September 10, 2016

MS No. soil-2016-34
To Dr. Fuensanta García-Orenes (Topical Editor, SOIL);

Please accept the following manuscript for resubmission to SOIL, entitled "**Soil Denitrifier Community Size Changes with Land Use Change to Perennial Bioenergy Cropping Systems**'', by authors Karen A. Thompson, Bill Deen and Kari E. Dunfield.

Thank you for the consideration of our original manuscript, and the constructive comments provided by the reviewers and editor. After careful consideration of the reviewer and editor comments, we have made minor revisions to the manuscript, and feel that it is greatly improved.

As specifically requested by reviewers we have added a section in the intro about our choice of the 16S rRNA gene target, made minor revisions to increase clarity, and have addressed reviewer #3's concerns in our point-by-point reply, as in the online discussion. We have also addressed concerns from the assessment of the original submission. Please refer to the Response to Reviewers for a detailed description of all of the revisions. We have also included a version of the manuscript with major changes highlighted.

Yours sincerely,

Karen Thompson and Kari Dunfield

Responses to Editor and Reviewer Comments re: SOIL MS No. soil-2016-34

Topical Editor Initial Decision: Publish subject to technical corrections (03 May 2016) by Fuensanta García-Orenes
Comments to the Author:
Please clarify how many real soil samples do you take to extract the ADN and analyze the chemical parameters, because:
the 10 samples in each treatment were mixed and two DNA samples extracted (then the two DNA extracts were mixed). This would prevent being able to see whether the variation within each treatment are larger or smaller than the variations between treatments. Also - two samples might be insufficient.

Response: this has been clarified in the methods section; the field trial consisted of 3 replicates of 10 treatment plots (n=30). Two subsamples were used for DNA extraction from each plot.

Second - there is no report by the authors on the quality assurance of the primers - in particular the 16S primers. Heterogenicity (different frgment size) in the soil DNA amplification products means that quantification may be inaccurate.

Response: This has been addressed in the methods, "*The 16S rRNA primers used are degenerate and have been cited as having 89-91% matching efficiency to all bacteria (Bergmark et al., 2012). The primers amplify one of two conserved regions in V3 of the SSU rRNA gene, resulting in a ca. 200 bp amplicon that is within small enough to amplify via qPCR methodology and amplifies for most bacterial taxa (Bakke et al., 2011).*"

Third- The statistical analysis are not enough, please try to provide the correlations between soil properties and some PCA or cannonical analysis.

Response: We have conducted a PCA analysis as recommended.

Anonymous Referee #1:

The manuscript represents a good contribution to scientific progress within the scope of SOIL; it includes a multidisciplinary approach and also this is good. The results are well discussed in a balanced way and conclusions are presented in a clear and cocise way; the English is appropriate. The approach and applied methods are valid even if I have some doubts about the choice of the gene used in qPCR, because of the reason I explain below. The authors aim was to compare the effects of LUC from corn-soybean to PG biomass production on the relative abundances of total (16S rRNA gene target) and denitrifier (nirS and nosZ gene 94 targets) soil bacterial communities. But, from literature (Case et al., 2007 Appl. Env. Microbiol. 278–288; Vetrovsky

and Baldrian, 2013 PLoSONE 8(2): e57923. doi:10.1371/journal.pone.0057923) we know that the 16S rRNA gene copy numbers per genome vary from 1 up to 15 or more copies. This limits the interpretation of 16S rRNA-derived results, specifically for a quantitative interpretation of the soil bacterial community. The use of a single-copy in this case would be more appropriate and could allow for a more accurate measurement of microbial community. Thus, I suggest to the author to add some more reasons about the choice of 16SrRNA gene for bacterial quantitative purposes.

Response:

Thank you for your review of our paper.

We will adjust the language we use regarding our interpretation of our 16S rRNA results in the discussion as advised. Additionally, we will add in some text regarding our choice of 16S rRNA as follows: 16S rRNA was chosen as a molecular target for the total bacterial community size; although 16S rRNA gene copies vary from 1-15 copies per genome, its use has continued to be the 'gold standard' for microbial ecology (Case et al., 2007; Vos et al., 2012). Although an alternate target, such as rpoB, which is a single copy gene would be valuable if assessing phylogenetic diversity, there are no universal primers for it (Adékambi et al., 2009) as rpoB is not conserved enough to be of use as a universal marker and only a subset of the microbial community can be targeted (Vos et al., 2012). Therefore in order to use rpoB as a target we would have had to design a suite of different primer sets to target several orders within the same bacterial phylum, which was not feasible for this paper, and would not have measured total bacterial abundance from our diverse environmental soil samples. Taking this into account, many studies have used 16S rRNA gene copy numbers as a proxy for the total bacterial community size; and some have found that the total estimated numbers of proteobacteria species was not significantly different if using rpoB or 16S rRNA markers (Vos et al., 2012). As this study has not assessed phylogenetic relationships of the microbial communities, 16S rRNA is an appropriate target for the relative comparison of the overall bacterial community size between environmental treatments/variables.

Specifically, this text has been added to the manuscript to address this concern:

"16S rRNA was chosen as a molecular target for the total bacterial community size; although 16S rRNA gene copies vary from 1-15 copies per genome, its use has continued to be the 'gold standard' for microbial ecology (Case et al., 2007; Vos et al., 2012). Although an alternate target, such as rpoB, which is a single copy gene would be valuable if assessing phylogenetic diversity, there are no universal primers for it (Adékambi et al., 2009) as rpoB is not conserved enough to be of use as a universal marker and only a subset of the microbial community can be targeted (Vos et al., 2012). Many studies have used 16S rRNA gene copy numbers as a proxy for the total bacterial community size, and some have found that the total estimated numbers of proteobacteria species was not significantly different if using rpoB or 16S rRNA markers (Vos et

al., 2012). As this study has not assessed phylogenetic relationships of the microbial communities, 16S rRNA is an appropriate target for the relative comparison of the overall bacterial community size between environmental treatments/variables."

With these additional references:

Adékambi, T., Drancourt, M., Raoult, D., 2009. The rpoB gene as a tool for clinical microbiologists. Trends Microbiol. 17, 37–45. doi:10.1016/j.tim.2008.09.008

Case, R.J., Boucher, Y., Dahllöf, I., Holmström, C., Doolittle, W.F., Kjelleberg, S., 2007. Use of 16S rRNA and rpoB genes as molecular markers for microbial ecology studies. Appl. Environ. Microbiol. 73, 278–88. doi:10.1128/AEM.01177-06

Vos, M., Quince, C., Pijl, A.S., Hollander, M. De, Kowalchuk, G.A., 2012. A Comparison of rpoB and 16S rRNA as Markers in Pyrosequencing Studies of Bacterial Diversity 7, 1–8. doi:10.1371/journal.pone.0030600

Anonymous Referee #2:

The manuscript from Thompson et al. is generally well structured, concise and informative. Results from their research, with higher biomass production from miscanthus but lower N2O flux, have great potential for soil science, agriculture, economics and climate change mitigations if their results can be further validated in future studies. As the authors mentioned in Conclusions, future measurements of N2O fluxes and other relevant N cycling processes is critical in linking microbial communities to actual N2O mitigation benefits during land use change. N2O fluxes are highly variable, which raises my concern on how to interpret the information from soil denitrifier community size.

Is 4 time samplings (May 9th, 2011; October 30th, 2011; May 2nd, 2012 and C1 SOILD Interactive comment Printer-friendly version Discussion paper October 20th, 2012) enough to represent the link between soil denitrifier community size and N2O fluxes, to differentiate seasonal changes?

Response: Thank you for this comment. We believe that the timing of our sampling encompasses both seasonal changes and changes that may occur due to cropping system/management practices. Our objective was not to directly link N_2O fluxes to these communities, but rather to assess whether biomass cropping systems and their management influenced the size of the denitrifier communities (ie the functional potential of these communities). Our sampling approach gave results showing significant changes in these communities based on cropping system and their management, validating our sampling choices.

Specific comments: 1, lines 61-65, confusing, need to clarify 2, lines 61-72, would it be better to add the reason why focus on N2O?

Response: Wording of lines 61-65 have been edited to clarify (comment #1) and a sentence at the start of this paragraph has been added to provide linkage between N2O and denitrification (comment #2):

"*N_2O is a potent greenhouse gas with a global warming potential 296x that of CO2 (IPCC 2007). However, measuring N_2O directly in the field is often difficult with chamber methods in cropping systems that produce large aboveground biomass. Additionally, including multiple field treatments (eg: RCBD design) make micrometeorological methods of N_2O flux impossible to obtain. Instead, relative abundances of denitrifier genes can be used to assess a soil's potential to produce (e.g. nirS or nirK) and consume (e.g. nosZ) N_2O via denitrification, representing a qualitative proxy of relative N_2O emission potential of a soil (Butterbach-Bahl et al., 2013; Hallin et al., 2009; Morales et al., 2010; Petersen et al., 2012; Philippot, 2002). Denitrifier community size has been correlated with denitrification process rates (Hallin et al., 2009; Wu et al., 2012), and denitrification potential (Attard et al., 2011; Cuhel et al., 2010; Enwall et al., 2010). Potential denitrifying activity and denitrifying community size have also been shown to be correlated with each other in some studies (Hallin et al., 2009; Morales et al., 2010; Szukics et al., 2010; Throback et al., 2007); suggesting community size may indicate potential differences in soil N processes after LUC. Particularly, the nosZ-bearing community may act as a N_2O sink and counter high N_2O production rates (Braker and Conrad, 2011; Philippot et al., 2011), therefore influencing N_2O emissions (Cuhel et al., 2010; Morales et al., 2010; Philippot et al., 2011)*."

3, line 97, there are only two N fertilization rates, 0 and 160 kgN ha-1, "multiple" is not appropriate

Response: Within the overall field trial, there are 4 N fertilization rates (0,80, 120 and 160 kgN ha-1). We chose two (unfertilized and 160N) for assessment within our study; however we will change the wording here.

4, line 115, add . after) **Response:** Thank you, done.

5, lines 119-120, N fertilization rates are confusing, "46-0-0" and "34-0-0" need further explanation **Response:** Thank you, we will add in "N-P-K" to denote chemical make-up.

6, line 127, capital words in subtitles are not coherent **Response:** Thank you, we will address this.

7, lines 155-156, strange position under 2.2 Soil sampling, suggest relocate to 2.1 Site Description **Response:** We agree, we will move this section accordingly.

8, lines 234-235, no context for Ho **Response:** Thank you, we will address this.

9, line 249, please explain "S. Ontario" **Response:** Done, we will write out "Southern Ontario".

10, lines 243-256, authors refer to Roy et al. 2014 for result of environmental conditions instead of Fig.1. Are precipitation and temperature taken from Roy et al. 2014 ? If so, it would be better to also mention it in the Figure caption. If only soil moisture is measured, it would be better to descript soil moisture conditions instead of only mention that soil moisture "could also impact soil N and soil bacterial communities".

Response: Thank you, we will edit for clarification. We use Roy et al. (2014) in text for 30 year average data, and whereas the data in figure 1 (precipitation and temperature) was collected from the Elora Research Station over the 2year study.

11**,** line 275, . after p **Response:** Thank you, we will check this.

12, line 339, "years 2 and 3", please specify what 2 and 3 refer to **Response:** We have adjusted the language to indicated 2 and 3 years after planting.

Anonymous Referee #3:

Thank you for your review of our work. Please find responses to your comments below.

First, the adopted DNA soil extraction method do not permit to discriminate between relic DNA pool and the intracellular poll, without considering the PMA approach to discriminate by qPCR between relic and living cells due to contradictory results on its efficiency on soil environment.

Response: Although this is true, at the time this research was conducted (2011-2012), there were no published PMA protocols for environmental matrices, such as soil. Additionally, although some studies have shown an impact of relic DNA on diversity meaC1 SOILD Interactive comment Printer-friendly version Discussion paper sures (Carini et al., 2016), others have shown that despite PMA decreasing extracted DNA yields, these decreases did not have a subsequent impact on fingerprinting measures, such as DGGE (Wagner et al., 2015). In this article, we aren't comparing taxonomic diversity etc. but making comparisons of gene abundances (functional potential) within one soil type between crop treatments. Therefore, the comparisons of gene abundances are still relevant. Finally, although the use of PMA in environmental matrices is still being improved upon, the efficiency of PMA on different taxa is unknown, and PMA permeability into cells might vary across taxa, indicating that we should interpret PMA-treated data with caution. There has also been some evidence (Taylor et al., 2014) that depending on the environmental matrix assessed and extraction method, at higher concentrations PMA may bind to DNA in viable cells, leaving only dormant state microbes, and therefore not be effective in differentiating viable and non-viable cells.

Paul Carini, Patrick J Marsden, Jonathan W Leff, Emily E Morgan, Michael S Strickland,Noah Fierer. Relic DNA is abundant in soil and obscures estimates of soil microbial diversitybioRxiv 043372; doi: http://dx.doi.org/10.1101/043372

Taylor MJ, Bentham RH, Ross KE. Limitations of Using Propidium Monoazide with qPCR to Discriminate between Live and Dead Legionella in Biofilm Samples. Microbiology Insights. 2014;7:15-24. doi:10.4137/MBI.S17723.

Wagner AO, Praeg N, Reitschuler C, Illmer P. Effect of DNA extraction procedure, repeated extraction and ethidium monoazide (EMA)/propidium monoazide (PMA) treatment on overall DNA yield and impact on microbial fingerprints for bacteria, fungi and archaea in a reference soil. Applied soil ecology: a section of Agriculture, Ecosystems & Environment. 2015;93:56-64. doi:10.1016/j.apsoil.2015.04.005.

Second, it is no possible to discriminate between the different nitrification/denitrification pathway and the related microbial community.

Response: I think you are inferring that we cannot connect functional potential (gene abundances) with community composition/identification, or process rates. This was not our intent, and we have not attempted to directly link the denitrification pathway with gene abundance data, but have instead assessed the sustainability of these cropping systems based on functional gene abundances involved in the denitrification pathway.

Third, It is also not possible to discriminate which of the detected species is active in the gene function without mRNA detection.

Response: We agree that it is not possible to assess potential activity with DNA-based methods. However, mRNA has a halflife of minutes and was thought to be inappropriate for assessment of denitrifier communities due to the timing of sampling in our study (which was over 2 years). It is more plausible to assess the potential functionality of the soil microbial community to cropping systems when sampling over the longterm than attempting to link differences in mRNA with edaphic factors or agricultural treatments as mRNA may be upregulated in response to short-term (in the order of minutes, hours) changes in soil and climatic properties, while DNA analyses may allow better differentiation of changes due to cropping systems.

Fourth, it is no possible to discriminate between the potential activity and the real activity of the nirS and nosK bacterial species.

Response: We have not attempted to, or claimed to, assess potential activity (mRNA) or real activity (enzymes) of denitrifiers in this study.

Finally result impossible to obtain extremely interesting data by coupling these data with those related to soil N2O/N2 emission.

Response: Although we would have preferred to measure N2O emissions, the fieldset up did not permit this. First, the plots were too small and numerous (N=36) to establish eddy covariance/flux towers. Secondly, due to the large root biomass, above ground biomass and overall ground coverage of miscanthus and switchgrass plants, after consulting with a

micrometeorologist, we were advised that it would be impossible to install chambers within our plots without highly disturbing the area, and therefore obtaining biased results. Our focus was in assessing the sustainability of the cropping systems by comparing the functional potential of the soils to produce or consume N2O by quantifying denitrifier gene targets.

Soil Denitrifier Community Size Changes with Land Use Change to Perennial Bioenergy Cropping Systems

Running Head: Soil Denitrifiers associated with Perennial Grasses

Karen A. Thompson[1], Bill Deen[2] and *Kari E. Dunfield[1]

[1]School of Environmental Sciences, University of Guelph, 50 Stone Road East, Guelph, Ontario N1G 2W1, Canada; [2]Department of Plant Agriculture, University of Guelph, 50 Stone Road East, Guelph, Ontario N1G 2W1, Canada.

*Correspondence to Kari E. Dunfield:

tel: (519) 824-4120 Ext.58088, *dunfield@uoguelph.ca*, *fax:*519-837-0756

Original Research Article

Abstract

Dedicated biomass crops are required for future bioenergy production. However, the effects of large-scale land use change (LUC) from traditional annual crops, such as corn-soybean rotations to the perennial grasses (PGs) switchgrass and miscanthus on soil microbial community functioning is largely unknown. Specifically, ecologically significant denitrifying communities, which regulate N_2O production and consumption in soils, may respond differently to LUC due to differences in carbon (C) and nitrogen (N) inputs between crop types and management systems. Our objective was to quantify bacterial denitrifying gene abundances as influenced by corn-soybean crop production compared to PG biomass production. A field trial was established in 2008 at the Elora Research Station in Ontario, Canada (n=30), with miscanthus and switchgrass grown alongside corn-soybean rotations at different N rates (0 and 160 kg N ha^{-1}) and biomass harvest dates within PG plots. Soil was collected on four dates from 2011-2012 and quantitative PCR was used to enumerate the total bacterial community (16S rRNA), and communities of bacterial denitrifiers by targeting nitrite reductase (*nirS*) and N_2O reductase (*nosZ*) genes. Miscanthus produced significantly larger yields and supported larger *nosZ* denitrifying communities than corn-soybean rotations regardless of management, indicating large-scale LUC from corn-soybean to miscanthus may be suitable in variable Ontario climatic conditions and under varied management, while potentially mitigating soil N_2O emissions. Harvesting switchgrass in the spring decreased yields in N-fertilized plots, but did not affect gene abundances. Standing miscanthus overwinter resulted in higher 16S rRNA and *nirS* gene copies than in fall-harvested crops. However, the size of the total (16S rRNA) and denitrifying bacterial communities changed differently over time and in response to LUC, indicating varying controls on these communities.

Comment [K1]: C McCall comment: variable management

Key Words: biomass, bioenergy, miscanthus, switchgrass, corn, soy

1. Introduction

Future energy needs require dedicated biomass crop production for bioethanol and combustion-based electricity generation. Corn (*Zea mays* L.) –soybean (*Glycine max* L.) rotations currently dominate the landscape across Ontario and the northern US Corn Belt (Gaudin et al., 2015), and corn grain is currently the primary feedstock for bioethanol production in Canada (Jayasundara et al., 2014). The C4 perennial grasses (PGs) switchgrass (*Panicum virgatum* L.) and miscanthus (*Miscanthus* spps.) have been proposed as alternate feedstock crops to corn for biomass-based bioenergy production due to their large biomass yields, reduced nitrogen (N) and water requirements, decreased nutrient leaching and potential for increased soil carbon (C) storage (Blanco-Canqui and Lal, 2009; Foster et al., 2013). Large scale production of C4 PGs in Ontario and the northern Corn Belt would require land use change (LUC) from existing corn-soybean rotations to PG biomass cropping systems (Deen et al., 2011; Kludze et al., 2013; Liang et al., 2012; Sanscartier et al., 2014).

Few studies have assessed how this LUC may influence soil microbial community functioning. In particular, soil denitrifying communities represent an ideal subset of the soil microbial community to target to assess changes in ecosystem functioning due to agricultural management and LUC. Denitrifying bacteria represent approximately 5% of the total soil microbial biomass (Braker and Conrad, 2011) and have been identified in over 60 genera (Philippot, 2006), encompassing a wide range of phylogenetic and functional diversity. Multiple studies have linked changes in denitrifier communities with plant types or development stage (Bremer et al., 2007; Hai et al., 2009; Petersen et al., 2012), N fertilization (Hallin et al., 2009; Yin et al., 2014), organic or conventional crop management (Reeve et al., 2010), perennial vs. annual crop land

use (Bissett et al., 2011) and C and N inputs (Bastian et al., 2009). These studies suggest that LUC from corn-soybean rotations to PG species may influence the soil bacterial communities which drive soil N_2O production and consumption.

N_2O is a potent greenhouse gas with a global warming potential 296x that of CO_2 (IPCC 2007). However, measuring N_2O directly in the field is often difficult with chamber methods in cropping systems that produce large aboveground biomass. Additionally, including multiple field treatments (eg: as in a RCBD design) make micrometeorological methods of N_2O flux impossible to obtain. Instead, relative abundances of denitrifier genes can be used to assess a soil's potential to produce (e.g. *nirS* or *nirK*) and consume (e.g. *nosZ*) N_2O via denitrification, representing a qualitative proxy of relative N_2O emission potential of a soil (Butterbach-Bahl et al., 2013; Hallin et al., 2009; Morales et al., 2010; Petersen et al., 2012; Philippot, 2002). Denitrifier community size has been correlated with denitrification process rates (Hallin et al., 2009; Wu et al., 2012), and denitrification potential (Attard et al., 2011; Cuhel et al., 2010; Enwall et al., 2010). Potential denitrifying activity and denitrifying community size have also been shown to be correlated with each other in some studies (Hallin et al., 2009; Morales et al., 2010; Szukics et al., 2010; Throback et al., 2007); suggesting community size may indicate potential differences in soil N processes after LUC. Particularly, the *nosZ*-bearing community may act as a N_2O sink and counter high N_2O production rates (Braker and Conrad, 2011; Philippot et al., 2011), therefore influencing N_2O emissions (Cuhel et al., 2010; Morales et al., 2010; Philippot et al., 2011).

Comment [K2]: Edited as per reviewer #2's comment

Denitrification *nirS* and *nosZ* gene targets represent the two most important steps in the denitrification pathway that produce gaseous by-products, and account for a large proportion of functional N genes in some studies (Stone et al., 2015). The first step in denitrification that

produces a gaseous N product is the reduction of nitrite (NO_2-) to nitric oxide (NO), catalyzed by nitrite reductases either encoded by the cytochrome cd_1 (*nirS*) or copper-containing (*nirK)* genes, which are equivalent but have not been detected within the same species (Zumft, 1997). We chose to quantify *nirS* because ¾ of cultured denitrifiers possess the *nirS* gene (Zumft, 1997) and some molecular reports indicate *nirS* may dominate in abundance over *nirK* in some natural environments (Deslippe et al., 2014; Nogales et al., 2002), indicating it may be a better-suited target for relative characterization of potential nitrite-reducing communities than *nirK*. Additionally, *nirK* has been recently identified in autotrophic ammonia-oxidizing species (Cantera and Stein, 2007; Casciotti and Ward, 2001), calling into question its utility in specifically targeting denitrifying communities. The *nosZ* target codes for nitrous oxide reductase, which catalyzes the reduction of N_2O to N_2 in the denitrification pathway, indicating *nosZ*-bearing communities help to complete the N cycle and determine the N_2O:N_2 balance. 16S rRNA was chosen as a molecular target for the total bacterial community size; although 16S rRNA gene copies vary from 1-15 copies per genome, its use has continued to be the 'gold standard' for microbial ecology (Case et al., 2007; Vos et al., 2012). Although an alternate target, such as rpoB, which is a single copy gene would be valuable if assessing phylogenetic diversity, there are no universal primers for it (Adékambi et al., 2009) as rpoB is not conserved enough to be of use as a universal marker and only a subset of the microbial community can be targeted (Vos et al., 2012). Many studies have used 16S rRNA gene copy numbers as a proxy for the total bacterial community size, and some have found that the total estimated numbers of proteobacteria species was not significantly different if using rpoB or 16S rRNA markers (Vos et al., 2012). As this study has not assessed phylogenetic relationships of the microbial

Comment [K3]: C McCall comment re: move to intro

104 communities, 16S rRNA is an appropriate target for the relative comparison of the overall
105 bacterial community size between environmental treatments/variables.

106 LUC resulting from displacement of corn-soybean rotations by PG production may alter soil
107 microhabitats and therefore soil microbial N-cycling due both to extensive root and rhizome
108 biomass and to large leaf litter inputs to soils in perennial vs. annual systems (Dohleman et al.,
109 2012). Within studies targeting soil microbial communities in biomass cropping systems to date
110 (Hedenec et al., 2014; Liang et al., 2012; Mao et al., 2013a, 2011; Orr et al., 2015), the effects of
111 various management practices (e.g. N fertilization and harvest) on soil microbial community
112 functioning have not been an area of focus. The effect this type of LUC may have on soil
113 microbial communities may depend on PG management practices in these systems.

114 Currently, there is no consensus regarding optimal N fertilization practices for increased yields in
115 PG production as yield responses can be highly variable depending on environmental conditions
116 and crop species (Deen et al., 2011). Depending on downstream use, miscanthus can be either
117 harvested in the fall pre-frost, harvested post-frost kill, or left to overwinter as standing biomass
118 for harvest in the spring. Switchgrass is commonly harvested in the fall, and is often windrowed
119 (cut, swathed and left on soil) over winter due to producers' limitations in collecting and storing
120 harvested biomass in winter (Resource Efficient Agricultural Production (REAP)-Canada, 2008;
121 Sokhansanj et al., 2009). Differences in N fertilizer requirements and harvest regimes may alter
122 C and N inputs (Attard et al., 2011) and may influence LUC impacts on soil denitrifier
123 community sizes.

124 Our objective was to compare the effects of LUC from corn-soybean to PG biomass production
125 on the relative abundances of total (16S rRNA gene target) and denitrifier (*nirS* and *nosZ* gene

targets) soil bacterial communities 3-4 years after PG planting. Soil was collected on four dates from 2011 to 2012 from a field trial established in Ontario in 2008. This study is unique in that it consists of two PG biomass crops produced in parallel with the existing common land use of corn-soybean rotation within the same field trial site. It also includes unfertilized and fertilized plots in both annual and perennial systems, and varied harvest practices within PG plots. We hypothesized that soils from PG plots would support larger total bacterial and denitrifier communities than soils from corn-soybean plots due to increased shoot residue return and root inputs to soils in PG systems, and that soils from PG plots with biomass harvested in the spring would support larger total bacterial and denitrifier communities than supported by soils from PGs harvested in the fall due to increased root inputs and leaf loss to soil over winter.

Comment [K4]: Re C McCall comment

Comment [K5]: As per reviewer #2's comment

2. Materials and Methods

2.1 Site Description and Experimental Design

A field trial was established in 2008 at the University of Guelph Research Station in Elora, ON (43°38'46.73" N and 80°24'6.66" W). The field site was cultivated on May 16th and June 6th, 2008. Switchgrass (*Panicum virgatum* L., Shelter variety) was planted on June 6th, 2008. Miscanthus (*M. sinensis* x *M. sacchariflorus*, Nagara-116 variety) was planted on June 12th, 2008, and soybean (*Glycine max* L.) was planted on June 24th, 2008 and annually rotated with corn (*Zea mays* L.). Corn was planted on May 5th, 2010; soy was planted on June 3rd, 2011, and corn was re-planted on May 18th, 2012, with annual light cultivation to prepare seedbeds for planting. In 2007, prior to trial establishment, the experimental area was planted to barley (*Hordeum vulgare* L.). The soil type is a London silt loam (Gray Brown Luvisol).

148 The field trial was a split-split strip plot design with three replicates. The main plot factor was
149 PG crop or annual rotation (miscanthus, switchgrass, and corn-soybean). Main treatment plots
150 measured 6.2 m x 26.0 m. Nitrogen fertilizer (0 or 160 kg N ha^{-1}) was applied in strips
151 randomly within replicates. 160 kg N ha^{-1} subplots received hand-broadcast urea fertilizer (46-0-
152 0; N-P-K) in May 2011 or hand-broadcast ammonium nitrate fertilizer (34-0-0; N-P-K) in May
153 2012, after soil sampling procedures described below. Main treatments were split into two
154 harvest timings (fall or spring) within the PG fertilizer strips only. Miscanthus plots were either
155 harvested in the late fall season after post-frost kill, or left standing to overwinter until spring
156 harvest. Switchgrass plots were harvested in the fall, or cut and assembled into windrows in the
157 field for biomass removal in the spring. Spring-harvest of PGs occurred before N fertilizer was
158 applied. Harvest methods of PG yields (dry harvested biomass (tonnes) ha^{-1}) are described in
159 Deen et al. (2011). Figure 1 illustrates the seasonal and annual variation in daily average air
160 temperature (°C), and daily precipitation (mm) measured at the Elora Research Station.

Comment [K6]: As per reviewer #2's comments

Comment [K7]: Moved as per reviewer #2's comment

161

162 **2.2 Soil Sampling and Analysis**

163 Baseline bulk density and carbon measurements were measured for each main plot on October
164 23rd, 2010. For bulk density, two soil cores per plot were collected at 0-5 cm depth using 2.5
165 cm-diameter cylindrical aluminum cores. Cores were weighed before and after drying for 24h at
166 105°C (Maynard & Curran, 2007). For soil carbon analysis, ten soil cores per plot were
167 collected from both 0-15 cm and 15-30 cm depths using a 5 cm-diameter soil corer on a zed-
168 shaped transect, and then composited per treatment plot for each depth. Total soil carbon and
169 inorganic carbon were analyzed with a Leco® Carbon Determinator CR-12 (Model No. 781-700,

Leco Instruments Ltd.) following the dry combustion technique (Périé and Ouimet, 2008) on approximately 0.300 g of dried, ground and homogenized soil (Table 1).

For molecular analyses, soil was sampled on 4 dates (May 9th, 2011; October 30th, 2011; May 2nd, 2012 and October 20th, 2012). October sampling dates occurred before fall harvest of PG crops, while May sampling dates occurred before N fertilizer application and after spring PG biomass removal (Fig. 1). Ten soil cores per plot were sampled aseptically to 15 cm depth using a 5 cm-diameter soil corer on a zed-shaped transect, composited and kept on ice until transport back to the laboratory. The transect shape was chosen to encompass plot heterogeneity; at a pre-trial study date initial analysis indicated gene abundances were not significantly different between bulk or rhizosphere soils in corn-soybean or PG plots, possibly due to the large root biomass/leaf loss to soils in perennial plots and residual soy/corn residue cover on soil in corn-soybean plots. Soil samples were divided for storage at 4˚C and -20˚C.

Mean values of gravimetric soil moisture (g g^{-1}) are shown in Figure 1. Soil exchangeable NO_3^--N and NH_4^+-N were determined for each of the soil samples by KCl extraction. Soil samples (10.0 g) were placed into 125 mL flasks and 100 mL of 2.0M KCl was added to each flask. Flasks were stoppered and shaken for 1h at 160 strokes per minute; solutions were allowed to settle and were then filtered through Whatman no. 42 filter paper (Whatman plc, ME, U.S.A). Extractable NO_3^--N and NH_4^+-N were determined colourmetrically with segmented flow analyses (AA3, SEAL Analytical, Wisconsin, USA) via a cadmium reduction (Technician Instrument Corporation, 1971), and a Berthelot reaction respectively (Fig. 2).

190 **2.3 Soil DNA Extraction**

191 Total DNA was extracted from field-moist soil sampled from each plot (3 field replicates, n=3;
192 total plots n=30). DNA was extracted in duplicate (ca. 0.250g) within 48 h of sampling as per
193 manufacturer's protocol using the DNA PowerSoil Kit (Mobio, Carlsbad, USA). Duplicate
194 extracts were then pooled, separated into aliquots, and stored at -80˚C until use in downstream
195 analyses.

196 **2.4 Quantification of total bacteria and functional genes**

197 Quantitative PCR (qPCR) assays were used to enumerate the total bacterial communities (16S
198 rRNA gene), and communities of denitrifiers by targeting nitrite reductase (*nirS*) and nitrous
199 oxide reductase (*nosZ*) genes, using primer pairs 338f/518r (16S rRNA, Fierer et al., 2005),
200 Cd3af/R3Cd (*nirS,* Throbäck et al., 2004) and 1F/1R *(nosZ,* Henry et al., 2006).

201 For each gene target analyzed, duplicate replicates were run in parallel on an IQ5 thermocycler
202 (Bio-Rad Laboratories, Hercules, CA, USA). qPCR reaction mixtures contained 12.5 μL of 1x
203 SYBR Green Supermix, each forward and reverse primer at a final concentration of 400 nM, 1
204 μL of DNA template and RNase/DNase-free water to a final volume of 25 μL. The SYBR Green
205 Supermix contained 100 nM KCl, 40 mM Tris-HCl, 0.4 mM dNTPs, 50 units mL^{-1} iTaq DNA
206 polymerase, 6 mM $MgCl_2$, SYBR Green 20 nM fluorescein, and stabilizer (Bio-Rad
207 Laboratories, Hercules, CA, USA).

208 Conditions for qPCR were an initiation step at 94°C for two minutes, followed by 35 cycles of
209 denaturing at 94°C for thirty seconds, annealing at 57°C for thirty seconds (16S rRNA) or at
210 55°C for sixty seconds (*nirS*), followed by elongation at 72°C for thirty (16S rRNA) or sixty
211 (*nirS*) seconds. For *nosZ,* a touchdown protocol adapted from Henry et al., (2006) was used.

212 Amplicon specificity was screened by running qPCR products on an ethidium bromide-stained
213 gel (1% agarose, 80 volts for 20 minutes) with a 100bp ladder, which resulted in clean bands for
214 all gene targets. The 16S rRNA primers used are degenerate and have been cited as having 89-
215 91% matching efficiency to all bacteria (Bergmark et al., 2012). The primers amplify one of two
216 conserved regions in V3 of the SSU rRNA gene, resulting in a ca. 200 bp amplicon that is within
217 small enough to amplify via qPCR methodology and amplifies for most bacterial taxa (Bakke et
218 al., 2011)

219 Known template standards were made from cloned PCR products from pure culture genomic
220 DNA (*Clostridium thermocellum (*16S*), Pseudomonas aeruginosa (nirS),* and *Pseudomonas*
221 *fluorescens (nosZ)*) and transformed into *Escherichia coli* plasmids (TOPO TA cloning kit);
222 plasmids were sequenced to confirm successful cloning and transformation of the target genes.
223 Amplicon specificity was screened by running PCR products on an ethidium bromide-stained gel
224 (1% agarose, 80 volts for 20 minutes) with a 100bp ladder. PCR amplicons of cloned gene
225 targets were sequenced by the Laboratory Services Department at the University of Guelph using
226 an ABI Prism 3720 (Applied Biosystems, Foster City, CA, USA) to confirm target identity.

227 In all qPCR assays, all unknown samples were amplified in parallel with a triplicate serial
228 dilution (10^1-10^8 gene copies per reaction) of control plasmids. PCR assays were optimized to
229 ensure efficiencies ranging from 93.0-106.4%, with R^2s ranging from 0.990-0.999 and standard
230 curve slopes of -3.177 to -3.408 by testing serial dilutions of DNA extracts in order to minimize
231 inhibition of amplification due to humic and fulvic contaminants. Duplicate no-template
232 controls were run for each qPCR assay, which gave null or negligible values. Melt curve
233 analysis was used to confirm amplicon specificity. Normalization of DNA concentrations to

gram of dry soil was used to give results on a biologically significant scale, which assumes similar DNA isolation efficiency across samples..

2.5 Statistical Analysis

Analysis of variance was conducted in SAS 9.3 (Carlsbad, NC, USA) using a generalized linear mixed model (PROC GLIMMIX). The Shapiro-Wilks test was used to test for normality of data; studentized test for residuals confirmed the absence of outliers. The probability distributions of gene abundance data sets were log normal or highly skewed and were analyzed using an overdispersed Poisson distribution for count data (Ver Hoef and Boveng, 2007). Bulk density, organic carbon, total carbon, nitrate and ammonium data were log transformed when required and fitted to the normal distribution.

Within each data set, sampling time was a repeated measure; independent and interactive fixed effects were associated with crop/crop rotation, nitrogen application rate and harvest timing within perennial grasses, while field replicate and its associated interactions were random effects. The residual maximum likelihood method was employed to fit the model for all data sets. Several covariance structures were entertained before the variance components structure was chosen based on convergence and model fitting criteria. Individual treatment means within data sets were compared using a post-hoc Tukey's test for all pairwise comparisons, with significance denoted at $p<0.05$.

Correlation analysis was used to assess nonparametric measures of statistical dependence between gene abundances and H_2O, NO_3^--N and NH_4^+-N measured over time (Supplementary Table 1). Correlation analysis resulted in multiple significant correlations between variables; as

such a principal component analysis was conducted in SAS (PROC FACTOR) on 120 samples using a VARIMAX rotation.

3. Results

3.1 Environmental and Soil Conditions

Environmental conditions varied during the periods prior to the four soil sampling dates (Figure 1). Average air temperatures over the growing seasons (May-October) were 16.9°C and 17.3°C in 2011 and 2012 respectively (Roy et al. 2014); average air temperatures in spring 2012 were warmer than normal and resulted in earlier emergence of PG crops compared to 2011. Cumulative monthly precipitation was above average prior to the May 2011 sampling date (101 mm vs. 72 mm 30-year average in April 2011 and 113 mm vs. 82 mm 30-year average in May 2011) (Roy et al., 2014). In comparison, Southern Ontario received very low cumulative precipitation in April 2012 (30 mm vs. 72 mm 30-year average) and May 2012 (28 mm vs. 82 mm 30-year average) (Roy et al., 2014). Cumulative precipitation levels were lower in 2012 compared to 2011 from May-August (391 mm in 2011 vs. 186 mm in 2012), however higher than normal precipitation levels occurred in October of 2011 (129 mm vs. 77 mm 30-year average) and both September (106 mm vs. 77 mm 30-year average) and October (127 mm vs. 77 mm 30-year average) of 2012 (Roy et al., 2014). Environmental conditions prior to soil sampling directly impact soil gravimetric content measured at time of sampling (Fig. 1), and could also impact soil N and soil bacterial communities.

Comment [K8]: Language amended to clarify – cumulative precip and average air temps from Roy et al.

Soil physical and chemical properties were assessed in October 2010, after only two years of contrasting management since crop establishment in 2008. The corn-soybean rotation had higher soil bulk density than soils from both miscanthus and switchgrass plots harvested in the fall. No differences in total or organic soil carbon were detected between the corn-soybean rotation and the PG treatments at either the 0-15cm or 15-30cm depth (Table 1). Soil NH_4-N levels did not

279 differ significantly between the corn-soybean rotation and the PG soils, however N fertilization
280 significantly increased NH_4-N levels in soils from fall-harvested miscanthus plots ($p<0.05$) (Fig.
281 2a). N fertilization also significantly increased NO_3-N levels in spring-harvested switchgrass
282 ($p<0.05$) (Fig. 2b). From May to October 2011, soil NH_4-N levels increased significantly and
283 soil NO_3-N levels decreased significantly in PG soils (data not shown); a similar trend was not
284 observed in 2012 or for soils from the corn-soybean rotation in either year.

285 **3.2 Biomass Yields**

286 Despite significant differences in precipitation between 2011 and 2012, biomass yields of
287 miscanthus and switchgrass did not differ between years. In comparison, corn grain yields were
288 higher in 2011 vs 2012 (Table 1). Miscanthus produced higher yields (12.7-18.3 dry tonnes ha^{-1})
289 than either switchgrass or corn grain, regardless of N fertilization rate or harvest timing (Table
290 1). When harvested in the fall and N-fertilized, switchgrass yields were not significantly lower
291 (10.5-11.1 dry tonnes ha^{-1}) than miscanthus yields. Switchgrass yields from unfertilized plots
292 were not significantly different if harvested in the fall or spring; however, switchgrass yields
293 from fertilized plots were significantly higher (ca. 3-4 dry tonnes ha^{-1}) when harvested in the fall
294 compared to yields obtained when switchgrass was windrowed over winter.

295 **3.3 Bacterial Responses to Annual and Perennial Crops and their Management**

296 There was no statistically significant effect of N fertilization or any significant interactions
297 between cropping system and sampling time on any of the targeted gene abundances. Therefore
298 we analyzed the impact of each biomass crop under specific harvest management on soil
299 bacterial gene abundances (Table 2). Denitrifying (*nosZ*) gene copy abundances were affected
300 by LUC; regardless of harvest or N management, mean *nosZ* gene copies were higher in

miscanthus plots than in the corn-soybean rotation, and *nirS:nosZ* ratios were higher in the corn-soybean soils than in miscanthus or switchgrass soils ($p<0.05$) (Table 2). Under fall harvesting management, biomass crop had no impact on total bacterial 16S rRNA gene copies or *nirS* gene copies. However, leaving miscanthus biomass standing overwinter until spring resulted in significantly higher 16S rRNA gene copies than observed in soils from fall-harvested biomass crops and significantly higher *nirS* gene copies than in fall-harvested switchgrass or the corn-soybean rotation (Table 2).

3.3 Temporal Changes in Bacterial Gene Abundances

Sampling date had a significant impact on gene abundances for all genes quantified (Fig. 3). Over both sampling years, 16S rRNA gene copies were significantly higher ($5.2\text{-}5.4 \times 10^9$ gene copies g^{-1} dry soil) at fall (October) sampling dates compared to the ca. $5.5\text{-}6.4 \times 10^8$ gene copies g^{-1} dry soil quantified at spring (May) sampling dates (Fig. 3). Populations of *nirS* and *nosZ* denitrifiers represented ca. 1.58% and 0.26% on a gene-to-gene basis *(nirS* or *nosZ to* 16S*)* of the total bacterial community (data not shown), and did not follow similar trends with time of sampling (Fig. 3). The abundance of *nirS* gene copies was significantly higher in 2012 (4.0×10^6 – 1.6×10^7 gene copies g^{-1} dry soil) compared to 2011 ($2.5\text{-}6.3 \times 10^5$ gene copies g^{-1} dry soil), with no significant differences between May and October sampling dates within each year (Fig. 3). The abundance of *nosZ* gene copies were approximately $1.3\text{-}3.2 \times 10^5$ gene copies g^{-1} dry soil, but increased significantly in May 2012 to approximately 3.2×10^6 gene copies g^{-1} dry soil and dropped back to previous levels by October 2012 (Fig. 3). Higher relative proportions of denitrifiers (*nirS* or *nosZ to*16S*)* were observed at spring sampling dates, when total bacterial 16S rRNA gene abundances decreased in comparison to fall sampling dates (Fig. 3).

323 Two factors were selected in the principal components analysis, which accounted for 67.73%
324 cumulative variance. A scree plot was examined for breaks and factors were retained when
325 eigenvalues ≥ 1. Soil NH_4-N^+, soil NO_3^--N, *nirS* and *nosZ* loaded on factor 1, which accounted
326 for 43.89% variance while soil gravimetric H_2O and 16S rRNA loaded on factor 2, which
327 accounted for 23.84% variance (Fig.4 a and b). Differences in soil NO_3^--N and NH_4^+-N were
328 strongly related to differences in *nirS* and *nosZ* gene abundances observed between May 2011
329 and May 2012 sampling dates (Fig. 3 and Fig. 4), while the size of the total bacterial community
330 (16S rRNA) was related to soil gravimetric moisture levels (Fig. 4).

331 **4. Discussion**

332 Denitrification is an important process contributing to the production and consumption of N_2O in
333 soils, and mitigation of GHGs such as N_2O is required to create sustainable biomass cropping
334 systems (Miller et al., 2008; Schlesinger, 2013). Changes in the potential functional abilities of
335 the soil microbial community may reflect changes in LUC or agricultural management and
336 should be considered to assess the ecological impact of biomass crop production (Hedenec et al.,
337 2014). Currently, few studies have assessed soil microbial community responses to PG biomass
338 production systems (Hedenec et al., 2014; Liang et al., 2012; Mao et al., 2013a, 2011; Orr et al.,
339 2015). The highest potential to reduce GHG emissions from biomass cropping systems is to
340 produce crops with high yields, such as PGs (Sanscartier et al., 2014), which offset the amount of
341 land required for crop production (Kludze et al., 2013). However, if PG biomass production
342 negatively affects soil health as indicated by changes in the potential functioning of microbial
343 communities, large-scale LUC from annual to perennial biomass production may not be as
344 sustainable as originally proposed. As such it is necessary to identify biomass cropping systems

that not only result in large biomass yields, but also ensure agroecosystem sustainability by maintaining or improving ecosystem services (Orr et al., 2015), such as soil N-cycling.

4.1 Biomass Yields of Annual and Perennial Crops

Miscanthus and switchgrass biomass yields were within the typical range of values reported previously in Ontario (Kludze et al., 2013; Resource Efficient Agricultural Production (REAP)-Canada, 2008) and Europe (Christian et al., 2008; Himken et al., 1997), despite differences in temperature and precipitation between the two study years. Corn grain yields were within the lower range for reported Ontario yields (Munkholm et al., 2013), potentially due to wetter (2011) and drier (2012) field conditions than normal over the two growing seasons (Roy et al., 2014). Deen et al. (2011) showed increases in PG biomass yields between the second and third years after PG planting at our site, whereas we measured similar yields in 2011 and 2012, indicating the PGs may have reached maximum yield potential.

Comment [K9]: As per reviewer #2's comment

Nitrogen fertilization significantly increased corn grain yields and fall-harvested switchgrass biomass yields, however no significant increases due to N fertilization were observed in miscanthus or spring-harvested switchgrass biomass yields. Potential yield increases from N fertilization in spring-harvested switchgrass may have been offset due to leaf loss over the winter season, as increases in switchgrass yields to N fertilization have been previously observed (Nikièma et al., 2011; Vogel et al., 2002). Similar to the present study, European and US field trials have also found no response of miscanthus yields to N (Lewandowski et al., 2000; Lewandowski et al., 2003; Behnke et al., 2012; Christian et al., 2008), and PG yields were minimally impacted by differences in growing season conditions compared to corn grain yields (Table 1).

Despite significant differences in biomass yields between miscanthus and corn-soybean systems, there were no significant differences in either total or organic soil carbon between any of cropping systems assessed (Table 1). Sampling of soil carbon occurred only two years after PG planting; PGs are expected to be productive for 20+ years, indicating future changes in soil carbon levels may occur. Additionally, Ontario-based land conversion modelling scenarios have estimated a soil carbon decrease of 2.5% upon miscanthus establishment (Sanscartier et al., 2014), which may have negated potential increases in soil organic carbon. However, high miscanthus yields most likely resulted in increases in above and below-ground plant residue return to soils (Mutegi et al., 2010; Soil Quality National, 2006); therefore our carbon measures may not have reflected short-term changes in labile carbon sources that had occurred. Regardless of management or climatic conditions, miscanthus consistently produced large yields, emphasizing its potential as a bioenergy crop suitable for production in variable Ontario conditions.

4.2 Bacterial Responses to Annual and Perennial Crops and their Management

Some studies in biomass cropping systems have not observed differences in soil microbial responses between perennial and annual crop types (Mao et al., 2011), while others have measured significant differences in microbial abundance, diversity and community structure between these cropping types (Liang et al., 2012; Morales et al., 2010; Watrud et al., 2013). Currently, we observed significantly higher *nosZ* gene copies in miscanthus soils compared to corn-soybean soils, illustrating a distinct effect of LUC from corn-soybean to miscanthus production on soil N cycling (Table 2).

Due to the large biomass produced by miscanthus compared to corn, a large amount of plant residues are returned to the soil; these residues provide surface cover, decrease soil bulk density, increase water retention and regulate temperatures (Blanco-Canqui and Lal, 2009). Previous work at the Elora Research Station found an inverse correlation between field-scale N_2O fluxes and *nosZ* transcript abundance in conventionally-tilled corn plots with residues returned to soils (Németh et al., 2014), and increased *nosZ* activity after residue amendment has also been observed in lab studies (Henderson et al., 2010). High C:N plant residues have been negatively correlated with cumulative N_2O emissions (Huang, 2004), and may encourage complete reduction of N_2O to N_2 as soil available NO_3-N is limiting, so bacterial populations with the ability to reduce N_2O to N_2 are favoured (Miller et al., 2008). Presently, the primers used for *nosZ* gene target amplification provided good coverage of γ -Proteobacteria (Henry et al., 2006), which are stimulated by surface-applied residues (Pascault et al, 2010). Increased residue return in miscanthus plots may have selected for bacterial populations harbouring enhanced catabolic capabilities, such as N_2O reduction (Pascault et al., 2010). This implies that producing biomass crops with large yields may indirectly alter soil N cycling and potentially mitigate soil N_2O emissions due to increased residue return influencing the soil microbial community. It is likely that differences in environmental conditions (e.g. temperature, H_2O and O_2 availability) and resource quality and availability between corn-soybean and miscanthus soils related to differences in microbial community structure (Cusack et al., 2011) and selected for different dominant taxa that filled different ecological niches (Stone et al., 2015).

N fertilization did not affect targeted gene abundances, however studies in other cropping systems have found that N fertilization affected the size of denitrifying communities (Hallin et al., 2009), nitrifying communities (He et al., 2007), and proportions of *nirS* to *nirK* communities

(ratio of *nirS:nirK* genes) (Hai et al., 2009). Elevated 16S rRNA and *nirS* gene copies were observed in soils from spring-harvested miscanthus and windrowed switchgrass (Table 2). Increased N return via senescent leaf loss in PG plots over winter contributes to the soil organic matter pool (Heaton et al., 2009), and may have contributed to elevated total (16S rRNA) bacterial populations in these soils, concomitantly increasing *nirS* abundances (Huang et al., 2011).

Comment [K10]: Re C McCall comment

4.3 Temporal Changes in Bacterial Gene Abundances

Total soil bacterial communities (16S rRNA) followed a seasonal trend, with elevated 16S rRNA gene copies at fall (October) compared to spring (May) sampling dates, possibly due to an increase in the availability and diversity of resources for microbial metabolism and growth over the growing season (Habekost et al., 2008). Denitrifying abundances changed differently than the total bacterial community, suggesting denitrifiers were influenced by different proximal regulators than the total bacterial community (Fig. 3 and 4). Seasonal dynamics of N-cycling microbial communities have been previously characterized (Boyer et al., 2006; Nemeth et al., 2014: Wolsing and Priemé, 2004; Dandie et al., 2008; Bremer et al., 2007), and are tightly coupled with seasonal changes in labile C and N pools, temperature and soil H_2O (Butterbach-Bahl et al. 2013; Rasche et al., 2011), indicating that local edaphic drivers may often take precedence over crop-specific drivers (Mao et al., 2013).

5.0 Conclusions

Miscanthus consistently produced large yields and supported larger *nosZ*-bearing communities than the corn-soybean rotation, emphasizing its influence on soil N cycling and its potential to mitigate soil N_2O emissions while being suitable for production in variable Ontario conditions.

Additionally, miscanthus yields were not increased with N fertilization, indicating a lower N input requirement for biomass production compared to switchgrass. Higher 16S rRNA and *nirS* gene copies were associated with reduced yields in spring-harvested PGs, indicating that harvesting PGs in the spring may increase populations of denitrifiers capable of producing N_2O emissions while simultaneously decreasing biomass yields. The size of both denitrifying (*nirS* and *nosZ)* and total bacterial (16S rRNA*)* communities changed over the sampling period, however changes in denitrifying gene abundances did not parallel changes in the total soil bacterial community, indicating denitrifying communities were regulated differently than the total bacterial community. Future work measuring N_2O emissions and denitrifier activity (mRNA) and community structure in these systems is required to link the effects of LUC on these communities directly with N_2O fluxes**.**

Author Contributions:

K. Thompson was the primary researcher and author on this study, conducting all field work, lab work, and manuscript preparation. Author B. Deen is credited for the use of his OMAFRA-funded field plots for this research, and valuable advice on experimental design, statistical analyses and manuscript focus. Author K. Dunfield is credited for her invaluable mentorship on molecular analyses and trouble-shooting on field sampling and sample preservation techniques, manuscript focus and preparation, and data interpretation.

Acknowledgements

Funding for this research was awarded through grants from the Natural Science and Engineering Research Council of Canada (NSERC), the Canada Research Chairs (CRC) Program and the Ontario Ministry of Agriculture and Food and the Ministry of Rural Affairs (OMAF/MRA).

456 K.T. was also supported through an Ontario Graduate Scholarship (OGS) and an Ontario
457 Graduate Fellowship (OGF). Special thanks to Henk Wichers (University of Guelph) for his
458 expertise in managing the Alternate Renewable Fuels trial at the Elora Research Station.

459 **References**

460 Adékambi, T., Drancourt, M., Raoult, D., 2009. The rpoB gene as a tool for clinical
461 microbiologists. Trends Microbiol. 17, 37–45. doi:10.1016/j.tim.2008.09.008

462 Attard, E., Recous, S., Chabbi, A., De Berranger, C., Guillaumaud, N., Labreuche, J., Philippot,
463 L., Schmid, B., Le Roux, X., 2011. Soil environmental conditions rather than denitrifier
464 abundance and diversity drive potential denitrification after changes in land uses. Glob.
465 Chang. Biol. 17, 1975–1989. doi:10.1111/j.1365-2486.2010.02340.x

466 Bakke, I., De Schryver, P., Boon, N., Vadstein, O., 2011. PCR-based community structure
467 studies of Bacteria associated with eukaryotic organisms: A simple PCR strategy to avoid
468 co-amplification of eukaryotic DNA. J. Microbiol. Methods 84, 349–351.
469 doi:10.1016/j.mimet.2010.12.015

470 Bastian, F., Bouziri, L., Nicolardot, B., Ranjard, L., 2009. Impact of wheat straw decomposition
471 on successional patterns of soil microbial community structure. Soil Biol. Biochem. 41,
472 262–275. doi:10.1016/j.soilbio.2008.10.024

473 Bergmark, L., Poulsen, P.H.B., Al-Soud, W.A., Norman, A., Hansen, L.H., Sørensen, S.J., 2012.
474 Assessment of the specificity of Burkholderia and Pseudomonas qPCR assays for detection
475 of these genera in soil using 454 pyrosequencing. FEMS Microbiol. Lett. 333, 77–84.
476 doi:10.1111/j.1574-6968.2012.02601.x

477 Bissett, A., Richardson, A.E., Baker, G., Thrall, P.H., 2011. Long-term land use effects on soil
478 microbial community structure and function. Appl. Soil Ecol. 51, 66–78.
479 doi:10.1016/j.apsoil.2011.08.010

480 Blanco-Canqui, H., Lal, R., 2009. Crop Residue Removal Impacts on Soil Productivity and
481 Environmental Quality. CRC. Crit. Rev. Plant Sci. 28, 139–163.
482 doi:10.1080/07352680902776507

483 Boyer, E.W., Alexander, R.B., Parton, W.J., Li, C., Butterbach-Bahl, K., Donner, S.D., Skaggs,
484 R.W., Del Grosso, S.J., 2006. Modeling denitrification in terrestrial and aquatic ecosystems
485 at regional scales. Ecol. Appl. 16, 2123–42.

486 Braker, G., Conrad, R., 2011. Diversity, structure, and size of N2O-producing microbial
487 communities in soils-what matters for their functioning?, Advances in applied
488 microbiology. Elsevier. doi:10.1016/B978-0-12-387046-9.00002-5

489 Bremer, C., Braker, G., Matthies, D., Reuter, A., Engels, C., Conrad, R., 2007. Impact of plant
490 functional group, plant species, and sampling time on the composition of nirK-type
491 denitrifier communities in soil. Appl. Environ. Microbiol. 73, 6876–84.
492 doi:10.1128/AEM.01536-07

493 Butterbach-Bahl, K., Baggs, E.M., Dannenmann, M., Kiese, R., Zechmeister-Boltenstern, S.,
494 2013. Nitrous oxide emissions from soils: how well do we understand the processes and
495 their controls? Philos. Trans. R. Soc. B Biol. Sci 368, 1–13. doi:10.1098/rstb.2013.0122

496 Cantera, J.J.L., Stein, L.Y., 2007. Role of nitrite reductase in the ammonia-oxidizing pathway of
497 Nitrosomonas europaea. Arch. Microbiol. 188, 349–54. doi:10.1007/s00203-007-0255-4

498 Casciotti, K.L., Ward, B.B., 2001. Dissimilatory Nitrite Reductase Genes from Autotrophic
499 Ammonia-Oxidizing Bacteria. Appl. Environ. Microbiol. 67, 2213–2221.

500 doi:10.1128/AEM.67.5.2213
501 Case, R.J., Boucher, Y., Dahllöf, I., Holmström, C., Doolittle, W.F., Kjelleberg, S., 2007. Use of
502 16S rRNA and rpoB genes as molecular markers for microbial ecology studies. Appl.
503 Environ. Microbiol. 73, 278–88. doi:10.1128/AEM.01177-06
504 Christian, D.G., Riche, a. B., Yates, N.E., 2008. Growth, yield and mineral content of
505 Miscanthus×giganteus grown as a biofuel for 14 successive harvests. Ind. Crops Prod. 28,
506 320–327. doi:10.1016/j.indcrop.2008.02.009
507 Cuhel, J., Simek, M., Laughlin, R.J., Bru, D., Cheneby, D., Watson, C.J., Philippot, L., Chèneby,
508 D., 2010. Insights into the effect of soil pH on N2O and N2 emissions and denitrifier
509 community size and activity. Appl. Environ. Microbiol. 76, 1870–8.
510 doi:10.1128/AEM.02484-09
511 Cusack, D.F., Silver, W.L., Torn, M.S., Burton, S.D., Firestone, M.K., 2011. Changes in
512 microbial community characteristics and soil organic matter with nitrogen additions in two
513 tropical forests. Ecology 92, 621–632. doi:10.1890/10-0459.1
514 Dandie, C.E., Burton, D.L., Zebarth, B.J., Henderson, S.L., Trevors, J.T., Goyer, C., 2008.
515 Changes in bacterial denitrifier community abundance over time in an agricultural field and
516 their relationship with denitrification activity. Appl. Environ. Microbiol. 74, 5997–6005.
517 doi:10.1128/AEM.00441-08
518 Deen, B.B., Young, D., Rowsell, J., Tubeileh, A., Engbers, H., Rosser, B., 2011. A comparative
519 study assessing variety and management effects on C 4 perennial grasses in a northern
520 climate. Asp. Appl. Biol. 112, 205–212.
521 Deslippe, J.R., Jamali, H., Jha, N., Saggar, S., 2014. Soil Biology & Biochemistry Denitrifier
522 community size , structure and activity along a gradient of pasture to riparian soils. Soil
523 Biol. Biochem. 71, 48–60. doi:10.1016/j.soilbio.2014.01.007
524 Dohleman, F.G., Heaton, E. a., Arundale, R. a., Long, S.P., 2012. Seasonal dynamics of above-
525 and below-ground biomass and nitrogen partitioning in Miscanthus × giganteus and
526 Panicum virgatum across three growing seasons. GCB Bioenergy 4, 534–544.
527 doi:10.1111/j.1757-1707.2011.01153.x
528 Enwall, K., Throbäck, I.N., Stenberg, M., Söderström, M., Hallin, S., 2010. Soil resources
529 influence spatial patterns of denitrifying communities at scales compatible with land
530 management. Appl. Environ. Microbiol. 76, 2243–50. doi:10.1128/AEM.02197-09
531 Fierer, N., Jackson, J.A., Vilgalys, R., Jackson, R.B., 2005. Assessment of Soil Microbial
532 Community Structure by Use of Taxon-Specific Quantitative PCR Assays. Appl. Environ.
533 Microbiol. 71, 4117–4120. doi:10.1128/AEM.71.7.4117
534 Foster, J.L., Guretzky, J.A., Huo, C., Kering, M.K., Butler, T.J., 2013. Effects of Row Spacing,
535 Seeding Rate, and Planting Date on Establishment of Switchgrass. Crop Sci. 53, 309–314.
536 doi:10.2135/cropsci2012.03.0171
537 Gaudin, A.C.M., Janovicek, K., Deen, B., Hooker, D.C., 2015. Wheat improves nitrogen use
538 efficiency of maize and soybean-based cropping systems. Agric. Ecosyst. Environ. 210, 1–
539 10. doi:10.1016/j.agee.2015.04.034
540 Habekost, M., Eisenhauer, N., Scheu, S., Steinbeiss, S., Weigelt, A., Gleixner, G., 2008.
541 Seasonal changes in the soil microbial community in a grassland plant diversity gradient

four years after establishment. Soil Biol. Biochem. 40, 2588–2595. doi:10.1016/j.soilbio.2008.06.019

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

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 J. 3, 597–605. doi:10.1038/ismej.2008.128

He, J.-Z., Shen, J.-P., Zhang, L.-M., Zhu, Y.-G., Zheng, Y.-M., Xu, M.-G., Di, H., 2007. Quantitative analyses of the abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea of a Chinese upland red soil under long-term fertilization practices. Environ. Microbiol. 9, 2364–74. doi:10.1111/j.1462-2920.2007.01358.x

Hedenec, P., Novotny, D., Ustak, S., Cajthaml, T., Slejska, A., Simackova, H., Honzik, R., Kovarova, M., Frouz, J., 2014. The effect of native and introduced biofuel crops on the composition of soil biota communities. Biomass and Bioenergy 60, 137–146. doi:10.1016/j.biombioe.2013.11.021

Henderson, S.L., Dandie, C.E., Patten, C.L., Zebarth, B.J., Burton, D.L., Trevors, J.T., Goyer, C., 2010. Changes in denitrifier abundance, denitrification gene mRNA levels, nitrous oxide emissions, and denitrification in anoxic soil microcosms amended with glucose and plant residues. Appl. Environ. Microbiol. 76, 2155–64. doi:10.1128/AEM.02993-09

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. Appl. Environ. Microbiol. 72, 5181–9. doi:10.1128/AEM.00231-06

Himken, M., Lammel, J., Neukirchen, D., Olfs, H., 1997. Cultivation of Miscanthus under West European conditions : Seasonal changes in dry matter production , nutrient uptake and remobilization. Plant Soil 189, 117–126.

Huang, S., Chen, C., Yang, X., Wu, Q., Zhang, R., 2011. Distribution of typical denitrifying functional genes and diversity of the nirS-encoding bacterial community related to environmental characteristics of river sediments. Biogeosciences 8, 3041–3051. doi:10.5194/bg-8-3041-2011

Huang, Y., 2004. Nitrous oxide emissions as influenced by amendment of plant residues with different C:N ratios. Soil Biol. Biochem. 36, 973–981. doi:10.1016/j.soilbio.2004.02.009

Intergovernmental Panel on Climate Change (IPCC) (2007) Climate Change 2007: The Physical Science Basis. Working Group I Contribution to the Fourth Assessment Report of the Inter- governmental Panel on Climate Change. Solomon et al. (eds). Cambridge University Press, Cambridge.

Jayasundara, S., Wagner-Riddle, C., Dias, G., Kariyapperuma, K. a., 2014. Energy and greenhouse gas intensity of corn (Zea mays L.) production in Ontario: A regional assessment. Can. J. Soil Sci. 94, 77–95. doi:10.4141/cjss2013-044

583 Kludze, H., Deen, B., Weersink, A., van Acker, R., Janovicek, K., De Laporte, A., 2013. Impact
584 of land classification on potential warm season grass biomass production in Ontario,
585 Canada. Can. J. Plant Sci. 93, 249–260. doi:10.4141/cjps2012-143

586 Lewandowski, I., Andersson, B., Basch, G., Christian, D.G., Jørgensen, U., Jones, M.B., Riche,
587 A.B., Schwarz, K.U., Tayebi, K., Teixeira, F., 2003. Environment and Harvest Time Affects
588 the Combustion Qualities of of Miscanthus Genotypes. Agron. J. 95, 1274–1280.

589 Lewandowski, I., Clifton-brown, J.C., Scurlock, J.M.O., Huisman, W., 2000. Miscanthus :
590 European experience with a novel energy crop. Biomass and Bioenergy 19, 209–227.

591 Liang, C., Jesus, E.D.C., Duncan, D.S., Jackson, R.D., Tiedje, J.M., Balser, T.C., 2012. Soil
592 microbial communities under model biofuel cropping systems in southern Wisconsin, USA:
593 Impact of crop species and soil properties. Appl. Soil Ecol. 54, 24–31.
594 doi:10.1016/j.apsoil.2011.11.015

595 Mao, Y., Yannarell, A.C., Davis, S.C., Mackie, R.I., 2013a. Impact of different bioenergy crops
596 on N-cycling bacterial and archaeal communities in soil. Environ. Microbiol. 15, 928–42.
597 doi:10.1111/j.1462-2920.2012.02844.x

598 Mao, Y., Yannarell, A.C., Davis, S.C., Mackie, R.I., 2013b. Impact of different bioenergy crops
599 on N-cycling bacterial and archaeal communities in soil: Impact of bioenergy crops on soil
600 N-cycling archaea and bacteria. Environ. Microbiol. 15, 928–942. doi:10.1111/j.1462-
601 2920.2012.02844.x

602 Mao, Y., Yannarell, A.C., Mackie, R.I., 2011. Changes in N-transforming archaea and bacteria
603 in soil during the establishment of bioenergy crops. PLoS One 6, 1–12.
604 doi:10.1371/journal.pone.0024750

605 Maynard, D.G. and Curran, M.P. 2007. Bulk Density Measurement in Forest Soil. Chapter 66.
606 In: Soil Sampling and methods of analysis. (Carter M.R. & Gregorich E.G.) Canadian
607 Society of Soil Science. CRC Press, Boca Raton, FL. 863-869.

608 Miller, M.N., Zebarth, B.J., Dandie, C.E., Burton, D.L., Goyer, C., Trevors, J.T., 2008. Crop
609 residue influence on denitrification, N2O emissions and denitrifier community abundance in
610 soil. Soil Biol. Biochem. 40, 2553–2562. doi:10.1016/j.soilbio.2008.06.024

611 Morales, S.E., Cosart, T., Holben, W.E., 2010. Bacterial gene abundances as indicators of
612 greenhouse gas emission in soils. ISME J. 4, 799–808. doi:10.1038/ismej.2010.8

613 Munkholm, L.J., Heck, R.J., Deen, B., 2013. Long-term rotation and tillage effects on soil
614 structure and crop yield. Soil Tillage Res. 127, 85–91. doi:10.1016/j.still.2012.02.007

615 Mutegi, J.K., Munkholm, L.J., Petersen, B.M., Hansen, E.M., Petersen, S.O., 2010. Nitrous
616 oxide emissions and controls as influenced by tillage and crop residue management
617 strategy. Soil Biol. Biochem. 42, 1701–1711. doi:10.1016/j.soilbio.2010.06.004

618 Németh, D.D., Wagner-Riddle, C., Dunfield, K.E., 2014. Abundance and gene expression in
619 nitrifier and denitrifier communities associated with a field scale spring thaw N2O flux
620 event. Soil Biol. Biochem. 73, 1–9. doi:10.1016/j.soilbio.2014.02.007

621 Nogales, B., Timmis, K.N., Nedwell, D.B., Osborn, A.M., 2002. Detection and Diversity of
622 Expressed Denitrification Genes in Estuarine Sediments after Reverse Transcription-PCR
623 Amplification from mRNA. Appl. Environ. Mcrobiology 68, 5017–5025.

624 doi:10.1128/AEM.68.10.5017
625 Ontario Ministry of Energy, The Government of Ontario (2014). Achieving Balance: Ontario's
626 Long-Term Energy Plan. [Online] Available: http://www.energy.gov.on.ca/
627 Orr, M.-J., Gray, M.B., Applegate, B., Volenec, J.J., Brouder, S.M., Turco, R.F., 2015.
628 Transition to second generation cellulosic biofuel production systems reveals limited
629 negative impacts on the soil microbial community structure. Appl. Soil Ecol. 95, 62–72.
630 doi:10.1016/j.apsoil.2015.06.002
631 Pascault, N., Nicolardot, B., Bastian, F., Thiébeau, P., Ranjard, L., Maron, P.-A.A., 2010. In situ
632 dynamics and spatial heterogeneity of soil bacterial communities under different crop
633 residue management. Microb. Ecol. 60, 291–303. doi:10.1007/s00248-010-9648-z
634 Périé, C., Ouimet, R., 2008. Organic carbon, organic matter and bulk density relationships in
635 boreal forest soils. Can. J. Soil Sci. 88, 315–325. doi:10.4141/CJSS06008
636 Petersen, D.G., Blazewicz, S.J., Firestone, M., Herman, D.J., Turetsky, M., Waldrop, M., 2012.
637 Abundance of microbial genes associated with nitrogen cycling as indices of
638 biogeochemical process rates across a vegetation gradient in Alaska. Environ. Microbiol.
639 14, 993–1008. doi:10.1111/j.1462-2920.2011.02679.x
640 Philippot, L., 2002. Denitrifying genes in bacterial and Archaeal genomes. Biochim. Biophys.
641 Acta 1577, 355–76.
642 Philippot, L., 2006. Use of functional genes to quantify denitrifiers in the environment. Biochem.
643 Soc. Trans. 34, 101–3. doi:10.1042/BST0340101
644 Philippot, L., Andert, J., Jones, C., Bru, D., Hallin, S., 2011. Importance of denitrifiers lacking
645 the genes encoding the nitrous oxide reductase for N2O emissions from soil. Glob. Chang.
646 Biol. 17, 1497–1504. doi:10.1111/j.1365-2486.2010.02334.x
647 Rasche, F., Knapp, D., Kaiser, C., Koranda, M., Kitzler, B., Zechmeister-Boltenstern, S.,
648 Richter, A., Sessitsch, A., 2011. Seasonality and resource availability control bacterial and
649 archaeal communities in soils of a temperate beech forest. ISME J. 5, 389–402.
650 doi:10.1038/ismej.2010.138
651 Reeve, J.R., Schadt, C.W., Carpenter-boggs, L., Kang, S., Zhou, J., Reganold, J.P., 2010. Effects
652 of soil type and farm management on soil ecological functional genes and microbial
653 activities. ISME J. 4, 1099–1107. doi:10.1038/ismej.2010.42
654 Resource Efficient Agricultural Production (REAP)-Canada, 2008. ARF07 P ROJECT
655 Optimization of Switchgrass Management for Commercial Fuel Pellet Production.
656 OMAFRA-Alternative Renew. Fuels Res. Dev. Fund.
657 Roy, A.K., Wagner-Riddle, C., Deen, B., Lauzon, J., Bruulsema, T., 2014. Nitrogen application
658 rate, timing and history effects on nitrous oxide emissions from corn (Zea mays L.). Can. J.
659 Soil Sci. 94, 563–573. doi:10.4141/cjss2013-118
660 Sanscartier, D., Deen, B., Dias, G., MacLean, H.L., Dadfar, H., McDonald, I., Kludze, H., 2014.
661 Implications of land class and environmental factors on life cycle GHG emissions of
662 Miscanthus as a bioenergy feedstock. GCB Bioenergy 6, 401–413. doi:10.1111/gcbb.12062
663 Schlesinger, W.H., 2013. An estimate of the global sink for nitrous oxide in soils. Glob. Chang.
664 Biol. 19, 2929–2931. doi:10.1111/gcb.12239

Soil Quality National, 2006. Crop Residue Removal For Biomass Energy Production: Effects on Soils and Recommendations. Tech. Note 19, 1–7.

Sokhansanj, S., Mani, S., Turhollow, A., Kumar, A., Bransby, D., Laser, L.L., Laser, M., 2009. Large-scale production, harvest and logistics of switchgrass (Panicum virgatum L .) – current technology and envisioning a mature technology. Biofuels Bioprod. Bioprocess. 124–141. doi:10.1002/bbb

Stone, M.M., Kan, J., Plante, A.F., 2015. Parent material and vegetation influence bacterial community structure and nitrogen functional genes along deep tropical soil profiles at the Luquillo Critical Zone Observatory. Soil Biol. Biochem. 80, 273–282. doi:10.1016/j.soilbio.2014.10.019

Szukics, U., Abell, G.C.J., Hödl, V., Mitter, B., Sessitsch, A., Hackl, E., Zechmeister-Boltenstern, S., 2010. Nitrifiers and denitrifiers respond rapidly to changed moisture and increasing temperature in a pristine forest soil. FEMS Microbiol. Ecol. 72, 395–406. doi:10.1111/j.1574-6941.2010.00853.x

Throbäck, I.N., Enwall, K., Jarvis, A., Hallin, S., 2004. Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiol. Ecol. 49, 401–17. doi:10.1016/j.femsec.2004.04.011

Ver Hoef, J.M. and Boveng, P.L., 2007. Quasi-Poisson vs. Negative Binomial Regression: How should we model overdispersed count data? Ecology 88, 2766–2772.

Vos, M., Quince, C., Pijl, A.S., Hollander, M. De, Kowalchuk, G.A., 2012. A Comparison of rpoB and 16S rRNA as Markers in Pyrosequencing Studies of Bacterial Diversity 7, 1–8. doi:10.1371/journal.pone.0030600

Watrud, L.S., Reichman, J.R., Bollman, M. a., Smith, B.M., Lee, E.H., Jastrow, J.D., Casler, M.D., Collins, H.P., Fransen, S., Mitchell, R.B., Owens, V.N., Bean, B., Rooney, W.L., Tyler, D.D., King, G. a., 2013. Chemistry and Microbial Functional Diversity Differences in Biofuel Crop and Grassland Soils in Multiple Geographies. BioEnergy Res. 6, 601–619. doi:10.1007/s12155-012-9279-y

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 Microbiol. Ecol. 48, 261–71. doi:10.1016/j.femsec.2004.02.002

Wu, L., Osmond, D.L., Graves, A.K., Burchell, M.R., Duckworth, O.W., 2012. Relationships Between Nitrogen Transformation Rates and Gene Abundance in a Riparian Buffer Soil. Environ. Manage. 50, 861–874. doi:10.1007/s00267-012-9929-z

Yin, C., Fan, F., Song, A., Li, Z., Yu, W., Liang, Y., 2014. Different denitrification potential of aquic brown soil in Northeast China under inorganic and organic fertilization accompanied by distinct changes of nirS - and nirK -denitrifying bacterial community. Eur. J. Soil Biol. 65, 47–56. doi:10.1016/j.ejsobi.2014.09.003

Zumft, W.G., 1997. Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. 61, 533–616.

Table 1. Mean soil properties measured at the Elora Research Station.

Cropping System/ Harvest		N Rate	*Bulk Density	% Organic Carbon		% Total Carbon		Yield (dry tonnes ha^{-1})		
		(kg ha^{-1})	(g cm^{-3})	0-15cm	15-30cm	0-15cm	15-30cm	2011	2012	†Mean
Corn-soybean	Fall	0	1.21 AB	1.88	1.06	2.22	1.86	5.341	2.912	E
Corn-soybean	Fall	160	1.27 A	1.79	1.47	2.25	2.11	9.92	7.882	BC
Miscanthus	Fall	0	1.10 B	2.06	1.44	2.27	1.72	17.62	12.77	A
Miscanthus	Fall	160	1.10 B	2.13	1.63	2.36	1.84	17.43	18.32	A
Miscanthus	Spring	0	1.13 AB	2.09	1.53	2.31	1.69	12.66	13.38	AB
Miscanthus	Spring	160	1.13 AB	2.24	1.42	2.47	1.89	14.33	14.56	A
Switchgrass	Fall	0	1.11 B	2.12	1.43	2.33	1.61	7.648	6.458	CD
Switchgrass	Fall	160	1.09 B	2.12	1.34	2.32	1.73	11.1	10.45	AB
Switchgrass	Spring	0	1.11 B	2.09	1.23	2.32	1.55	6.33	4.146	DE
Switchgrass	Spring	160	1.21 AB	1.92	1.33	2.23	1.7	6.905	6.441	CD

*****Means of bulk density (n=6) followed by the same letter within one column are not significantly different according to a post-hoc Tukey's means comparison ($p<0.05$); carbon measurements (n=3) were not significantly different between treatments. †Mean yields (n=3) followed by the same letter are not significantly different according to a post-hoc Tukey's means comparison ($p< 0.05$).

Table 2. Mean gene abundance responses to crop and harvest management, averaged over nitrogen application rate and time at the Elora Research Station.

Cropping System	Management	†Total soil bacteria (gene copy g^{-1} soil)	†Soil denitrifying bacteria (gene copy g^{-1} soil)		
		16S	*nirS*	*nosZ*	*nirS:nosZ* ($x10^{-2}$)
Corn-Soybean	Fall Harvest	1.35×10^{9}b	1.95×10^{6}b	2.63×10^{5}b	7.42
Miscanthus	Fall Harvest	1.38×10^{9}b	2.30×10^{6}ab	4.47×10^{5}a	5.15
Miscanthus	Spring Harvest	1.91×10^{9}a	3.02×10^{6}a	5.25×10^{5}a	5.75
Switchgrass	Fall Harvest	1.41×10^{9}b	2.19×10^{6}b	3.55×10^{5}ab	6.17
Switchgrass	Spring Windrow	1.48×10^{9}ab	2.46×10^{6}ab	3.98×10^{5}ab	6.18

†Means followed by the same letter within one column are not significantly different according to post-hoc Tukey's means comparison at $p < 0.05$ ($n = 24$).

Figure Captions

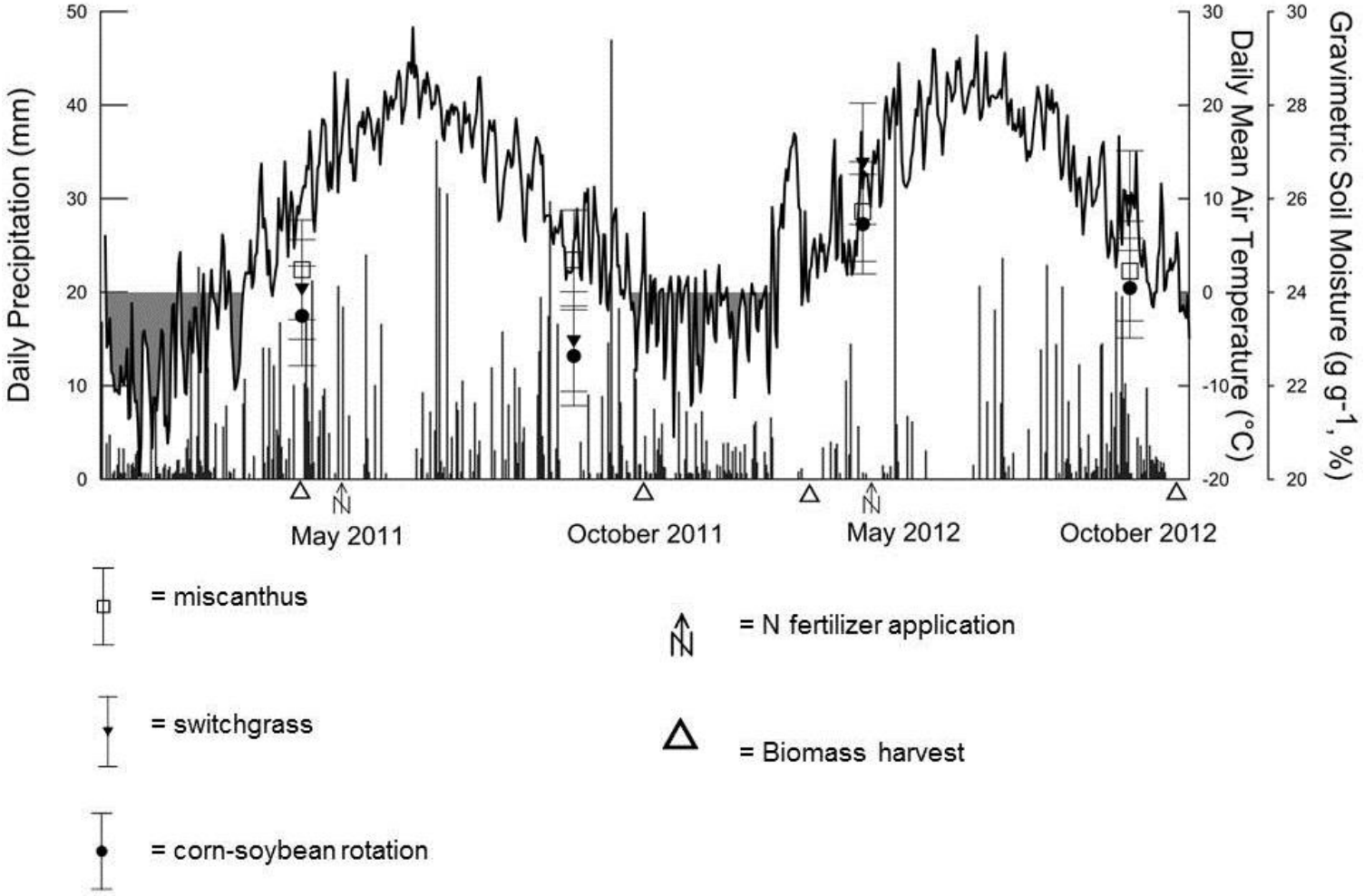
Figure 1. Mean daily air temperature (°C) and daily precipitation (mm) at the Elora Research Station from January 2011 to November 2012. Soil gravimetric H_2O was measured on a per-sample basis and is shown as crop means (±SE) for each sampling date (May 9th, 2011; October 30th, 2011; May 2nd, 2012 and October 20th, 2012) (n=12 in perennial grasses, n=6 in corn-soybean rotation).

Figure 2. Mean soil NH_4-N and NO_3-N (mg g^{-1} dry soil ±SE) in annual and perennial biomass cropping systems under varied harvest and N management at the Elora Research Station. CS = corn-soybean, SF = fall-harvested switchgrass, SS = spring-harvested switchgrass, MF = fall-harvested miscanthus and MS = spring-harvested miscanthus. Different letters within panels indicate significant differences according to a post-hoc Tukey's test ($p<0.05$).

Figure 3. Mean log gene copies (g^{-1} dry soil ±SE) in annual and perennial biomass cropping systems under varied harvest management at the Elora Research Station (n=6) over time. Different letters within panels indicate significant differences according to a post-hoc Tukey's test ($p<0.05$).

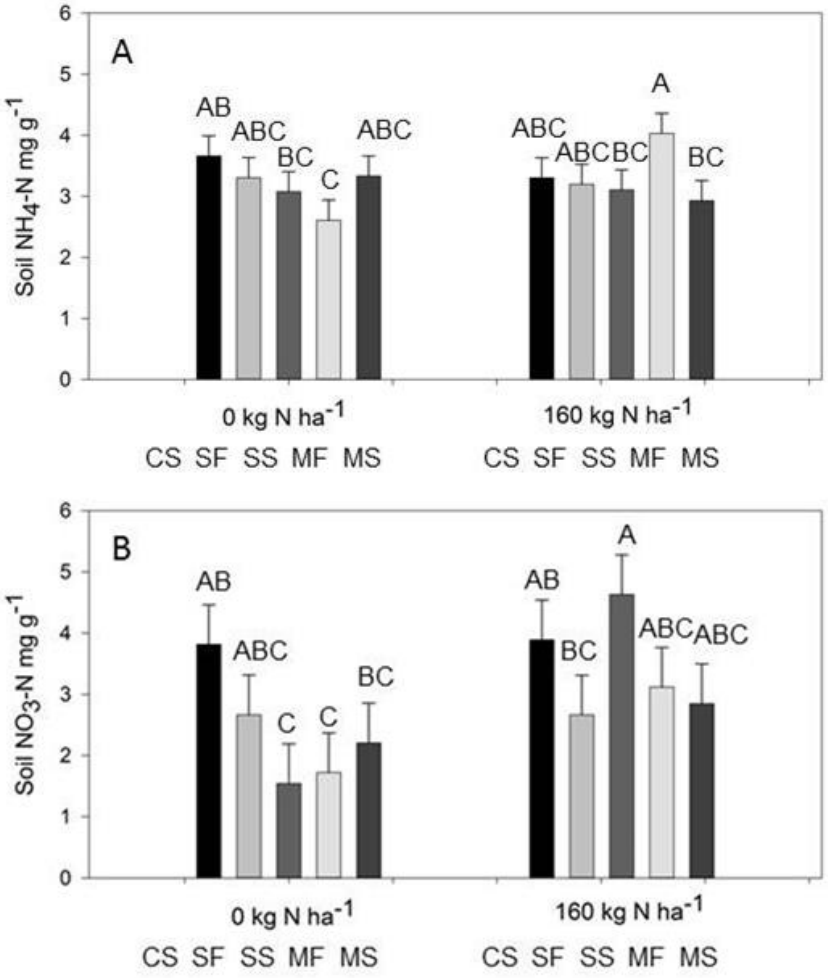
Figure 4a. Principal Component Analysis; factor 1 accounted for 43.89% variance and factor 2 accounted for 23.84% variance. **4b**. Loading plot for principal components of response variables (*nirS*, *nosZ* and 16S rRNA gene copies, and soil NO_3-N, soil NH_4-N, gravimetric soil H_2O)..

736 Figure 1.



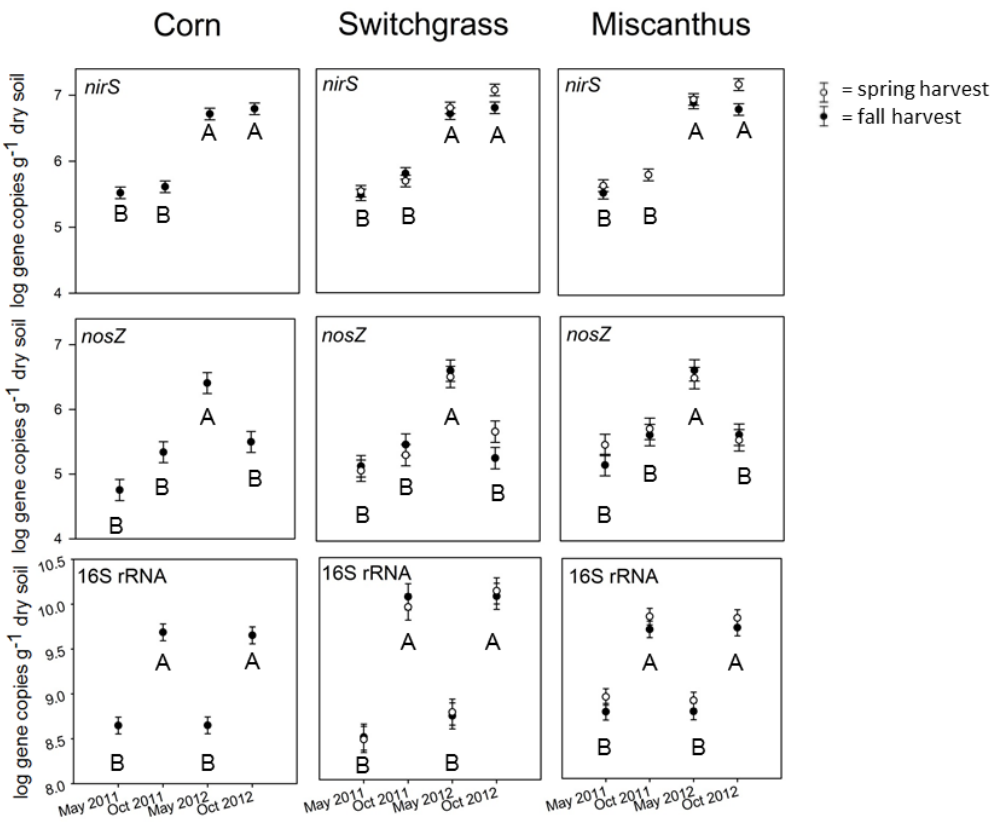
737

738 Figure 2.

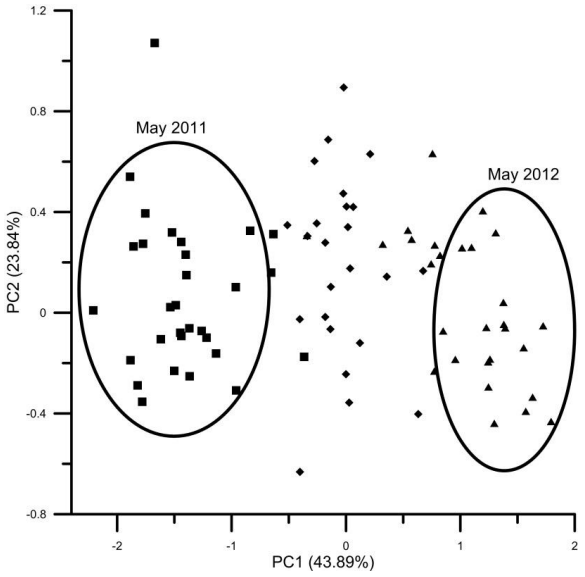


739

Figure 3.

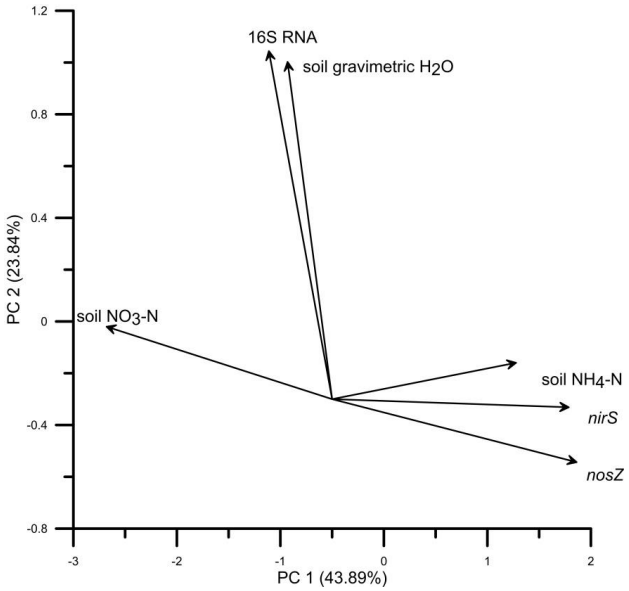

Corn
Switchgrass
Miscanthus
nirS
nosZ
16S rRNA
log gene copies g-1 dry soil
= spring harvest
= fall harvest
May 2011
Oct 2011
May 2012
Oct 2012

742 Figure 4a.



743

744 Figure 4b.



745

746