential approximately 300 times that of carbon dioxide over a 100-year horizon (Forster et al, 2007). It accounts for 6% of the total radiative forcing of long-lived greenhouse gases (Forster et al., 2007). N_2O is also the most significant ozone-depleting substance when ozone depletion potential is considered (Ravishankara et al., 2009). N_2O emissions continue to increase linearly each year (Forster et al., 2007). Unlike carbon dioxide, the majority of N_2O emissions are not sourced from industrial or chemical processes. The majority of direct and indirect N_2O emissions in the U.S. come from agricultural soil management of croplands (EPA, 2013). Agricultural management of land is a significant contributor to N_2O emissions because nitrogen (N) fertilizer additions act as substrate for the microbial processes of denitrification and

nitrification (Davidson, 2009). These soil microbial processes emit N_2O as either a by-product or intermediate of the enzymatic pathway, and are considered the two main sources of N_2O emissions from soil. It is important to consider N_2O in climate mitigation strategies, as some management strategies that sequester carbon (C) can still exacerbate climate change due to N_2O emissions (Stöckle et al., 2012). In no-till cropping management, for example, global warming potential has been reported to increase initially despite C sequestration due to increases in N_2O emissions, with reduced global warming potential only achieved in the long term (Six et al., 2004).

Since N_2O emissions from soil are mainly biologically mediated, they are regulated by environmental conditions such as temperature, oxygen availability, soil pH, as well as inorganic N substrate availability. The activity of heterotrophic denitrifying microorganisms, and consequently N_2O emissions, is also increased by the availability of labile organic C (Skiba and Smith, 2000). Studies have indicated that N_2O emissions increase with greater water-filled pore space, or lower oxygen availability (Linn and Doran, 1984; Smith et al., 1998). Denitrification is considered the primary microbial process generating N_2O emissions in anaerobic conditions, since nitrification can not occur in the absence of oxygen. In addition, denitrification increases in anaerobic conditions because denitrifiers are able to utilize oxidized forms of N as an alternative electron acceptor, making denitrification an advantageous energy generating process in the absence of oxygen.

Predicting and modeling denitrification

Because denitrification is affected by soil environmental properties (such as the availability of nitrate and labile C as well as the temperature and oxygen availability of the soil),

several models use easily measurable soil properties to predict denitrification. However, useful soil predictors of denitrification differ among studies. A field-scale study of cultivated soil developed a predictive model for denitrification based solely on soil water content, nitrate availability, and respiration of soil cores (Hénault and Germon, 2000). Another study developed a model to predict denitrification rates of Saskatchewan soils from measurements of water-film thickness, air porosity, organic C, nitrate, and maximum daily ambient temperature (Elliot and Jong, 1993). Further, another model was developed for denitrification utilizing soil moisture content, distributions of aggregate size and oxygen reduction potential, nitrate, and moisture tension (Arah and Smith, 1989). These models overlap in their predictive characteristics, but they also differ greatly. The advantage of these models is that they only require a few soil measurements to predict denitrification rates. However, due to the complex spatio-temporal variability of denitrification (Folorunso and Rolston, 1984; Groffman and Tiedje, 1989; Parsons et al., 1991; Cambardella et al., 1994) these models are limited in their ability to predict denitrification accurately. Differences among studies in the predictive characteristics utilized are perhaps also a result of spatio-temporal variation. It has been demonstrated that drivers of denitrification can shift between seasons. A study of the seasonal variation of denitrification found that nitrate and C were limiting factors in summer, but that lower variation of denitrification in the spring and fall was likely due to more uniform soil structure, C, and aeration observed in those seasons (Groffman and Tiedje, 1989). Considering that spatial variation exists for denitrification drivers, the value of certain soil properties in predicting denitrification would likely change seasonally as well. However, an analysis of spatio-temporal variation of denitrification drivers has not been fully developed. An increased understanding of how the drivers of denitrification change with space and time could assist in creating better

predictive models for denitrification. Predicting denitrification with greater accuracy would lead to better estimation of N_2O emissions and the development of appropriate mitigation strategies. It is the goal of this study to ascertain whether spatial and temporal variation of denitrification drivers exists and, if present, to identify those drivers for different topographical positions and seasons.

METHODS

Description of study site

The study site was located at the Washington State University R.J. Cook Agronomy Farm (CAF) in the dryland Palouse region of Washington State, USA (46°46′44″ N, 117°05′19″ W) . The farm has been utilized for no-tillage cropping systems research since 1999 by the USDA-ARS and Washington State University. Based on 30-year normals (1981-2010) from the nearby PULLMAN 2 NW National Weather Service station (46° 45' 37"N, 117° 11' 10" W), average annual precipitation is 20.4" while the mean annual high temperature is 14.7 °C and the mean annual low temperature is 2.8°C . Soils of the study location were either Palouse silt loam, a fine-silty, mixed, superactive, mesic Pachic Ultic Haploxeroll; or Thatuna silt loam, which is classified as a fine-silty, mixed, superactive, mesic Oxyaquic Argixerolls (USDA, 1978). The field of study is under a winter wheat, spring wheat, spring grain legume rotation that is characteristic of regional dryland agriculture.

The rolling topography of the Palouse provides an ideal location for studying field-scale topographical variation. Estimation of N_2O emissions from this region is currently being developed (Kostyanovsky et al., unpublished results). Studying denitrification and its drivers

across topographical positions can help describe the emissions observed for this region.

Soil sampling

Samples were taken within a field from summit, backslope and footslope topographical positions (Ruhe, 1969) at four separate sampling events. Each topographic position was sampled at six different locations during a sampling event, with the same locations used each time (Figure 1). For each sample, approximately 500 g of soil was collected from a 0 to 5 cm depth using a sharpshooter shovel. Soil was passed through a 4mm sieve and stored without drying in plastic zippered bags at 4°C until analyzed.

Sampling events occurred on November 4, 2012; February 26, 2013; May 6, 2013 and July 29, 2013. These sampling dates were chosen to represent the seasons of autumn, winter, spring and summer, respectively. The summit and backslope are planted to a winter wheat, spring wheat, spring legume rotation; the footslope is planted to a winter wheat, winter wheat, spring legume rotation. All three topographical positions were harvested for winter wheat in summer of 2012. The summit and backslope positions of the field were planted to spring wheat (*Triticum aestivum* **L.** var. "Louise") in early April 2013. The footslope was planted to Clearfield soft winter wheat (*Triticum aestivum* **L.** var. "AP 700") in October 2012. Both winter and spring wheat were fertilized at the time of planting with 135kg N ha^{-1} urea ammonium nitrate that was banded below the seed at a 10 cm depth. The fertilizer placement for all topographical positions was 5 cm below the sampling depth of this study. Harvest occurred after the final soil sampling event.

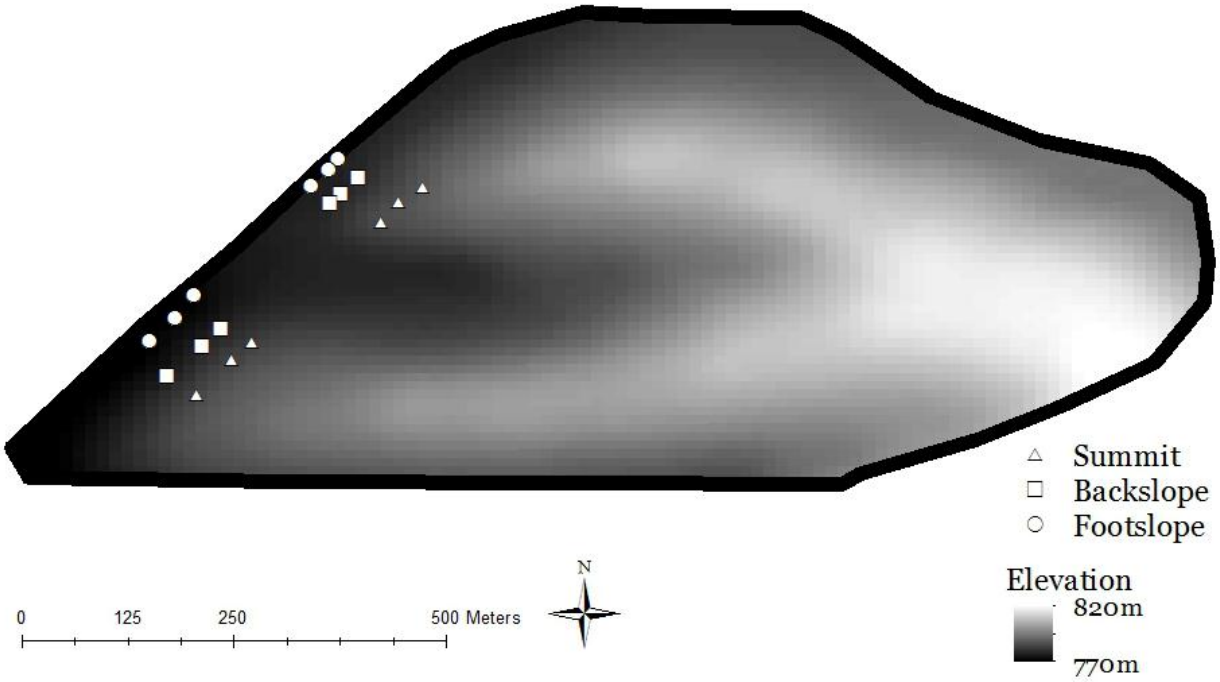

Figure 1. Digital elevation map of the CAF showing study site sampling locations.

Soil analyses

Soil moisture content was determined from an oven-dry (105°C for at least 48 hours) mass of fresh soil samples. Soil pH and electrical conductivity were determined using a Denver Instruments Model 250 pH ISE conductivity meter with a 1:1 soil:water extract (Denver Instruments Company, Denver, Colorado). Nitrate (NO_3-N) and ammonium (NH_4-N) concentrations were determined by 2 M KCl extraction (Keeney and Nelson, 1982). A 6 g moist soil sample was shaken in 25 mL 2 M KCl solution for 30 minutes and then filtered through a Whatman #40 filter. The filtered solution was then analyzed for NO_3-N and NH_4-N concentrations with a Technicon flow-injection auto-analyzer (Lachat Quickchem. FIA 8000 series, Lachat Instruments, Milwuakee, WI). Soil samples were prepared for analysis of soluble non-purgeable organic carbon (NPOC) and total N concentration by diluting in 3 volumes at

least 17 mΩ nanopure water and centrifuging, filtering the resultant supernatant to 0.2 μm using a vacuum filter, and diluting the filtered supernatant as needed with at least 17 mΩ nanopure water. Soluble NPOC and total N concentration was then measured by a total C and N analyzer (TOC-VCSH, Shimadzu, Kyoto, Japan). Soils were homogenized and air-dried before C and N analysis. Particulate organic matter (POM) and mineral fractions were separated by shaking in 5g L^{-1} sodium hexametaphosphate solution for 24 hours and then sieving to 53 μm (the mineral fraction was <53 μm and the POM fraction was >53 μm). Total, POM and mineral C and N concentrations were analyzed by dry combustion using a Costech Elemental Combustion System 4010 (Valencia, CA).

Short-term denitrification incubations

Potential and basal denitrification in soils were measured using the acetylene inhibition method (Yoshinari et al, 1977; Smith and Tiedje, 1979). Soils were pre-incubated overnight at 20°C prior to the tests, which were performed in triplicate for each soil sample. Twenty g dry-weight equivalent moist soil was sealed in half-pint wide-mouth canning jars with a septum in the lid. The headspace of each jar was flushed with N_2 gas to create an anaerobic environment. For the potential denitrification incubation, each jar received 25 mL solution with 300 μg glucose-C g $soil^{-1}$ and 50 μg NO_3-N g $soil^{-1}$ prior to the addition of acetylene. Incubation began when acetylene was injected into the headspace to a 10 Pa partial pressure of acetylene. Acetylene at this concentration inhibits the reduction of N_2O in the denitrification pathway and also completely inhibits autotrophic nitrification (Berg et al, 1982; Klemedtsson et al.,1988). The acetylene method does not inhibit heterotrophic nitrification, but its contribution to N_2O emissions is considered negligible (De Boer and Kowalchuk, 2001).

Chloramphenicol, which has been suggested to inhibit the synthesis of new enzymes, was not utilized due to evidence that it may inhibit existing enzymes and underestimate denitrification (Pell et al., 1996). Instead, the assay was performed under 5 hrs at 20°C to ensure that a significant amount of new enzymes were not synthesized (Luo et al., 1996). The short incubation also ensured that the inhibition of nitrification by acetylene did not affect the rate of denitrification (Drury et al., 2008). Gas samples were taken from the headspace at 2 and 4 hrs. The gas samples and standards were analyzed on a gas chromatograph equipped with an automated valve system for routing gas samples to the electron capture (ECD) and flame ionization (FID) detectors. Gas samples and standards were initially injected with an autosampler into a vented 1 mL loop connected to a 10 port valve, which allowed samples to normalize to atmospheric pressure when injected into the gas chromatograph. The amount of N_2O in the gas samples was determined using calculations described in Drury et al. (2008) and with a Bunsen coefficient of 0.632 at 20°C (Tiedje, 1982). N_2O flux was determined as the change in N_2O between the 2 hr and 4 hr measurements.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) with PROC MIXED (SAS Institute, Cary, N.C., version 9.3) using season and topographical position as main effects, with repeated measures. Normality was tested using the Anderson-Darling test at p=0.05. A natural log transformation was applied to basal denitrification, potential denitrification, and electrical conductivity; a square root transformation was applied to nitrate and soluble total N; and a reciprocal transformation was applied to soluble NPOC to address normality for ANOVA and multivariate regression. In addition, pH values were converted to hydrogen ion concentration for

ANOVA and multivariate regression. As a repeated measures study, a compound symmetric covariance structure could not be assumed for the ANOVA and had to be assessed. The suitability of a compound symmetric covariance structure was tested by Mauchly's Sphericity test at a *p*-value of 0.05. If a compound symmetric covariance structure was found to be inappropriate (*p*-value<0.05 by Mauchly's Sphericity test), the most appropriate covariance structure was selected by choosing the covariance structure with the lowest Bayesian Information Criterion (BIC). BIC rates the covariance structures based upon likelihood but also penalizes a greater number of parameters. The first-order unstructured covariance structure was used for nitrate and ammonium, the first-order heterogeneous autoregressive covariance structure was used for total inorganic N, and the first-order autoregressive covariance structure was used for particulate organic matter N. To develop a more rigorous and conservative estimate of spatio-temporal variability, Tukey's pairwise comparisons were selected to compare topography or season with a *p*-value of 0.05. A multivariate analysis of denitrification to identify denitrification drivers was performed by a stepwise multivariate regression analysis. The stepwise multivariate regression analysis was performed for both potential and basal denitrification using measured soil characteristics (described in section 2.3) as possible explanatory factors. POM and mineral fraction C and N were excluded from analysis due to a smaller sample size. For the stepwise multivariate regression analysis, PROC REG was used with a significance level of $p<0.05$ for explanatory factors to enter and to stay in the model. The parameter estimates for soluble NPOC and pH in the stepwise multivariate regression models are the opposite direction of actual values, as soluble NPOC was under a reciprocal transformation and pH was converted to hydrogen ion concentration.

RESULTS

Spatial and temporal variation of soil characteristics

Significant differences were observed among topographical positions and seasons for all soil characteristics except total N and mineral C and N; these appeared to be similar within the field throughout the year (Table 1,2). Soil water content was significantly less in summer samples (3.1 – 3.8%) than in other seasons. Total inorganic N ranged from 0.1 ± 0.02 μg g^{-1} in the summer to 9.6 ± 1.9 μg g^{-1} in the autumn. Franzluebbers et al. (1995) found a similar trend for both no-till and conventional cropping systems, with inorganic N lowest toward the end of the growing season and peaking during post-harvest due to N mineralization. Soil pH in the autumn (5.96 ± 0.13) was significantly greater than in other seasons, which ranged from 5.19 ± 0.05 in the summer to 5.30 ± 0.13 in the winter. Conyers et al. (1995) studied seasonal variation of soil pH in New South Wales, Australia and found that metabolic processes, with N cycling dominant, accounted for most of the variation of soil pH. Our observed peak in soil pH during autumn is possibly due to decreased nitrification rates in proportion to N mineralization.

Soluble NPOC was greater at the footslope (69.7 ± 13.1 mg kg^{-1}) and backslope (68.9 ± 10.4 mg kg^{-1}) than at the summit (58.4 ± 9.8 mg kg^{-1}). In addition, soluble total N was greater at the footslope (22.2 ± 4.8 mg kg^{-1}) than at the backslope (14.0 ± 3.1 mg kg^{-1}) and summit (12.5 ± 1.7 mg kg^{-1}) (Table 1). Similarly, Wood et al. (1991) found organic C and N to be greater at the footslope than at the summit and backslope in no-till fields. Smith and Halvorson (2011) studied field-scale variation of soil characteristics in Palouse silt loam, and found that soil pH was less variable with a field scale than was total C or electrical conductivity. Our results followed a similar trend, as soil pH did not significantly differ among topographical positions within a

season, while total C significantly differed among topographical positions in the summer and electrical conductivity significantly differed among topographical positions in the autumn. Sherrod et al. (2005) found that POM total C was greater at lower slope positions in no-till dryland fields. Although no significant topographical differences were observed within a season for POM C in our study, the footslope soil had less POM N than backslope soil in the autumn.

Table 1. Soil environmental measurements of each topographical position for each season. Values given are arithmetic means ± standard error. Values sharing the same letter in the same column are not significantly different with a *p*-value of 0.05 (n=5-6).

		Soil Water Content (%)	Soluble NPOC (mg/kg soil)	Soluble Total N (mg/kg soil)	NO_3-N (µg/g soil)	NH_4-N (µg/g soil)	Total Inorganic N (µg/g soil)	pH	Electrical Conductivity (dS/m)
Autumn	Summit	29.5 ± 1.6 ^{bc}	44.2 ± 3.8 ^{bcd}	11.6 ± 3.4 ^{bc}	0.26 ± 0.15 ^{cd}	2.60 ± 0.61 ^{bc}	3.87 ± 1.14 ^{bc}	6.10 ± 0.33 ^{ab}	0.22 ± 0.04 ^{b}
	Backslope	28.3 ± 1.3 ^{bcd}	46.7 ± 6.5 ^{cd}	12.2 ± 4.0 ^{bc}	2.55 ± 1.52 ^{bcd}	2.73 ± 1.42 ^{bc}	7.04 ± 2.87 ^{abcd}	5.60 ± 0.07 ^{ab}	0.18 ± 0.02 ^{bc}
	Footslope	34.1 ± 1.1 ^{ab}	31.6 ± 4.5 ^{d}	56.8 ± 8.0 ^{a}	6.83 ± 1.43 ^{ab}	9.70 ± 1.31 ^{a}	17.77 ± 2.15 ^{a}	6.17 ± 0.09 ^{a}	0.40 ± 0.01 ^{a}
Winter	Summit	33.8 ± 1.5 ^{ab}	35.0 ± 5.4 ^{d}	6.3 ± 1.4 ^{bc}	6.31 ± 0.72 ^{a}	1.13 ± 0.08 ^{bd}	7.48 ± 0.70 ^{b}	5.46 ± 0.40 ^{abc}	0.21 ± 0.03 ^{ab}
	Backslope	33.8 ± 1.4 ^{ab}	31.4 ± 6.2 ^{d}	6.1 ± 0.8 ^{bc}	5.92 ± 0.85 ^{ab}	1.49 ± 0.14 ^{b}	7.41 ± 0.92 ^{b}	5.14 ± 0.07 ^{abc}	0.17 ± 0.02 ^{bc}
	Footslope	36.6 ± 3.0 ^{a}	30.3 ± 3.5 ^{d}	8.2 ± 0.7 ^{bc}	8.18 ± 0.69 ^{a}	1.29 ± 0.20 ^{bd}	9.47 ± 0.79 ^{b}	5.33 ± 0.14 ^{abc}	0.19 ± 0.02 ^{bc}
Spring	Summit	25.4 ± 1.1 ^{cde}	29.7 ± 3.7 ^{d}	15.0 ± 4.4 ^{bc}	0.61 ± 0.19 ^{c}	0.26 ± 0.18 ^{bc}	0.87 ± 0.37 ^{c}	5.28 ± 0.05 ^{abc}	0.19 ± 0.03 ^{bc}
	Backslope	20.7 ± 2.4 ^{de}	50.7 ± 11.0 ^{cd}	24.5 ± 11.1 ^{b}	0.63 ± 0.19 ^{c}	0.08 ± 0.55 ^{bc}	1.23 ± 0.71 ^{c}	5.10 ± 0.14 ^{c}	0.19 ± 0.05 ^{bc}
	Footslope	19.8 ± 1.8 ^{e}	42.9 ± 6.0 ^{cd}	3.9 ± 0.9 ^{c}	0.05 ± 0.03 ^{d}	0.07 ± 0.01 ^{cd}	0.12 ± 0.04 ^{c}	5.21 ± 0.19 ^{cb}	0.10 ± 0.01 ^{c}
Summer	Summit	3.4 ± 0.2 ^{f}	133.1 ± 17.9 ^{abc}	17.0 ± 2.7 ^{bc}	0.09 ± 0.03 ^{d}	0.02 ± 0.01 ^{c}	0.10 ± 0.03 ^{c}	5.31 ± 0.10 ^{abc}	0.22 ± 0.02 ^{ab}
	Backslope	3.1 ± 0.2 ^{f}	146.6 ± 11.7 ^{ab}	13.5 ± 1.2 ^{bc}	0.04 ± 0.01 ^{d}	0.02 ± 0.00 ^{c}	0.06 ± 0.01 ^{c}	5.28 ± 0.05 ^{abc}	0.16 ± 0.01 ^{bc}
	Footslope	3.8 ± 0.5 ^{f}	173.8 ± 12.7 ^{a}	19.8 ± 2.9 ^{c}	0.08 ± 0.03 ^{d}	0.12 ± 0.03 ^{c}	0.19 ± 0.06 ^{c}	4.99 ± 0.07 ^{c}	0.22 ± 0.03 ^{b}

Table 2. Measurements of total, mineral and POM C and N of each topographical position for each season. The POM fraction is defined as >53 μm, while the mineral fraction is <53 μm. Values given are arithmetic means ± standard error. Values sharing the same letter in the same column are not significantly different with a *p*-value of 0.05 (n=5-6, *n=3).

		Total C (%)	Total N (%)	Mineral* Total C (%)	Mineral* Total N (%)	POM* Total C (%)	POM* Total N (%)
Autumn	Summit	2.48 ± 0.24 b	0.20 ± 0.02 a	0.18 ± 0.04 a	0.44 ± 0.06 a	7.40 ± 0.71 ab	0.44 ± 0.06 abc
	Backslope	2.18 ± 0.18 b	0.17 ± 0.01 a	0.17 ± 0.01 a	0.48 ± 0.05 a	7.95 ± 0.44 ab	0.48 ± 0.05 ab
	Footslope	2.14 ± 0.06 b	0.19 ± 0.01 a	0.17 ± 0.04 a	0.25 ± 0.01 a	3.63 ± 0.43 b	0.25 ± 0.01 c
Winter	Summit	2.54 ± 0.15 b	0.19 ± 0.01 a	0.18 ± 0.02 a	0.46 ± 0.04 a	6.51 ± 0.55 ab	0.46 ± 0.04 abc
	Backslope	2.54 ± 0.20 b	0.19 ± 0.01 a	0.13 ± 0.01 a	0.48 ± 0.03 a	7.19 ± 1.22 ab	0.48 ± 0.03 ab
	Footslope	2.43 ± 0.16 b	0.19 ± 0.01 a	0.15 ± 0.01 a	0.25 ± 0.06 a	4.12 ± 0.96 ab	0.25 ± 0.06 bc
Spring	Summit	2.46 ± 0.12 b	0.18 ± 0.01 a	0.13 ± 0.02 a	0.46 ± 0.03 a	6.70 ± 0.57 ab	0.46 ± 0.03 abc
	Backslope	2.36 ± 0.19 b	0.18 ± 0.01 a	0.15 ± 0.00 a	0.56 ± 0.00 a	8.38 ± 0.30 a	0.56 ± 0.00 a
	Footslope	3.11 ± 0.22 ab	0.21 ± 0.01 a	0.16 ± 0.01 a	0.54 ± 0.05 a	8.74 ± 1.52 a	0.54 ± 0.05 ab
Summer	Summit	2.81 ± 0.22 b	0.18 ± 0.01 a	0.16 ± 0.00 a	0.49 ± 0.03 a	8.28 ± 0.26 ab	0.49 ± 0.03 ab
	Backslope	2.98 ± 0.17 b	0.18 ± 0.01 a	0.17 ± 0.00 a	0.53 ± 0.01 a	8.69 ± 0.86 a	0.53 ± 0.01 a
	Footslope	4.01 ± 0.39 a	0.22 ± 0.01 a	0.17 ± 0.03 a	0.48 ± 0.04 a	8.94 ± 1.57 a	0.48 ± 0.04 ab

Spatial and temporal variation of denitrification

Potential denitrification exhibited significant differences seasonally, with the summer potential denitrification rate significantly greater than the other seasons at every topographical position (Figure 2A). The summer potential denitrification rate ranged from 1080 ± 126 ng N_2O-N $hr^{-1}g^{-1}$ at the summit to 1270 ± 47 ng N_2O-N $hr^{-1}g^{-1}$ at the backslope, while potential denitrification rates for the other seasons ranged from 66 ± 18 ng N_2O-N $hr^{-1}g^{-1}$ at the summit in the winter to 502 ± 136 ng N_2O-N $hr^{-1}g^{-1}$ at the footslope in the autumn. .Summer potential denitrification rates reported by Dandie et al. (2011) in a Nova Scotia soil cropped to maize were similar in range to our summer potential denitrification rates. Spring potential denitrification rates were comparable to those found in a rice paddy soil in China during March (Chen et al., 2012) and autumn potential denitrification rates were comparable those found in a study of soil cropped to corn, soybean, and winter wheat in Ontario during November (Guo et al., 2011). Basal denitrification also differed significantly among seasons at every topographical position (Figure 2B). The seasonal trend varied depending on the topographical position, except winter rates were consistently lower than other seasons. Basal denitrification rates were sometimes orders of magnitude lower than potential denitrification rates, and ranged from 0.08 ± 0.04 ng N_2O-N $hr^{-1}g^{-1}$ in the winter to 4.9 ± 2.2 ng N_2O-N $hr^{-1}g^{-1}$ in the autumn.

Potential denitrification was consistently greater at the footslope (563 ± 95 ng N_2O-N $hr^{-1}g^{-1}$) than at the summit (373 ± 92 ng N_2O-N $hr^{-1}g^{-1}$) for every season, albeit the differences were significant in the autumn and winter (Figure 2C). The greatest basal denitrification rates were seen at the footslope in the autumn (Figure 2D). This is likely due to the large concentrations of total inorganic N seen at the end of the season (Table 1). Basal denitrification was also greatest at the footslope in the summer, although this trend was not observed in winter and spring (Figure

2D). Greater denitrification rates observed at lower topographical positions was consistent with other studies (van Kessel et al., 1993; Florinsky et al., 2004).

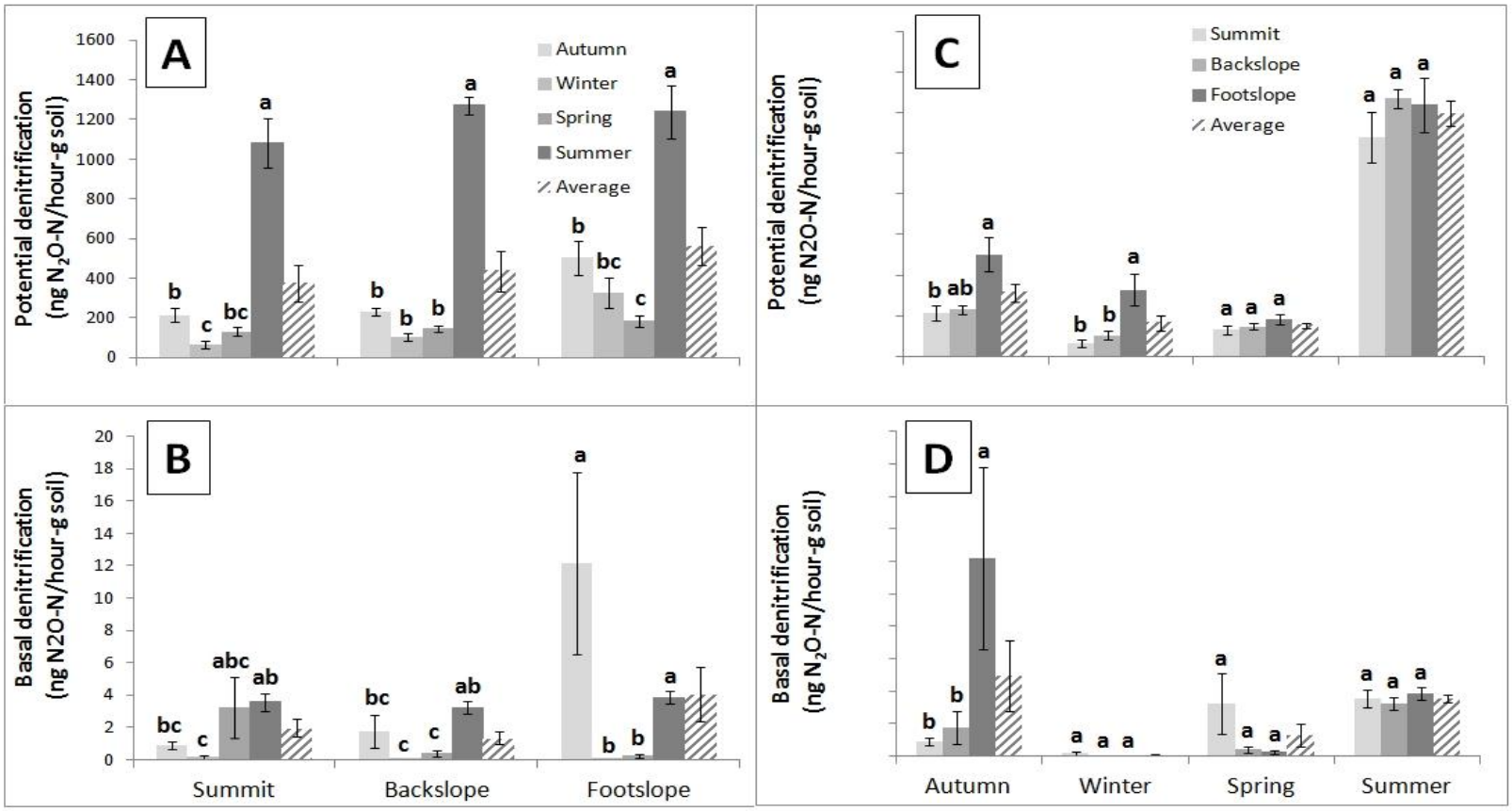

Figure 2. Potential and basal denitrification rates of each season at each topographical position (A and B) and of each topographical position during each season (C and D). Values are arithmetic means and error bars are standard error. Bars sharing the same letter are not

Identifying drivers of denitrification

Soil water content was the primary explanatory variable and accounted for 48% of the variation in potential denitrification across all seasons and topographical positions (Table 3). Significant explanatory factors of potential denitrification differed depending upon the season considered, and no significant explanatory factors were shared among seasons. The highest seasonal model R-square was seen for autumn, where soluble total N and electrical conductivity collectively explained 83% of the variance of potential denitrification. This exceeded the R-square of the model developed from all samples0.69). Nitrate was the only significant explanatory term for winter, total C was the only significant explanatory term for spring, and soluble NPOC was the only significant explanatory term for summer. Spatial variation was also evident for explanatory factors of potential denitrification, although to a lesser extent, as significant explanatory factors were shared among topographical positions. For the summit and backslope, soil water content was the primary explanatory variable and explained 67 and 66% of the variance of potential denitrification, respectively. The soil water content was still a significant explanatory term for the footslope, but electrical conductivity was the primary explanatory factor. The summit and backslope models had a greater R-square than the overall model. . When included in the model, soil water content was negatively correlated to potential denitrification due to the large potential denitrification rates and low soil water content of the summer samples (Figure 2A; Table 1). Attard et al. (2011) also studied soil environmental factors of potential denitrification and found that water-filled pore space, nitrate, and soil organic C were significant in explaining the variance of potential denitrification, but whether they were significant at *p*-value=0.05 was dependent upon which order they were entered into the model. Similarly, our study found soil water content to be the primary explanatory variable across all

samples, and measurements of N and C were also significant explanatory variables but in different forms (total inorganic N and total C) than those of Attard et al. (2011).

For basal denitrification, soluble total N and soluble NPOC collectively explained 54% of the variance across all samples (Table 4) which was less robust than the potential denitrification model (Table 3. Basal denitrification also exhibited seasonal variation in explanatory factors. Ammonium was the only significant explanatory variable in autumn and soil water content was the only significant explanatory variable in spring; no significant explanatory factors were found in winter and summer. Moreover, when the model significance level was raised to $p<0.10$, there were still no soil characteristics that made significance for the winter and summer basal denitrification models. The autumn model had an R-square of 0.58, which was larger than the R-square for the overall model (0.54). Basal denitrification exhibited more topographical variation than potential denitrification, as no explanatory factors were shared among topographical positions. Total inorganic N and electrical conductivity were significant explanatory factors at the summit; soluble NPOC was the only significant explanatory at the backslope; and soluble total N, soil water content, ammonium and pH were significant explanatory factors at the footslope. The most robust topographical model for basal denitrification was for the footslope, which had an R-square of 0.95. The backslope and footslope models had a greater R-square than the overall model.

Table 3. Stepwise multivariate regression analysis for potential denitrification across all samples as well as for each season and topographical position. Variables are listed in the order they were entered into the model. Each variable entered into the model and stayed in the model if its corresponding *p*-value was < 0.05. The parameter estimates for soluble NPOC and pH in the stepwise multivariate regression models are the opposite direction of actual values, as soluble NPOC was under a reciprocal transformation and pH was converted to hydrogen ion concentration.

Explanatory variable	Parameter estimate	R^2	p-value	n
All samples				
Soil water content	-0.071	48%	<.0001	
Total inorganic N	0.066	10%	0.0002	
Total C	0.33	7%	0.0071	
pH	-6.55E+04	4%	0.0002	
Total		**69%**		64
Temporal variation				
Autumn				
Soluble total N	0.37	53%	0.0021	
Electrical conductivity	-1.0	29%	0.0007	
Total		**83%**		15
Winter				
Nitrate	0.96	**29%**	0.0472	14
Spring				
Total C	0.34	**26%**	0.0289	18
Summer				
Soluble NPOC	-83	**38%**	0.0088	17
Spatial variation				
Summit				
Soil water content	-0.087	67%	<.0001	
Total N	15	16%	0.0009	
Total		**83%**		20
Backslope				
Soil water content	-0.059	66%	<.0001	
pH	-6.44E+04	10%	0.0088	
Total C	0.57	7%	0.0114	
Total		**84%**		22
Footslope				
Soil water content	-0.043	28%	0.0112	
Electrical conductivity	1.1	39%	0.0001	
Total		**67%**		22

Table 4. Stepwise multivariate regression analysis for basal denitrification across all samples as well as for each season and topographical position. Variables are listed in the order they were entered into or removed from the model. Each variable entered into the model and stayed in the model if its corresponding *p*-value was < 0.05. The parameter estimates for soluble NPOC and pH in the stepwise multivariate regression models are the opposite direction of actual values, as soluble NPOC was under a reciprocal transformation and pH was converted to hydrogen ion concentration.

Explanatory variable	Variable Removed	Parameter estimate	R^2	p-value	n
All samples					
Soluble total N		0.51	23%	0.0001	
Soluble NPOC		-64	22%	<.0001	
pH		-1.27E+05	6%	0.0125	
Soil water content		-0.75	5%	0.0202	
	Soluble NPOC	-29	-2%	0.1342	
			54%		57
Temporal variation					
Autumn					
Ammonium		0.25	**58%**	0.0010	15
Winter					
No significant variables					7
Spring					
Soil water content		0.15	**29%**	0.0205	18
Summer					
No significant variables					17
Spatial variation					
Summit					
Total inorganic N		-0.29	30%	0.0161	
Electrical conductivity		1.8	20%	0.0244	
Total			**49%**		19
Backslope					
Soluble NPOC		-94	**55%**	0.0003	19
Footslope					
Soluble total N		-0.16	56%	0.0002	
Soil water content		0.30	28%	<.0001	
Ammonium		0.33	9%	0.0004	
pH		-7.76E+04	2%	0.0279	
Total			**95%**		19

DISCUSSION

This study agrees with others in that denitrification exhibited both spatial and temporal variation (Folorunso and Rolston, 1984; Groffman and Tiedje, 1989; Parsons et al., 1991; Cambardella et al., 1994). The larger potential denitrification rates in the summer were expected because dryer antecedent soil conditions have been shown to increase denitrification, as measured by $N_2 + N_2O$ fluxes and N_2O-to-N_2 ratio (Bergstermann et al., 2011). In that study, the increased rates following wetting of dry soil were considered a result of increased C availability, as nitrate was similar between samples (Bergstermann et al., 2011). Considering the relatively low inorganic N and the relatively large soluble NPOC concentration observed in the summer samples, this is likely the case for our soil samples as well.

Explanatory factors of potential denitrification were found to shift seasonally as well as topographically. These shifts in explanatory factors indicate it is important to consider samples separately by season and topographical positions; otherwise, important local relationships in space and time may be overlooked. Moreover, the model R-square was at times improved when seasons and topographical positions were analyzed separately. The model R-square for both potential and basal denitrification was greater in the autumn than for the overall model. In addition, the potential denitrification models for the summit and backslope had a greater R-square the overall model and the basal denitrification models for the backslope and footslope had a greater R-square than for the overall model. Thus, the ability to predict denitrification can be improved at times if topographical positions and seasons are considered separately.

In addition, seasonal variation may be important for understanding pH as a denitrification driver. Šimek et al. (2002) found that short-term denitrification enzyme activity (equivalent to our study's potential denitrification measurement) was greatest at the natural pH of soil (defined

as the pH of soil slurry after addition of NO_3^- and glucose amendments for optimizing conditions for denitrifying activity); the authors did not find a specific range of pH values in which short-term denitrification was optimum. For both potential and basal denitrification, pH was observed as a significant explanatory variable for the overall model as well as certain topographical models. Considering we observed pH as an explanatory variable for models that were across all seasons, it is likely seasonal variation of pH is affecting denitrification rates; this seasonal variation deviates from the 'natural pH' for which the denitrifier population is adapted.

Predicting basal denitrification appears to be more complex than predicting potential denitrification. For basal denitrification, predicting activity within a season for the winter and summer was not possible with a *p*-value significance level up to 0.10 with the measured soil characteristics, and thus it was not possible to develop a predictive model for every season with the given soil measurements. Other possible explanatory variables for basal denitrification that were not included in the study are measurements of the denitrifier community size and structure; these could possibly assist in predicting denitrification rates where soil environmental measurements could not. In addition, none of the explanatory factors of basal denitrification were shared among topographical positions. Basal denitrification seems to be less predictable than potential denitrification.

Spatial and temporal variation can help explain why prior studies that only consider certain seasons and topographical positions identified different soil characteristics as drivers of denitrification. Although the model developed by Hénault and Germon (2000) was calibrated by samples across all seasons, topographical variation was not considered; the model developed by Elliot and de Jong (1993) considered spatial variation but only sampled during the growing season to calibrate the model. Accounting for both spatial and temporal variation can not only

contribute to better predictive models of denitrification, but can also allow for more consistency among studies as to which drivers are predictive of denitrification.

It has been found that fertilizer additions, freeze-thaw cycles, and precipitation events can cause significant spikes in N_2O emissions (Wagner-Riddle et al., 1997; Teepe et al., 2001; Dobbie and Smith, 2003; Vilain et al., 2010). The basal denitrification rate measured is an underestimation of average field rates, although it is representative of rates outside of hot spots and hot moments; the potential denitrification rate is an overestimation of average field rates, although it is representative of spikes observed in hot spots and hot moments. Whether N_2O emissions for the Palouse region is comprised mainly of basal rates or spikes from fertilizer, thawing, or precipitation events remains to be seen; thus, the relative importance of predicting basal or potential denitrification rates for estimating N_2O emissions for the region has yet to be determined.

It is important to note that although this study found significant differences among seasons and topographical positions, there are limitations to the assessment of both of these variables. Each season had only one sampling date; having multiple sampling dates within each season would have developed more robust seasonal data. In addition, topographical variation was within a field; to establish topographical differences at a more regional scale would require sampling across different fields. During the study, the footslope was cropped to winter wheat while the summit and backslope were cropped to spring wheat; this may have caused the results to be differentiated due to the crop rather than topographical position, although it had not been established as to whether soil denitrification rates differ between crops as closely related as winter wheat and spring wheat. . Because of the difference in crops, there was also a difference in fertilizer events (winter wheat was fertilized in the fall, while spring wheat was not), and

possibly a difference in soil moisture from crop uptake. Fertilizer was banded in 30 cm intervals at 10 cm depth during seed placement and samples were collected at the top 0-5 cm. If the soil sampling had inadvertently captured the banded fertilizer, we would expect significant variability in the inorganic N measurements between the replicate soil samples after the fertilization events. It does not appear that the fertilizer influenced the soil chemistry results since the standard error for inorganic N in the autumn and winter was not greater at the footslope compared to the summit and backslope. Moreover, there were no significant differences in soil moisture content between topographical positions for any season. Regardless, it would be preferable to assess topographical positions that are simultaneously in the same crop. Despite these limitations, there was still a demonstrable need to consider spatial variation just from the summit and backslope, as the potential denitrification models for the summit and backslope had a greater R-square than for the overall model. Thus, this study demonstrates the importance of considering spatio-temporal variation of drivers for developing accurate predictive models of denitrification.

SPATIO-TEMPORAL VARIATION OF DENITRIFIER AND NITRIFIER MICROBIAL COMMUNITIES IN SOIL, AND INFLUENCE ON DENITRIFICATION RATE

ABSTRACT

Nitrous oxide is a potent greenhouse gas that is mediated by the soil microbial processes of denitrification and nitrification. Predicting denitrification rates contributes to estimating soil nitrous oxide emissions, but whether denitrifier population size and structure contributes to predicting denitrification rates has not been established. This study assesses whether population size and structure of denitrifiers can help predict denitrification across seasonal and topographical variation. Samples were taken within a field from different topographical positions (summit, backslope, footslope) and seasons (autumn, winter, spring, summer). Gene abundance of nitrate reductase (*nirK*), and bacterial and archaeal ammonia monooxygenase *(amoA)* were determined by quantitative PCR and the community structure of the same populations was determined by T-RFLP. Potential and basal denitrification rates and soil environmental measurements of the same soil samples has been described by the authors. Stepwise multivariate regression models of denitrification already described by the authors were modified by adding *nirK* abundance and community structure to existing soil environmental explanatory factors. Significant spatio-temporal variation was observed for the denitrifier and nitrifier populations studied, warranting separate seasonal and topographical stepwise multivariate regression models. Adding *nirK* abundance increased the R-square for the potential denitrification summit model (from 0.83 to 0.88), but *nirK* abundance was not included as a significant explanatory variable for the other potential denitrification models. The *nirK* abundance was not a significant

explanatory variable for any of the basal denitrification models, and *nirK* community structure was not a significant explanatory variable for either the potential or basal denitrification models (*p*-value=0.05). The results of this study indicate that spatio-temporal variation must be considered in studying denitrifier and nitrifier abundances, as abundances can differ significantly depending upon the season and topographical position. We found that *nirK* abundance may contribute to predicting potential denitrification rates if spatio-temporal variation is considered. However, considering *nirK* abundance did not contribute to predictive models of basal denitrification, and *nirK* community structure did not contribute to predicting either potential or basal denitrification.

INTRODUCTION

Sources of nitrous oxide

$N_2O$is a potent greenhouse gas with emissions that continue to increase linearly each year (Forster et al., 2007). The majority of N_2O emissions come from the soil microbial processes of nitrification and denitrification (EPA, 2013). N_2O production from denitrification occurs when the last reduction step is not completed, such that N_2O is not reduced to dinitrogen (Philippot, 2002). N_2O production from nitrification occurs as a byproduct of the oxidation of hydroxylamine or from the reduction of nitric oxide through a nitrifier denitrifier pathway (Wrage et al., 2001; Arp and Stein, 2003).

Since N_2O emissions from soil are mediated by microbial activity, they are affected by environmental conditions such as temperature, oxygen availability, pH, as well as substrate availability in the form of inorganic N (Skiba and Smith, 2000). It has been indicated by several studies that N_2O emissions increase with increasing water-filled pore space, or low oxygen

availability (Linn and Doran, 1984; Smith et al., 1998). Nitrification is thought to be the primary contributor for N_2O flux in unsaturated soil conditions (Mummey et al., 1997).

The study of denitrifier and nitrifier communities

Before the advent of accessible and high-throughput molecular methods to study denitrifiers and nitrifiers, the role of microbial communities in N_2O production had been estimated by studies in controlled environments. In potential denitrification incubations, for example, critical environmental factors can be homogenized so that any observed differences in denitrification rates are assumed to be a result of differences in the microbial community and its enzymatic activity (Drury et al., 2008). Further, a study of nitrate reduction and N_2O reduction found differences in enzymatic kinetics among soils in the same environmental conditions, although these differences dissipated with anaerobic conditioning. The authors concluded that the differences in enzyme kinetics were a result of microbial community structure (Holtan-Hartwig et al., 2000). In these instances, it has been suggested that the microbial community is responsible for observed differences in denitrification under consistent environmental conditions. It is now possible to assess whether population size and structure influences denitrification or nitrification rates through molecular studies.

Denitrifiers and nitrifiers are extremely diverse communities of organisms. Denitrifiers include over 60 genera that spans over archaea, bacteria, and fungi; nitrification can be performed by both archaea and bacteria (Leininger et al., 2006; Philippot et al., 2007). Because of the diversity observed in denitrifiers and nitrifiers, it is more practical for molecular studies to target denitrifier and nitrifier populations by their functional genes rather than taxonomically with 16S or 18S ribosomal DNA (Philippot, 2002). Nitrification involves three different

enzymatic steps, while denitrification has four (Wrage et al., 2001). Nitrifiers are typically targeted by the bacterial or archaeal *amoA* gene, which encodes the ammonia monooxygenase enzyme responsible for ammonia oxidation. Denitrifiers can be targeted by a diversity of genes involved in the denitrification pathway: *napA* targets periplasmic nitrate reductase and *narG* targets membrane bound periplasmic nitrate reductase, and both enzymes perform reduction of nitrate; *nirK* targets copper nitrite reductase and *nirS* targets cytochrome cd_1 nitrate reductase, and both enzymes perform reduction of nitrite; *norB* targets nitric oxide reductase and *qnorB* targets quinol nitric oxide reductase, and both enzymes perform reduction of nitric oxide; and *nosZ* targets nitrous oxide reductase reductase, which performs reduction of N_2O to dinitrogen gas (Philippot et al., 2007). It is important to note that because of the diversity of genetically distinct enzymes involved in the denitrification pathway, targeting a single gene does not capture the entire denitrifier population.

A common molecular technique for determining population size is quantitative polymerase chain reaction, or qPCR (Heid et al., 1996). By amplifying a functional gene of interest in a sample while simultaneously amplifying the same gene of interest in standards of known population size, a population of interest (such as denitrifiers and nitrifiers) can be quantified by qPCR. To determine community structure, terminal restriction length fragment polymorphism (T-RFLP) is a widely-used method. T-RFLP utilizes polymerase chain reaction (PCR) amplicons that are fluorescently labeled on at least one end and digested by restriction enzymes, such that each fragment represents a taxonomically distinct individual. The T-RFLP analysis generates an electropherogram composed of peaks, with each peak representing a different terminal restriction fragment or taxonomic unit (Liu et al., 1997). Denaturing gradient gel electrophoresis (DGGE) is another common molecular method to measure community

structure. In DGGE, PCR amplicons are separated by sequence using gel electrophoresis with a gradient of denaturing chemical. The result is a series of bands across the gel, with each band representing a genetically distinct individual (Ercolini, 2004). Quantitative PCR, T-RFLP and DGGE can be utilized to study DNA or RNA, although studies of RNA are less common for studies of soils.

Relationship of denitrifier population size and structure to denitrification

The influence that denitrifier population size has on N_2O emissions or denitrification rates is still debatable, as recent molecular studies give contrasting results. A study comparing adjacent riparian and agricultural areas found that there was no correlation between *nirK* and *nirS* denitrifier population abundance and N_2O flux, denitrification or denitrification enzyme activity (Dandie et al., 2011). A study at a long-term agricultural research site in Sweden found that *nosZ* population abundance was correlated to denitrification rates, although *nirK* and *nirS* population abundances were not correlated to denitrification rates (Hallin et al., 2009). From these studies it may seem that it depends on which gene is targeted as to whether a correlation is found, and that *nosZ* is better correlated to denitrification rates than *nirK* and *nirS*. However, Miller et al. (2008) did not find any significant correlation between *nosZ* and $cnorB_P$ denitrifier population size and N_2O emissions or cumulative denitrification (defined as the headspace N_2O concentration in the presence of acetylene). Further, *nirK* and *nirS* denitrifier populations have been correlated to denitrification in other studies. For example, Chen et al. (2012) found that the abundance of *narG, nirK, nirS* and *nosZ* genes was correlated to potential denitrification activity (Pearson's correlation test, p-value<0.01), but the *qnorB* gene abundance was not correlated to potential denitrification activity (Pearson's correlation test, p-value>0.05). The abundance of

nirS has been correlated to N_2O emissions as well as the gaseous emission of CH_4-C and NO_3-N in another study (Morales et al., 2010). From the aforementioned studies, *nirK, nirS* and *nosZ* abundances have been found to be correlated to denitrification as well as uncorrelated to denitrification, depending on the study. Since the functional gene target does not seem to be responsible for whether a correlation is found between denitrifier abundance and denitrification, there is likely another variable responsible for these conflicting results.

Some studies have found that soil environmental factors are a better determinant of denitrification and N_2O emissions than is denitrifier population size. In one study of agricultural soil in a potato rotation, *nirK, nosZ, cnorB*$_B$ and *cnorB*$_P$ abundances were found to be unrelated to both N_2O emissions and denitrification rates, but N_2O emissions and denitrification rate were positively correlated to inorganic N and water filled pore space (Dandie et al, 2008). Further, in a study of land use changes it was found that soil environmental conditions were a greater determinant of potential denitrification rate than *nirS* and *nirK* population size (Attard et al., 2011).

Discrepancies among studies as to whether denitrifier abundance and denitrification rates are related may be due to yet another variable, the relative enzymatic activity of different denitrifier populations. Wertz et al. (2009) found that the "active" community of denitrifiers (indicated by mRNA transcripts of *nirK*) contained the most abundant and common denitrifiers found through DGEE analysis of DNA, but concluded that not all members of the denitrifier community detected by DNA were active. Unfortunately, mRNA studies of soil microbial communities are more difficult than DNA-based methods, and are thus less common. In the future it is possible that new approaches such as whole-community RNA amplification may make mRNA studies more amenable in environmental samples (Gao et al., 2007).

Another possible explanation for the disagreement among studies as to whether denitrifier abundance and denitrification are correlated is spatio-temporal variation. It has been demonstrated that denitrification exhibits both spatial and temporal variation (Folorunso and Rolston, 1984; Groffman and Tiedje, 1989; Parsons et al., 1991; Cambardella et al., 1994). Moreover, the authors have demonstrated that soil environmental drivers of denitrification measurements differ among seasons and topographical positions (Chapter 1). It is possible that denitrifier abundance also exhibits spatio-temporal variation as a driver of denitrification, and thus seasonal or topographical differences may be responsible for the current discrepancy in the literature.

In contrast, many studies agree that denitrifier community structure and denitrification are not correlated, and this appears to be consistent across various soil types and cropping systems. In a study of denitrifiers at various slope positions along agrarian and riparian zones, Dandie et al. (2011) found that the structure of *nirK* and *nirS* communities (as determined by DGGE analysis) was uncorrelated to denitrification, denitrification enzyme activity and N_2O emissions; however the structure of *nirK* communities was most correlated to soil pH, while *nirS* community structure was not correlated to any soil environmental variable. Another spatial study of denitrifiers in adjacent riparian soil and creek sediment found that the community structure of *nosZ* denitrifiers was not correlated to denitrification across the habitats studied, and relationships differed depending on the ecosystem studied (Rich and Myrold, 2004). A study of soil cropped to potatoes found that there was not a strong relationship between changes in *nirK* community composition (determined by DGGE) and changes in environmental factors or denitrification (Wertz et al., 2009). In a study of rice paddy soil, the community composition of denitrifiers as determined by T-RFLP was unrelated to potential denitrification activity (Chen et

al., 2012).

Despite the lack of correlation between community structure and denitrification in field studies, it has been demonstrated in laboratory conditions that it is possible for community structure to affect denitrification rate. Philippot et al. (2013) found that by decreasing the diversity richness of denitrifiers by 75% through serial dilutions, soil potential denitrification activity was lowered significantly. Although field studies find no correlation between denitrifier community structure and denitrification, this may be due to denitrifier diversity not reaching low enough levels to have an observable effect on denitrification.

Purpose of current study

Currently studies disagree as to whether denitrifier abundance is correlated to denitrification. The cause of the disagreement among studies may be due to spatio-temporal variation in the relationship between denitrifier abundance and denitrification. Previously, how soil environmental measurements inform denitrification among different seasons and topographical positions has been assessed (Chapter 1). Measurements of denitrifier abundance and community structure will be added to these assessments to determine whether DNA-based molecular measurements can be useful in predicting denitrification rates, as well as how their usefulness may change seasonally and topographically. In addition, there have been very few studies of denitrifier and nitrifiers with topography. Dandie et al. (2011) studied denitrifier populations across seven different slope positions and found significant differences in *nosZ* and *nirS* population abundances among slope positions on certain dates, with larger populations often found at lower slope positions. It has yet to be established as to whether nitrifier populations are sensitive to topography. Considering that N_2O emissions has been shown to differ

topographically by several studies (van Kessel, 1993; Ambus, 1998; Vilain et al., 2010), understanding the population dynamics of nitrifiers and denitrifiers with topography may help explain these differences in emissions. It is the goal of this study to assess the usefulness of DNA-based measurements of denitrifier population size and structure in predicting denitrification rates, and to determine whether seasonal and topographical variation exists for denitrifier and nitrifier population size and structure.

METHODS

Study site and soil sampling

The study site was located at the R.J. Cook Agronomy Farm in the dryland Palouse region of Washington State, USA (Chapter 1). Samples were taken from three topographical positions (summit, backslope, footslope) within a field during the autumn, winter, spring and summer, as described in Chapter 1. Soil samples were stored on ice for up to 2 hrs before being stored in the laboratory at 4°C. An unsieved field moist subsample was taken from each soil sample at the laboratory for molecular applications, and was stored at -20°C until DNA extraction.

Soil characteristics and denitrification

Analyses of soil samples included moisture content, nitrate (NO_3-N) concentration, ammonium (NH_4-N) concentration, soil pH, electrical conductivity, soluble non-purgeable organic carbon (NPOC), soluble total N, total C, total N, particulate organic matter (POM) C and N (>53 μm), and mineral fraction C and N (Chapter 1).

Potential denitrification and basal denitrification short-term incubations were performed as described in Chapter 1.

Molecular analyses of denitrifiers and nitrifiers

a. DNA extraction

DNA was extracted from field moist soil at the equivalent of 0.2g oven-dry weight soil using PowerLyzer PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). Extraction was performed according to manufacturer's instructions except that DNA was eluted from the column first with buffer heated to 80°C then re-eluted with the eluant. Extracts were visualized by 1% agarose gel electrophoresis with a 1kb ladder (New England Biolabs, Ipswich, MA). DNA concentration was measured by a Qubit ds DNA HS Assay (Life Technologies, Grand Island, NY).

b. Quantitative PCR

Gene abundance of nitrate reductase (*nirK*), and bacterial and archaeal ammonia monooxygenase *(amoA)* were determined by quantitative PCR. DNA extracts were first tested for the presence of PCR inhibitors using a method described previously (Reardon et al., 2013). Briefly, the C_T of qPCR reactions containing pUC19 plasmid (Invitrogen) and M13F/ M13R primers was compared in the presence and absence of soil DNA. The mean cycle threshold values for reactions containing soil extracts varied from -1.1 to 0.8 of the mean cycle threshold of the extract-free control, indicating little to no significant inhibition. The primer pair used for bacterial *nirK* was nirK876F (5' -ATY GGC GGV CAY GGC GA - 3') and nirK1040R (5' - GCC TCG ATC AGR TTR TGG TT - 3') (Henry et al., 2004; Henry et al., 2006). The bacterial

nitrifier population was amplified using primers *amoA*-1F (5' - GGG GTT TCT ACT GGT GGT - 3') and *amoA*-2R (5' - CCC CTC KGS AAA GCC TCC TCC - 3') (Rotthauwe et al., 1997), and the archaeal nitrifier population was amplified using the primers Arch-amoAF (5' - STA ATG GTC TGG CTT AGA CG - 3') and Arch-amoAR (5' - GCG GCC ATC CAT CTG TAT GT - 3') (Francis et al., 2005). The qPCR reaction volume was 10 µL, which contained 0.8× Power SYBR Green PCR Master Mix (Life Technologies), 0.1 µg μL^{-1} bovine serum albumin (Roche Applied Science, Indianapolis, IN), 0.1 µM (*nirK*) or 0.2 µM (*amoA*) each primer, and 1 µL DNA diluted 1:20 in H_2O. The standard curves were generated by 10-fold dilution of plasmids containing cloned amplification product over 4 (*nirK*) or 5 (*amoA*) orders of magnitude. Plasmids were constructed using the TOPO-TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA) and the inserts were sequenced to ensure that the gene-specific primer sequences were complete and correct (Strauss et al., 2014). Thermal cycling was performed using a StepOnePlus Real-Time PCR System (Life Technologies) with either a two-step or three-step thermal cycling. A two-step thermal cycling was used for *nirK* and bacterial *amoA* and consisted of 10 minutes at 95°C, 40 cycles of amplification for 15 seconds at 95°C, and 1 minute at the annealing temperature (55°C for *nirK*, 60°C for bacterial *amoA*). A three-step thermal cycling was used for archaeal *amoA* and consisted of 10 minutes at 95°C, 40 cycles of denaturation for 15 seconds at 95°C, 1 minute at the annealing temperature followed by a 72°C extensions step for 30 seconds. The melt curve consisted of 15 seconds at 95°C and 1 minute at 60°C with an increase of 0.3°C to a final temperature of 95°C. The standard curve efficiencies were above 80% for all targets and the cycle threshold standard deviation was below 0.5 for all samples.

c. PCR and terminal restriction fragment length polymorphism analysis

The DNA extracts from the replicate samples collected at each site and season were pooled prior to T-RFLP analysis. The *nirK* gene was amplified using the primers nirK1F (5' - GGM ATG GTK CCS TGG CA - 3') and nirK5R (5' - GCC TCG ATC AGR TTR TGG - 3') (Braker et al., 1998) in which the forward primer was labeled on the 5' end with WellRED D3-PA fluor (Sigma Aldrich, St. Louis, MO). The bacterial and archaeal *amoA* gene was amplified using the same primers as qPCR but with the forward primers labeled with the WellRED D2-PA fluor or WellRED D3-PA, respectively. PCR was performed in duplicate for each sample using a Veriti 96 Well Thermal Cycler (Life Technologies). The 25 µL (20 µL for *nirK*) reaction volume contained 0.1 ug μL^{-1} bovine serum albumin, 0.2 µM primers, 1× PCR Master Mix (Promega), and 0.5 µL DNA. The PCR cycling consisted of an initial denaturing step for 2 minutes at 94°C; 35 cycles (40 cycles for *nirK*) of amplification that consisted of 30 seconds of denaturing at 94°C, 30 seconds at the annealing temperature of 55°C (52°C for *nirK*), and 1 minute of extension at 72°C; and a final extension of 7 minutes at 72°C. PCR product duplicates were combined and analyzed for amplification using 1% agarose gel electrophoresis with a 100 kb ladder (New England Biolabs). PCR products were purified with the UltraClean PCR Clean-Up Kit (MO BIO Laboratories, Inc.) per manufacturer's instructions except the elution buffer was heated to 80°C.

Restriction enzymes were selected based upon their use in previous studies and lab optimizations in which the enzymes yielding the most T-RFs were selected. The profiles generated using the different enzymes were compared and the enzyme yielding the most T-RFs was selected. The enzymes tested were MspI, HhaI and HaeIII for *nirK;* the combination RsaI and HaeIII as well as HhaI for bacterial *amoA*; and the combination HhaI and HaeIII as well as RsaI for archaeal *amoA.* The *nirK* amplicon was digested with HaeIII (Braker et al., 2000;

Avrahami et al., 2002; Wolsing and Priemé, 2004; Bremer et al., 2007; Bremer et al., 2009), the bacterial *amoA* amplicon with HhaI (Ye and Zhang, 2011; Strauss et al., 2014), and the archaeal *amoA* amplicon with HhaI and HaeIII (Strauss et al., 2014). The 20 µL digestion mixture contained 10 U of restriction enzyme, 2 µL of 10× buffer, and 5µL of purified PCR product. Digests were carried out for 6 hours at 37°C, after which enzymes were inactivated with an 80°C step for 15 minutes. The digested products were purified with an ethanol precipitation step as per Beckman Coulter protocol 608019. The pellet was dried at 65°C for 10 minutes in a thermocycler before being suspended in 40 µl SLS buffer containing 0.25 µl GenomeLab™ Size Standard 600 (Beckman Coulter, Indianapolis, IN). The purified PCR products were analyzed using a Beckman-Coulter CEQ 8800 Genetic Analysis System. The analysis occurred under the following conditions: denaturation for 120 seconds at 90 °C, injection for 15 seconds at 2 kV, separation for 90 min at 4.8 kV and a capillary temperature of 50 °C. The T-RFLP profiles were analyzed using the Fragment Analysis Model of the CEQ 8800 Genetic Analysis Software with a slope threshold of one, 0.5% relative peak height threshold, 95% confidence level, quartic model, time migration variable, peak calculation by height, and PA ver. 1 mobility calibration using calculated dye spectra (Reardon et al., 2014). Profiles were trimmed to 60 to 550 bp for *nirK*, 60 to 500 bp for bacterial *amoA*, and 60 to 600 bp for archaeal *amoA*. Peaks were manually edited and the data was further analyzed using T-REX software (Culman et al., 2008). Noise was filtered by a standard deviation multiplier of 1 and T-RFs were aligned using a clustering threshold of 1 (Reardon et al., 2014). Only peaks observed in both duplicates were considered present. The sum of T-RF peaks present is equal to T-RF richness.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) with PROC MIXED (SAS Institute, Cary, N.C., version 9.3) using season and topographical position as main effects, with repeated measures. Normality was tested using the Anderson-Darling test at p=0.05. A natural log transformation was applied to bacterial and archaeal *amoA* abundance to address normality for ANOVA and multivariate regression. As a repeated measures study, a compound symmetric covariance structure could not be assumed for the ANOVA and had to be assessed. The suitability of a compound symmetric covariance structure was tested by Mauchly's Sphericity test at a p-value of 0.05. None of the variables were found to be significant by Mauchly's Sphericity test; as a result, a compound symmetric covariance structure was applied to all ANOVAs. To develop a more rigorous and conservative estimate of spatio-temporal variability, Tukey's pairwise comparisons were selected to compare topography or season with a p-value of 0.05. A multivariate analysis of denitrification to identify denitrification drivers was performed by a stepwise multivariate regression analysis, as described in Chapter 1. The stepwise multivariate regression analysis had been previously performed for both potential and basal denitrification using soil environmental measurements as possible explanatory factors; for this study, *nirK* population size and structure (T-RF richness) was added to the denitrification models. When *nirK* T-RF richness was incorporated into the models, all other variables had to be averaged over topographical position and season to accommodate the community structure measurement being from a pooled sample of each topographical position and season. For the stepwise multivariate regression analysis, PROC REG was used with a significance level of p<0.05 for explanatory factors to enter and to stay in the model. T-RF richness and β diversity were used to describe diversity of community structure. The β diversity is defined as S/α-1,

where S = total number of T-RFs and α = average number of T-RFs (Magurran, 1988). A greater β diversity value indicates more dissimilarity among samples. A moving window analysis of % change of structural diversity was calculated as % change = 100 - Jaccard similarity % (Pereira e Silva et al., 2011). Jaccard similarity indices and β diversity were obtained from the PAST statistical program (Hammer et al., 2001).

RESULTS

Spatial and temporal variation of denitrifier and nitrifier abundance

The seasonal trend of *nirK* abundance was consistent at each topographical position, with the greatest abundance observed in the autumn and the lowest abundance observed in the winter and summer (Figure3A). In addition, the *nirK* abundance was typically greater in the autumn than in the other seasons (average population size in the autumn was $1.7x10^6 \pm 1.9x10^5$ copies g^{-1}, while the next greatest seasonal average population size was in the winter with $9.3x10^5 \pm 1.1x10^5$ copies g^{-1}). In contrast, the seasonal trend of bacterial and archaeal *amoA* abundance varied depending upon the topographical position (Figure3B,C). Bacterial *amoA* exhibited significant seasonal differences across all topographical positions (Figure 3E), but no significant seasonal differences were observed when topographical positions were analyzed separately (Figure 3B). For archaeal *amoA*, significant seasonal differences were observed at the backslope and footslope in the winter (Figure 3F).

The *nirK* and bacterial *amoA* abundance were significantly greater at the footslope than at the summit and backslope across all seasons (Figure 3A,B). The footslope was also consistently greater than higher topographical positions when each season was analyzed separately, although differences were only significant in the autumn (Figure 3D,E). For both *nirK* and bacterial *amoA*,

significant topographical differences were not observed when the winter, spring and summer were each analyzed separately. Archaeal *amoA* abundance was greater at the summit than at the backslope and footslope in the autumn, spring and summer (Figure 3F). Significant topographical differences for archaeal *amoA* abundance were only observed in the winter, where abundance at the backslope was significantly less than the footslope even though the footslope had a high degree of variability between replicate samples. Archaeal *amoA* showed the greatest variability and ranged over 1.5 magnitudes, from $6.2x10^5 \pm 2.9x10^5$ copies g^{-1} at the footslope in the spring to $2.7x10^7 \pm 1.6x10^7$ copies g^{-1} at the footslope in the winter.

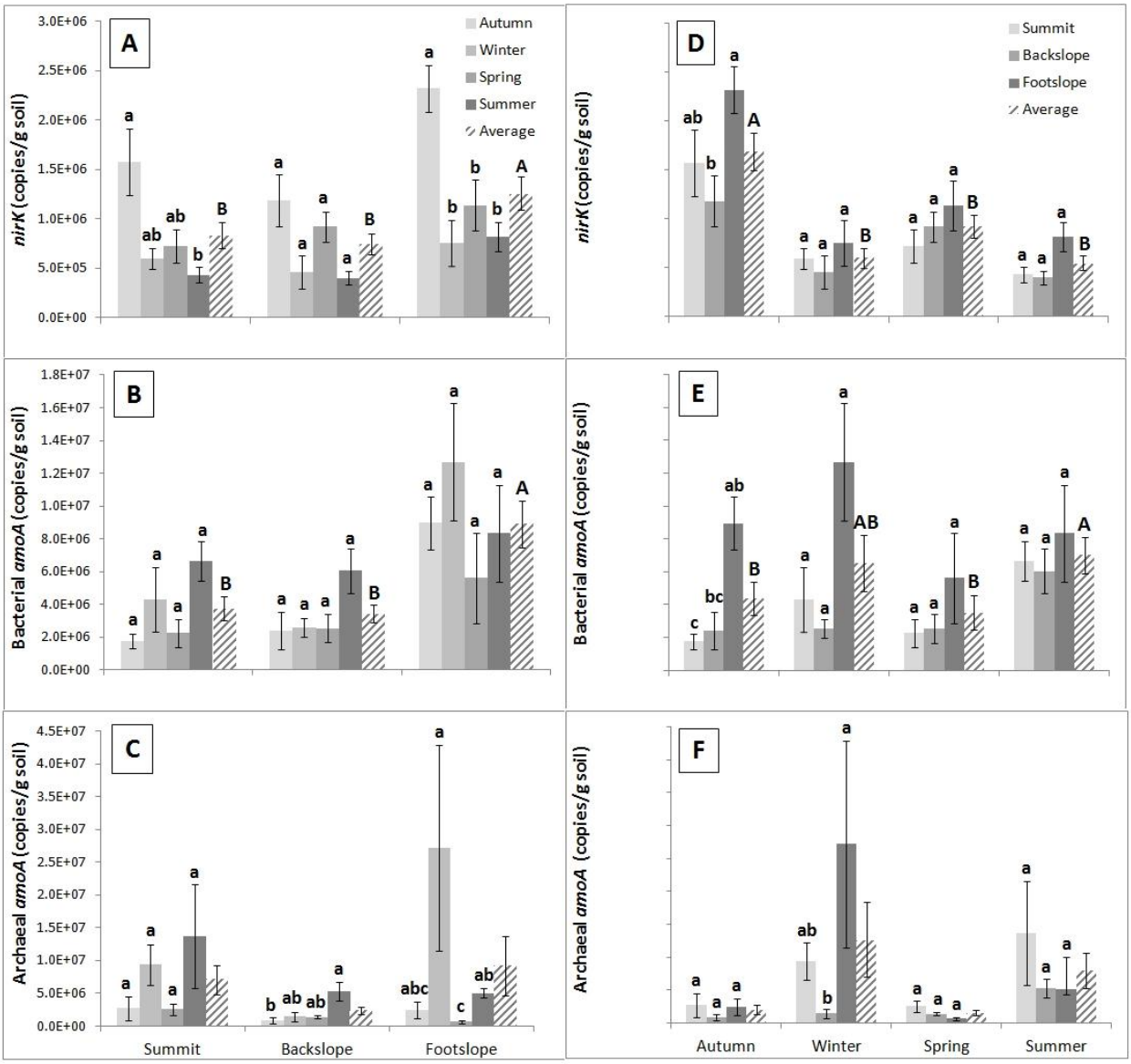

Figure 3. Gene abundance of *nirK*, bacterial *amoA* and archaeal *amoA* abundance for each season at each topographical position (A, B and C) and at each topographical position during each season (D, E and F). Values are arithmetic means and error bars are standard error. Hatched bars indicate the average value for a season or topographical position. Bars sharing the same lowercase letter are not significantly different within a season (A, B and C) or topographical position (D, E and F) based on a *p*-value of 0.05. Hatched average bars sharing the same uppercase letter are not significantly different. Due to an interaction between season and topography for archaeal *amoA*, differences among seasons and topographical positions overall were not statistically differentiated.

nirK *abundance and community structure as predictors of denitrification*

The *nirK* abundance and community structure were added to the stepwise multivariate regression analyses of potential and basal denitrification performed in Chapter 1. The *nirK* abundance was a significant explanatory variable for certain potential denitrification models (Table 5), but was not a significant variable for any of the basal denitrification models at a *p*-value of 0.05. The *nirK* community structure (T-RF richness) was not a significant explanatory variable for any of the basal or potential denitrification models at a *p*-value of 0.05.

The *nirK* abundance was only included in the summit model for potential denitrification, where it explained 4% of the variance, although it was also a significant explanatory variable in the autumn model before soil water content and nitrate were added as significant explanatory variables (Table 5). The addition of *nirK* abundance improved the summit model R-square from 83% to 88% (Chapter 1, Table 5).

Table 5. Stepwise multivariate regression models for potential denitrification overall as well as for each season and topographical position. Variables are listed in the order they were entered into or removed from the model. Variables were entered into the model and stayed in the model if they had a corresponding *p*-value less than 0.05. The parameter estimates for soluble NPOC and pH in the stepwise multivariate regression models are the opposite direction of actual values, as soluble NPOC was under a reciprocal transformation and pH was converted to hydrogen ion concentration.

Explanatory variable	Variable Removed	Parameter estimate	R^2	p-value	n
All samples					
Soil water content		-0.71	48%	<.0001	
Total inorganic N		0.066	10%	0.0003	
pH		-6.46E+04	7%	0.0007	
Total C		0.33	4%	0.0071	
Total			**69%**		64
Temporal variation					
Autumn					
nirK abundance		4.02E-07	55%	0.0015	
Nitrate		0.30	18%	0.0152	
Soil water content		0.093	8%	0.0463	
	nirK abundance	1.90E-07	-4%	0.158	
Electrical conductivity		-0.45	9%	0.0179	
Total			**87%**		15
Winter					
Nitrate		0.96	**29%**	0.0472	14
Spring					
Total C		0.34	**26%**	0.0289	18
Summer					
Soluble NPOC		-83	**38%**	0.0088	17
Spatial variation					
Summit					
Soil water content		3.93E-07	67%	<.0001	
Total N		-0.092	16%	0.0009	
nirK abundance		12	4%	0.0288	
Total			**88%**		20
Backslope					
Soil water content		-0.059	66%	<.0001	
pH		-6.44E+04	10%	0.0088	
Total C		0.57	7%	0.0114	
Total			**84%**		22
Footslope					
Soil water content		-0.043	28%	0.0112	
Electrical conductivity		1.1	39%	0.0001	
Total			**67%**		22

Potential drivers of denitrifier and nitrifier population size and structure

Ammonium, soil water content and nitrate explained 40, 9, and 6% of variance of *nirK* abundance, respectively (Table 6). Dandie et al. (2011) found *nirSp* (a gene specific to *Pseudomonas mandelii* and closely related species) abundance to be correlated to nitrate and soil water content as well. Soil water content was the primary explanatory variable in the autumn and summer models, and explained 56% and 67% of variance of *nirK* abundance, respectively. No significant explanatory variables were observed for winter and spring models of *nirK* abundance. Ammonium was the only significant explanatory variable for all three topographical positions, explaining 26%, 22%, and 54% of variance of *nirK* abundance for the summit, backslope and footslope, respectively. Potential drivers of *nirK* community structure, as measured by T-RF richness, were also assessed. Soil pH was the only significant explanatory variable and explained 85% of variance. Similarly in Dandie et al. (2011), *nirK* structure was found to be correlated to soil pH and no other soil environmental measurement. Moreover, Lauber et al. (2009) has found that soil pH is significantly correlated to overall bacterial community structure and diversity.

Bacterial *amoA* abundance had only one weak significant predictor overall (electrical conductivity) which accounted for 8% of variance (Table 7). However, stronger predictors were seen when topography and season were analyzed separately. Ammonium was the only significant explanatory variable in autumn and explained 73% of variance, pH was the only significant explanatory variable in the winter and explained 37% of variance, total C was the only significant explanatory variable in the spring and explained 36% of variance, and total inorganic N was the only significant explanatory variable in the summer and explained 62% of variance of bacterial *amoA* abundance. At the summit, electrical conductivity was the only significant explanatory variable and explained 30% of variance; at the backslope, total C

explained 33% of variance while nitrate explained 18% of variance for bacterial *amoA* abundance*;* no significant explanatory variables were seen at the footslope. Potential drivers of bacterial *amoA* community structure were also assessed. None of the measured soil characteristics were significant explanatory variables for bacterial *amoA* community structure.

Archaeal *amoA* abundance did not have any significant explanatory variable overall (Table 8). For spring, total N was the only significant explanatory variable and explained 25% of variance for archaeal *amoA* abundance. Autumn, winter and summer did not have any significant explanatory variables. For the backslope, soil water content was the only significant explanatory variable and explained 30% of variance of archaeal *amoA* abundance. The summit and footslope did not have any significant explanatory variables. Potential drivers of archaeal *amoA* community structure were also assessed. None of the measured soil characteristics were significant explanatory variables for archaeal *amoA* community structure.

Table 6. Stepwise multivariate regression models for *nirK* abundance overall as well as for each season and topographical position. Variables are listed in the order they were entered into the model. Variables were entered into the model and stayed in the model if they had a corresponding *p*-value less than 0.05.

Explanatory variable	Parameter estimate	R^2	p-value	n
All samples				
Ammonium	1.79E+05	40%	<.0001	
Nitrate	-3.25E+05	6%	0.0098	
Soil water content	2.14E+04	9%	0.0010	
Total		**55%**		64
Temporal variation				
Autumn				
Soil water content	1.50E+05	**56%**	0.0012	15
Winter				
No significant variables		-	-	14
Spring				
No significant variables		-	-	18
Summer				
Soil water content	2.10E+05	67%	<.0001	
Ammonium	1.77E+06	9%	0.0331	
Total		**77%**		17
Spatial variation				
Summit				
Ammonium	2.63E+05	**26%**	0.0220	20
Backslope				
Ammonium	1.32E+05	**22%**	0.0283	22
Footslope				
Ammonium	1.35E+05	**54%**	0.0001	22

Table 7. Stepwise multivariate regression models for bacterial *amoA* abundance overall as well as for each season and topographical position. Variables are listed in the order they were entered into the model. Variables were entered into the model and stayed in the model if they had a corresponding *p*-value less than 0.05. Soil pH was a significant explanatory variable in the summer model but was removed due to multicollinearity (square root of the ratio of the largest eigenvalue to each individual eigenvalue > 100). The parameter estimates for soluble NPOC and pH in the stepwise multivariate regression models are the opposite direction of actual values, as soluble NPOC was under a reciprocal transformation and pH was converted to hydrogen ion concentration.

Explanatory variable	Parameter estimate	R^2	p-value	n
All samples				
Electrical conductivity	3.73E+06	**8%**	0.0275	63
Temporal variation				
Autumn				
Ammonium	9.02E+05	**73%**	<.0001	15
Winter				
pH	-1.23E+12	**37%**	0.0211	14
Spring				
Total C	4.68E+06	**36%**	0.0115	17
Summer				
Total inorganic N	4.77E+07	63%	0.0002	
Total		**63%**		17
Spatial variation				
Summit				
Electrical conductivity	5.89E+06	**30%**	0.0121	20
Backslope				
Total C	-1.18E+06	33%	0.0066	
Nitrate	2.70E+06	18%	0.0197	
Total		**51%**		21
Footslope				
No significant variables				22

Table 8. Stepwise multivariate regression models for archaeal *amoA* abundance overall as well as for each season and topographical position. Variables are listed in the order they were entered into the model. Variables were entered into the model and stayed in the model if they had a corresponding *p*-value less than 0.05.

Explanatory variable	Parameter estimate	R^2	p-value	n
All samples				
No significant variables				63
Temporal variation				
Autumn				
No significant variables				
Winter				
No significant variables				14
Spring				
Total N	-2.74E+07	**25%**	0.0395	17
Summer				
No significant variables				17
Spatial variation				
Summit				
No significant variables				20
Backslope				
Soil water content	-1.17E+05	**30%**	0.0085	22
Footslope				
No significant variables				21

Denitrifier and nitrifier community structure

Seasonally, *nirK*, bacterial *amoA* and archaeal *amoA* exhibited greater structural diversity in the autumn, as this is when *nirK* had the greatest number of T-RFS and bacterial and archaeal *amoA* had the greatest β diversity value (Table 9). In considering topographical trends in diversity, *nirK* had the greatest structural diversity at the summit, as this is where richness and β diversity was greatest. For bacterial and archaeal *amoA*, greater structural diversity was seen at the footslope, as both exhibited a greater β diversity value and bacterial *amoA* also exhibited a greater number of T-RFs.

The percent change between seasons and topographical positions was typically greater than 40% for *nirK*, bacterial *amoA* and archaeal *amoA* (Figure 4). This indicates that the structure of denitrifier and nitrifier populations shifts dramatically between seasons and also exhibits large variability topographically within a field. The *nirK* communities at the summit and footslope positions diversified over the year study from autumn to summer whereas the backslope communities exhibited little change (Figure 4A). The bacterial *amoA* communities showed greater diversification from winter to spring than from autumn to winter or spring to summer regardless of position (Figure 4B). Archaeal *amoA* communities exhibited different seasonal trends depending on the topographical position and the backslope and footslope showed the most diversification (Figure 4C). The trend in topographical percent change differed among the populations studied. The *nirK* communities showed the least amount of change in diversity across topographical positions in the autumn, with relatively more topographic difference in the winter and spring (Figure 4D). The bacterial *amoA* communities showed little difference in diversity between topographical positions in the spring (Figure 4E). Archaeal *amoA*

communities exhibited differences in diversity between topographical positions for every season (Figure 4F).

Table 9. Average richness and β diversity for each topographical position and season. Richness = total number of T-RFs. The β diversity = S/α-1, where S = total number of T-RFs and α = average number of T-RFs. A greater value indicates greater dissimilarity among samples.

		nirK		**Bacterial *amoA***		**Archeal *amoA***	
		Richness	β Diversity	Richness	β Diversity	Richness	β Diversity
Season	Autumn	44	0.63	12	1.06	27	1.10
	Winter	30	0.87	10	1.00	31	0.74
	Spring	29	0.88	12	0.70	21	0.90
	Summer	31	0.63	10	0.86	33	1.07
Topographical position	Summit	37	1.18	9	1.17	23	1.09
	Backslope	31	0.97	11	0.95	36	1.23
	Footslope	33	0.78	13	1.26	26	1.31

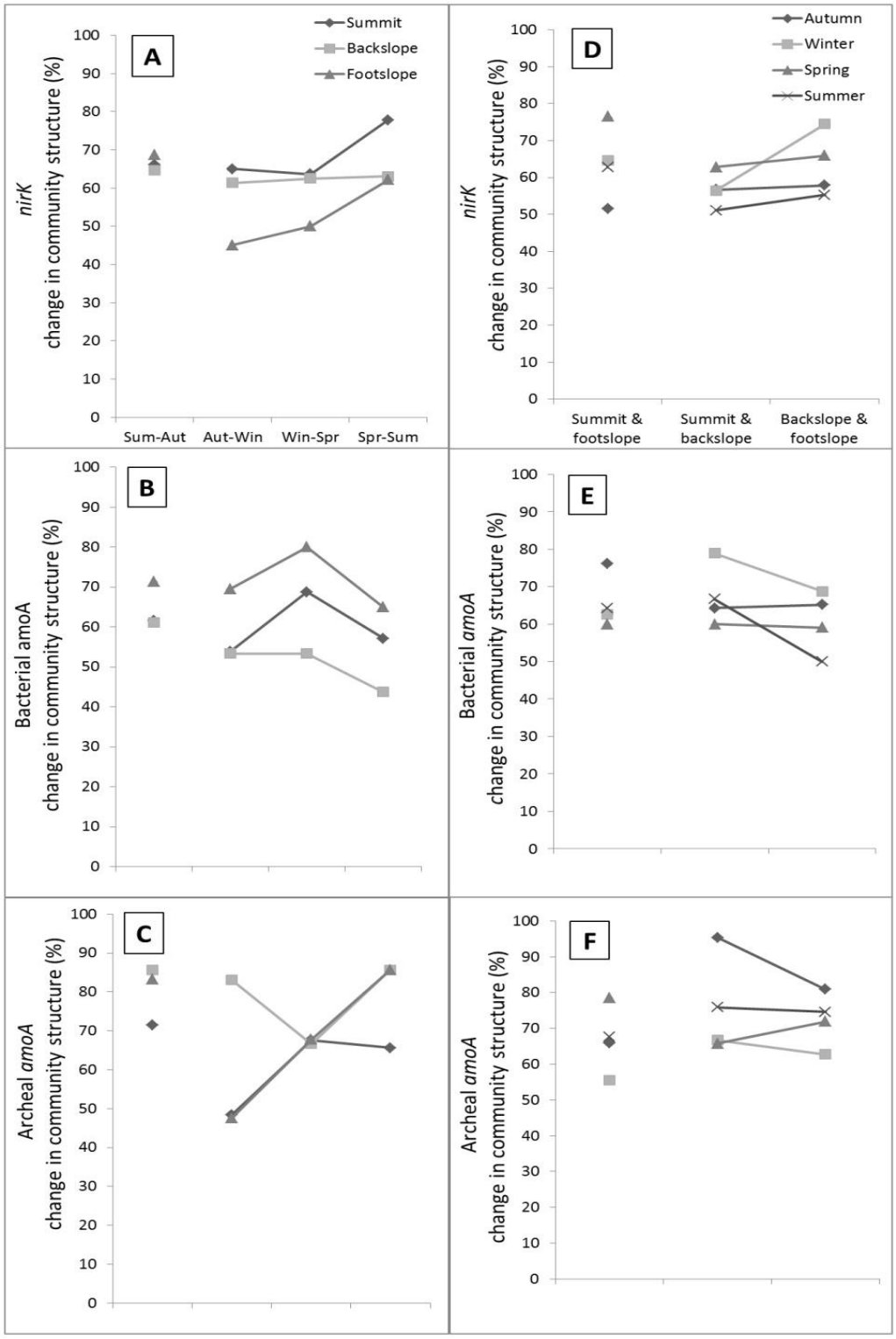

Figure 4. Moving window analysis of *nirK*, bacterial *amoA* and archaeal *amoA* percent change in community structure between seasons at each topographical position (A, B, and C) and between topographical positions for each season (D,E, and F). % change = 100% - Jaccard similarity.

DISCUSSION

The seasonal and topographical variation of denitrifier and nitrifier population size had not yet been studied extensively. Considering that we found significant seasonal and topographical variation for denitrifier and nitrifier abundance, it is recommended to analyze seasons and topographical positions separately when studying these populations. This allows for results to be appropriately compared among studies. In addition, this finding informed our decision to identify soil environmental drivers of denitrifier and nitrifier abundance by season and topographical position, as significant seasonal and topographical variation in abundance may be the result of seasonal or topographical shifts in drivers.

Spatio-temporal variation was observed in potential drivers of denitrifier and nitrifier abundance, as explanatory factors were not shared among seasons or topographical positions. The abundances of the populations studied did not have any significant explanatory variables (at a *p*-value of 0.05) for certain seasons and topographical positions with the given soil environmental measurements. In addition, bacterial and archaeal *amoA* community structure did not have any significant explanatory variables at a *p*-value of 0.05 from the given soil environmental measurements. The drivers of denitrifier and nitrifier abundance for certain seasons and topographical positions, as well as the drivers of bacterial and archaeal *amoA* community structure, were not described by the measured soil environmental characteristics. For both *nirK* and bacterial *amoA*, the R-square of the separate seasonal and topographical models were sometimes greater than that of the overall model, indicating season and topography may be useful variables for studying these populations.

Spatio-temporal variation was also observed for *nirK* abundance as a predictor of

potential denitrification. Seasonal variation explains in part the discrepancy among other studies regarding the correlation between denitrifier abundance and denitrification. In our study, *nirK* abundance and potential denitrification were significantly correlated in the autumn, but not in the winter, spring and summer (Pearson's correlation test, *p*-value=0.05). Several studies that did not find a correlation between potential denitrification and denitrifier abundance either contained samples that were taken in the summer or only sampled in the summer, which could have masked or did not capture correlation that may have been occurring in other seasons (Dandie at al., 2008; Dandie et al., 2011). Likewise, a study that did find a correlation between denitrification and denitrifier abundance sampled mainly in the spring and autumn (Attard et al., 2011).

We found *nirK* abundance was only useful in predicting potential denitrification if topographical variation was considered: *nirK* abundance was able to improve prediction of potential denitrification for the summit model, although it did not improve prediction for the other models. The *nirK* abundance did not contribute to prediction of basal denitrification and *nirK* community structure (T-RF richness) did not contribute to prediction of basal or potential denitrification. This agrees with other studies that have also found *nirK* community structure and denitrification rates to be uncorrelated (Rich and Myrold, 2004; Wertz et al., 2009; Dandie et al., 2011; Chen et al., 2012). Intentional laboratory modifications of the denitrifier community has been shown to alter denitrification rates (Philippot et al., 2013), indicating the lack of correlation between community structure and denitrification is possibly because denitrifier diversity does not reach levels low enough to cause observable changes in denitrification.

Community structure of the denitrifier and nitrifier populations studied was shown to differ among season and topographical positions. Seasonally, *nirK*, bacterial *amoA* and archaeal

amoA exhibited greater structural diversity in the autumn. Topographically, *nirK* had the greatest structural diversity was at the summit while bacterial and archaeal *amoA* had greater structural diversity at the footslope. The community structure of the denitrifier and nitrifier populations studied was found to shift between seasons and between topographical positions within a field, as the percent change between seasons and topographical positions was typically above 40%.

Although this study found significant differences among seasons and topographical positions for denitrifier and nitrifier abundance, there are limitations to the assessment of both of these variables. Each season only had one sampling date; having more sampling dates within each season would have developed more robust seasonal data. Topographical variation was within a field; to establish topographical differences at a more regional scale would require sampling across fields. In addition, during the study the footslope was cropped to winter wheat while the summit and backslope were cropped to spring wheat; this may have caused the results to be differentiated due to the crop rather than difference in topographical position. However, it has not been established as to whether these populations differ between crops as similar as winter wheat and spring wheat. Because of the difference in crops, there was also a difference in fertilizer events (winter wheat was fertilized in the fall, while spring wheat was not), and possibly a difference in soil moisture from crop uptake. Fertilizer was banded in 30 cm intervals at 10 cm depth during seed placement and samples were collected at the top 0-5 cm. If the soil sampling had inadvertently captured the banded fertilizer, we would expect significant variability in the inorganic N measurements between the replicate soil samples after the fertilization events. It does not appear that the fertilizer influenced the soil chemistry results since the standard error for inorganic N in the autumn and winter was not greater at the footslope compared to the

summit and backslope (Chapter 1). Moreover, there were no significant differences in soil moisture content between topographical positions for any season (Chapter 1). Regardless, it would be preferable to assess topographical positions that are simultaneously in the same crop.

This study found that *nirK* abundance can help in prediction of potential denitrification, but only when topographical variation was considered. This should assist future studies in what biological variables are useful in predicting denitrification. Since only *nirK* was studied, studying other denitrifier functional gene targets may have differing predictive capability for denitrification. Studying *nirS* as well would capture more of the denitrification community, as these two genes encode enzymes that perform the same function in the denitrification pathway but have evolved independently (Philippot et al., 2007). Other studies, however, have correlated different genes involved in denitrification to denitrification activity (Miller et al., 2008; Hallin et al., 2009; Dandie et al., 2011; Chen et al., 2012).

REFERENCES

Ambus, P., 1998. Nitrous oxide production by denitrification and nitrification in temperate forest, grassland and agricultural soils. European Journal of Soil Science 49, 495–502.

Arah, J.R.M., Smith, K.A., 1989. Steady-state denitrification in aggregated soils: a mathematical model. Journal of Soil Science 40, 139–149.

Arp, D.J., Stein, L.Y., 2003. Metabolism of inorganic N compounds by ammonia-oxidizing bacteria. Critical Reviews in Biochemistry & Molecular Biology 38, 471–495.

Attard, E., Recous, S., Chabbi, A., De Berranger, C., Guillaumaud, N., Labreuche, J., Philippot, L., Schmid, B., Le Roux, X., 2011. Soil environmental conditions rather than denitrifier abundance and diversity drive potential denitrification after changes in land uses. Global Change Biology 17, 1975–1989.

Avrahami, S., Conrad, R., Braker, G., 2002. Effect of soil ammonium concentration on N_2O release and on the community structure of ammonia oxidizers and denitrifiers. Applied Environmental Microbiology 68, 5685–5692.

Berg, P., Klemedtsson, L., Rosswall, T., 1982. Inhibitory effect of low partial pressures of acetylene on nitrification. Soil Biology and Biochemistry 14, 301–303.

Bergstermann, A., Cárdenas, L., Bol, R., Gilliam, L., Goulding, K., Meijide, A., Scholefield, D., Vallejo, A., Well, R., 2011. Effect of antecedent soil moisture conditions on emissions and isotopologue distribution of N_2O during denitrification. Soil Biology and Biochemistry 43, 240–250.

Braker, G., Fesefeldt, A., Witzel, K.-P., 1998. Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. Applied Environmental Microbiology 64, 3769–3775.

Braker, G., Zhou, J., Wu, L., Devol, A.H., Tiedje, J.M., 2000. Nitrite reductase genes (*nirK* and *nirS*) as functional markers to investigate diversity of denitrifying bacteria in Pacific Northwest marine sediment communities. Applied Environmental Microbiology 66, 2096–2104.

Bremer, C., Braker, G., Matthies, D., Reuter, A., Engels, C., Conrad, R., 2007. Impact of plant functional group, plant species, and sampling time on the composition of *nirK*-type denitrifier communities in soil. Applied Environmental Microbiology 73, 6876–6884.

Bremer, C., Braker, G., Matthies, D., Beierkuhnlein, C., Conrad, R., 2009. Plant presence and species combination, but not diversity, influence denitrifier activity and the composition of nirK-type denitrifier communities in grassland soil. FEMS Microbiology Ecology 70, 377–387.

Cambardella, C.A., Moorman, T.B., Parkin, T.B., Karlen, D.L., Novak, J.M., Turco, R.F., Konopka, A.E., 1994. Field-scale variability of soil properties in central Iowa soils. Soil Science Society of America Journal 58, 1501-1511.

Chen, Z., Liu, J., Wu, M., Xie, X., Wu, J., Wei, W., 2012. Differentiated response of denitrifying communities to fertilization regime in paddy soil. Microbial Ecology 63, 446–459.

Conyers, M.K., Uren, N.C., Helyar, K.R., 1995. Causes of changes in pH in acidic mineral soils. Soil Biology and Biochemistry 27, 1383–1392.

Culman, S.W., Gauch, H.G., Blackwood, C.B., Thies, J.E., 2008. Analysis of T-RFLP data using analysis of variance and ordination methods: A comparative study. Journal of Microbiological Methods 75, 55–63.

Dandie, C.E., Burton, D.L., Zebarth, B.J., Henderson, S.L., Trevors, J.T., Goyer, C., 2008. Changes in bacterial denitrifier community abundance over time in an agricultural field and

their relationship with denitrification activity. Applied Environmental Microbiology 74, 5997–6005.

Dandie, C.E., Wertz, S., Leclair, C.L., Goyer, C., Burton, D.L., Patten, C.L., Zebarth, B.J., Trevors, J.T., 2011. Abundance, diversity and functional gene expression of denitrifier communities in adjacent riparian and agricultural zones. FEMS Microbiology Ecology 77, 69–82.

Davidson, E.A., 2009. The contribution of manure and fertilizer nitrogen to atmospheric nitrous oxide since 1860. Nature Geoscience 2, 659–662.

De Boer, W., Kowalchuk, G.., 2001. Nitrification in acid soils: micro-organisms and mechanisms. Soil Biology and Biochemistry 33, 853–866.

Dobbie, K.E., Smith, K.A., 2003. Nitrous oxide emission factors for agricultural soils in Great Britain: the impact of soil water-filled pore space and other controlling variables. Global Change Biology 9, 204–218.

Drury, C.F., Myrold, D.D., Beauchamp, E.G., Reynolds, W.D., 2008. Denitrification techniques for soils. In: Carter, M.R., Gregorich, E.G. (Eds.), Soil sampling and methods of analysis. Canadian Society of Soil Science, Boca Raton, Fla, pp. 471-493.

Elliott, J.A., de Jong, E., 1993. Prediction of field denitrification rates: a boundary-line approach. Soil Science Society of America Journal 57, 82.

Environmental Protection Agency USA. 2014. Inventory of U.S. greenhouse gas emissions and sinks: 1990-2011. Washington D.C.

Ercolini, D., 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. Journal of Microbiological Methods 56, 297–314.

Florinsky, I.V., McMahon, S., Burton, D.L., 2004. Topographic control of soil microbial activity: a case study of denitrifiers. Geoderma 119, 33–53.

Folorunso, O.A., Rolston, D.E., 1984. Spatial variability of field-measured denitrification gas fluxes. Soil Science Society of America Journal 48, 1214.

Forster, P., Ramaswamy, V., Artaxo, P., Berntsen, T., Betts, R., Fahey, D.W., Haywood, J., Lean, J., Lowe, D.C., Myhre, G., Nganga, J., Prinn, R.G., Raga, G., Schulz, M., Van Dorland, R., 2007. Changes in atmospheric constituents and in radiative forcing. In: Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Avery, K.B., Tignor, M., Miller, H.L. (Eds.), Climate Change 2007: the Physical Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom, pp. 129–234.

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

Franzluebbers, A.J., Hons, F.M., Zuberer, D.A., 1995. Tillage and crop effects on seasonal soil carbon and nitrogen dynamics. Soil Science Society of America Journal 59, 1618.

Gao, H., Yang, Z.K., Gentry, T.J., Wu, L., Schadt, C.W., Zhou, J., 2007. Microarray-based analysis of microbial community RNAs by whole-community RNA amplification. Applied Environmental Microbiology 73, 563–571.

Gleeson, D.B., Herrmann, A.M., Livesley, S.J., Murphy, D.V., 2008. Influence of water potential on nitrification and structure of nitrifying bacterial communities in semiarid soils. Applied Soil Ecology 40, 189–194.

Groffman, P.M., Tiedje, J.M., 1989. Denitrification in north temperate forest soils: Spatial and temporal patterns at the landscape and seasonal scales. Soil Biology and Biochemistry 21, 613–620.

Gubry-Rangin, C., Nicol, G.W., Prosser, J.I., 2010. Archaea rather than bacteria control nitrification in two agricultural acidic soils. FEMS Microbiology Ecology 74, 566–574.

Guo, X., Drury, C.F., Yang, X., Reynolds, W.D., Zhang, R., 2011. Influence of current and previous crops on soil basal and potential denitrification rates. Biology and Fertility of Soils 47, 937–947.

Hallin, S., Jones, C.M., Schloter, M., Philippot, L., 2009. Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. ISME Journal 3, 597–605.

Hammer, Ø., Harper, D.A.T., Ryan., P.D., 2001. PAST: Paleontological statistics software package for education and data analysis, Palaeontologia Electronica 4, 9.

Heid, C.A., Stevens, J., Livak, K.J., Williams, P.M., 1996. Real time quantitative PCR. Genome Research 6, 986–994.

Hénault, C., Germon, J.C., 2000. NEMIS, a predictive model of denitrification on the field scale. European Journal of Soil Science 51, 257–270.

Henry, S., Baudoin, E., López-Gutiérrez, J.C., Martin-Laurent, F., Brauman, A., Philippot, L., 2004. Quantification of denitrifying bacteria in soils by *nirK* gene targeted real-time PCR. Journal of Microbiological Methods 59, 327–335.

Henry, S., Bru, D., Stres, B., Hallet, S., Philippot, L., 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. Applied Environmental Microbiology 72, 5181–5189.

Holtan-Hartwig, L., Dörsch, P., Bakken, L.R., 2000. Comparison of denitrifying communities in organic soils: kinetics of NO−3 and N_2O reduction. Soil Biology and Biochemistry 32, 833–843.

Kaiser, E.-A., Kohrs, K., Kücke, M., Schnug, E., Heinemeyer, O., Munch, J.C., 1998. Nitrous oxide release from arable soil: importance of N-fertilization, crops and temporal variation. Soil Biology and Biochemistry 30, 1553–1563.

Keeney, D.R., Nelson, D.W., 1982. Inorganic forms of nitrogen. In: Page, A.L. (Ed.), Methods of soil analysis. Part 2. Chemical and microbiological properties. American Society of Agronomy, Inc., Soil Science Society of America, Inc., Madison, WI, pp. 643-698.

Klemedtsson, L., Svensson, B.H., Rosswall, T., 1988. A method of selective inhibition to distinguish between nitrification and denitrification as sources of nitrous oxide in soil. Biology and Fertility of Soils 6, 112–119.

Lauber, C.L., Hamady, M., Knight, R., Fierer, N., 2009. Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. Applied Environmental Microbiology 75, 5111–5120.

Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G.W., Prosser, J.I., Schuster, S.C., Schleper, C., 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442, 806–809.

Linn, D.M., Doran, J.W., 1984. effect of water-filled pore space on carbon dioxide and nitrous oxide production in tilled and nontilled soils. Soil Science Society of America Journal 48, 1267–1272.

Liu, W.T., Marsh, T.L., Cheng, H., Forney, L.J., 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. . Applied Environmental Microbiology 63, 4516–4522.

Luo, J., White, R.E., Roger Ball, P., Tillman, R.W., 1996. Measuring denitrification activity in soils under pasture: optimizing conditions for the short-term denitrification enzyme assay and effects of soil storage on denitrification activity. Soil Biology and Biochemistry 28, 409–417.

Magurran, A.E., 1988. Ecological diversity and its measurement. Princeton University Press, Princeton, New Jersey, p. 91.

Miller, M.N., Zebarth, B.J., Dandie, C.E., Burton, D.L., Goyer, C., Trevors, J.T., 2008. Crop residue influence on denitrification, N_2O emissions and denitrifier community abundance in soil. Soil Biology and Biochemistry 40, 2553–2562.

Morales, S.E., Cosart, T., Holben, W.E., 2010. Bacterial gene abundances as indicators of greenhouse gas emission in soils. ISME J 4, 799–808.

Mummey, D.L., Smith, J.L., Bolton Jr, H., 1994. Nitrous oxide flux from a shrub-steppe ecosystem: Sources and regulation. Soil Biology and Biochemistry 26, 279–286.

Mummey, D.L., Smith, J.L., Bolton Jr, H., 1997. Small-scale spatial and temporal variability of N_2O flux from a shrub-steppe ecosystem. Soil Biology and Biochemistry 29, 1699–1706.

Nicol, G.W., Leininger, S., Schleper, C., Prosser, J.I., 2008. The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. Environmental Microbiology 10, 2966–2978.

Parsons, L.L., Smith, M.S., Murray, R.E., 1991. Soil denitrification dynamics: spatial and temporal variations of enzyme activity, populations, and nitrogen gas loss. Soil Science Society of America Journal 55, 90.

Pell, M., Stenberg, B., Stenström, J., Torstensson, L., 1996. Potential denitrification activity assay in soil—with or without chloramphenicol? Soil Biology and Biochemistry 28, 393–398.

Pereira e Silva, M.C., Semenov, A.V., van Elsas, J.D., Salles, J.F., 2011. Seasonal variations in the diversity and abundance of diazotrophic communities across soils. FEMS Microbiology Ecology 77, 57–68.

Philippot, L., 2002. Denitrifying genes in bacterial and archaeal genomes. Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression 1577, 355–376.

Philippot, L., Hallin, S., Schloter, M., 2007. Ecology of denitrifying prokaryotes in agricultural soil. Advances in Agronomy 96, 135-190.

Philippot, L., Spor, A., Hénault, C., Bru, D., Bizouard, F., Jones, C.M., Sarr, A., Maron, P.A., 2013. Loss in microbial diversity affects nitrogen cycling in soil. ISME Journal 7, 1609–1619.

Ravishankara, A.R., Daniel, J.S., Portmann, R.W., 2009. Nitrous oxide (N_2O): the dominant ozone-depleting substance emitted in the 21st Century. Science 326, 123–125.

Reardon, C.L., Strauss, S.L., Mazzola, M., 2013. Changes in available nitrogen and nematode abundance in response to Brassica seed meal amendment of orchard soil. Soil Biology and Biochemistry 57, 22–29.

Reardon, C.L., Strauss, S.L., Mazzola, M., 2014. Diazotroph community structure and abundance in wheat-fallow and wheat-pea crop rotation. Soil Biology and Biochemistry 69, 1-7.

Rich, J.J., Myrold, D.D., 2004. Community composition and activities of denitrifying bacteria from adjacent agricultural soil, riparian soil, and creek sediment in Oregon, USA. Soil Biology and Biochemistry 36, 1431–1441.

Rotthauwe, J.H., Witzel, K.P., Liesack, W., 1997. The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Applied Environmental Microbiology 63, 4704–4712.

Ruhe, R.V., 1969. Quaternary landscapes in Iowa.

Sherrod, L.A., Peterson, G.A., Westfall, D.G., Ahuja, L.R., 2005. Soil organic carbon pools after 12 Years in no-till dryland agroecosystems. Soil Science Society of America Journal 69, 1600.

Šimek, M., Jíšová, L., Hopkins, D.W., 2002. What is the so-called optimum pH for denitrification in soil? Soil Biology and Biochemistry 34, 1227–1234.

Six, J., Ogle, S.M., Jay breidt, F., Conant, R.T., Mosier, A.R., Paustian, K., 2004. The potential to mitigate global warming with no-tillage management is only realized when practised in the long term. Global Change Biology 10, 155–160.

Skiba, U., Smith, K.., 2000. The control of nitrous oxide emissions from agricultural and natural soils. Chemosphere - Global Change Science 2, 379–386.

Smith, J.L., Halvorson, J.J., 2011. Field scale studies on the spatial variability of soil quality indicators in Washington state, USA. Applied and Environmental Soil Science 2011.

Smith, K.., Thomson, P.., Clayton, H., Mctaggart, I.., Conen, F., 1998. Effects of temperature, water content and nitrogen fertilisation on emissions of nitrous oxide by soils. Atmospheric Environment 32, 3301–3309.

Smith, M.S., Tiedje, J.M., 1979. Phases of denitrification following oxygen depletion in soil. Soil Biology and Biochemistry 11, 261–267.

Slattery, W., Ronnfeldt, G., 1992. Seasonal variation of pH, aluminium, and manganese in acid soils from north-eastern Victoria. Aust. J. Exp. Agric. 32, 1105–1112.

Stöckle, C., Higgins, S., Kemanian, A., Nelson, R., Huggins, D., Marcos, J., Collins, H., 2012. Carbon storage and nitrous oxide emissions of cropping systems in eastern Washington: A simulation study. Journal of Soil and Water Conservation 67, 365–377.

Strauss, S.L., Reardon, C.L., Mazzola, M., 2014. The response of ammonia-oxidizer activity and community structure to fertilizer amendment of orchard soils Soil Biology & Biochemistry 68, 410-418.

Teepe, R., Brumme, R., Beese, F., 2001. Nitrous oxide emissions from soil during freezing and thawing periods. Soil Biology and Biochemistry 33, 1269–1275.

Tiedje, J.M., 1982. Denitrification. In: Page, A.L., Miller, R.H., Keeney, D.R. (Eds.), methods of soil analysis. part 2. chemical and microbiological properties. American Society of Agronomy, Madison, WI, pp. 1011–1026.

Torsvik, V., Goksøyr, J., Daae, F.L., 1990. High diversity in DNA of soil bacteria. Applied Environmental Microbiology 56, 782–787.

USDA, 1978. Palouse cooperative river basin study. SCS, Forest Service, and Economics, Statistics, and Cooperative Extension Service. U.S. Government Print, Washington, D.C.

Van Kessel, C., Pennock, D.J., Farrell, R.E., 1993. Seasonal variations in denitrification and nitrous oxide evolution at the landscape scale. Soil Science Society of America Journal 57, 988–995.

Vilain, G., Garnier, J., Tallec, G., Cellier, P., 2010. Effect of slope position and land use on nitrous oxide (N_2O) emissions (Seine Basin, France). Agricultural and Forest Meteorology 150, 1192–1202.

Wagner-Riddle, C., Thurtell, G.W., Kidd, G.K., Beauchamp, E.G., Sweetman, R., 1997. Estimates of nitrous oxide emissions from agricultural fields over 28 months. Canadian Journal of Soil Science 77, 135–144.

Wertz, S., Dandie, C.E., Goyer, C., Trevors, J.T., Patten, C.L., 2009. Diversity of *nirK* denitrifying genes and transcripts in an agricultural soil. Applied Environmental Microbiology 75, 7365–7377.

Wolsing, M., Priemé, A., 2004. Observation of high seasonal variation in community structure of denitrifying bacteria in arable soil receiving artificial fertilizer and cattle manure by determining T-RFLP of *nir* gene fragments. FEMS Microbiology Ecology 48, 261–271.

Wood, C.W., Westfall, D.G., Peterson, G.A., 1991. Soil carbon and nitrogen changes on initiation of no-till cropping systems. Soil Science Society of America Journal 55, 470.

Wrage, N., Velthof, G.., van Beusichem, M.., Oenema, O., 2001. Role of nitrifier denitrification in the production of nitrous oxide. Soil Biology and Biochemistry 33, 1723–1732.

Yao, H., Gao, Y., Nicol, G.W., Campbell, C.D., Prosser, J.I., Zhang, L., Han, W., Singh, B.K., 2011. Links between ammonia oxidizer community structure, abundance, and nitrification potential in acidic soils. Applied Environmental Microbiology. 77, 4618–4625.

Ye, L., Zhang, T., 2011. Ammonia-oxidizing bacteria dominates over ammonia-oxidizing archaea in a saline nitrification reactor under low DO and high nitrogen loading. Biotechnology and Bioengineering 108, 2544–2552.

Yoshinari, T., Hynes, R., Knowles, R., 1977. Acetylene inhibition of nitrous oxide reduction and measurement of denitrification and nitrogen fixation in soil. Soil Biology and Biochemistry 9, 177–183.