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

Running Head: Soil Denitrifiers associated with Perennial Grasses

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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.

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).

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

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

126 targets) soil bacterial communities 3-4 years after PG planting. Soil was collected on four dates
127 from 2011 to 2012 from a field trial established in Ontario in 2008. This study is unique in that
128 it consists of two PG biomass crops produced in parallel with the existing common land use of
129 corn-soybean rotation within the same field trial site. It also includes unfertilized and fertilized
130 plots in both annual and perennial systems, and varied harvest practices within PG plots. We
131 hypothesized that soils from PG plots would support larger total bacterial and denitrifier
132 communities than soils from corn-soybean plots due to increased shoot residue return and root
133 inputs to soils in PG systems, and that soils from PG plots with biomass harvested in the spring
134 would support larger total bacterial and denitrifier communities than supported by soils from
135 PGs harvested in the fall due to increased root inputs and leaf loss to soil over winter.
136
137 ## 2. Materials and Methods
138 ### 2.1 Site Description and Experimental Design
139 A field trial was established in 2008 at the University of Guelph Research Station in Elora, ON
140 (43°38'46.73" N and 80°24'6.66" W). The field site was cultivated on May 16th and June 6th,
141 2008. Switchgrass (*Panicum virgatum* L., Shelter variety) was planted on June 6th, 2008.
142 Miscanthus (*M. sinensis* x *M. sacchariflorus*, Nagara-116 variety) was planted on June 12th,
143 2008, and soybean (*Glycine max* L.) was planted on June 24th, 2008 and annually rotated with
144 corn (*Zea mays* L.). Corn was planted on May 5th, 2010; soy was planted on June 3rd, 2011, and
145 corn was re-planted on May 18th, 2012, with annual light cultivation to prepare seedbeds for
146 planting. In 2007, prior to trial establishment, the experimental area was planted to barley
147 (*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.

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,

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

172 For molecular analyses, soil was sampled on 4 dates (May 9th, 2011; October 30th, 2011; May
173 2nd, 2012 and October 20th, 2012). October sampling dates occurred before fall harvest of PG
174 crops, while May sampling dates occurred before N fertilizer application and after spring PG
175 biomass removal (Fig. 1). Ten soil cores per plot were sampled aseptically to 15 cm depth using
176 a 5 cm-diameter soil corer on a zed-shaped transect, composited and kept on ice until transport
177 back to the laboratory. The transect shape was chosen to encompass plot heterogeneity; at a pre-
178 trial study date initial analysis indicated gene abundances were not significantly different
179 between bulk or rhizosphere soils in corn-soybean or PG plots, possibly due to the large root
180 biomass/leaf loss to soils in perennial plots and residual soy/corn residue cover on soil in corn-
181 soybean plots. Soil samples were divided for storage at 4˚C and -20˚C.

182 Mean values of gravimetric soil moisture (g g^{-1}) are shown in Figure 1. Soil exchangeable NO_3^--
183 N and NH_4^+-N were determined for each of the soil samples by KCl extraction. Soil samples
184 (10.0 g) were placed into 125 mL flasks and 100 mL of 2.0M KCl was added to each flask.
185 Flasks were stoppered and shaken for 1h at 160 strokes per minute; solutions were allowed to
186 settle and were then filtered through Whatman no. 42 filter paper (Whatman plc, ME, U.S.A).
187 Extractable NO_3^--N and NH_4^+-N were determined colourmetrically with segmented flow
188 analyses (AA3, SEAL Analytical, Wisconsin, USA) via a cadmium reduction (Technician
189 Instrument Corporation, 1971), and a Berthelot reaction respectively (Fig. 2).

2.3 Soil DNA Extraction

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

2.4 Quantification of total bacteria and functional genes

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

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

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

Amplicon specificity was screened by running qPCR products on an ethidium bromide-stained gel (1% agarose, 80 volts for 20 minutes) with a 100bp ladder, which resulted in clean bands for all gene targets. 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)

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

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

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

236 **2.5 Statistical Analysis**

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

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

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

255 such a principal component analysis was conducted in SAS (PROC FACTOR) on 120 samples
256 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.

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

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

3.2 Biomass Yields

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

3.3 Bacterial Responses to Annual and Perennial Crops and their Management

There was no statistically significant effect of N fertilization or any significant interactions between cropping system and sampling time on any of the targeted gene abundances. Therefore we analyzed the impact of each biomass crop under specific harvest management on soil bacterial gene abundances (Table 2). Denitrifying (*nosZ*) gene copy abundances were affected 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 (5.2-5.4 x 10^9 gene copies g^{-1} dry soil) at fall (October) sampling dates compared to the ca. 5.5-6.4 x 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 x 10^6 – 1.6 x 10^7 gene copies g^{-1} dry soil) compared to 2011 (2.5-6.3 x 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 1.3-3.2 x 10^5 gene copies g^{-1} dry soil, but increased significantly in May 2012 to approximately 3.2 x 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.

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).

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

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

411 (ratio of *nirS:nirK* genes) (Hai et al., 2009). Elevated 16S rRNA and *nirS* gene copies were
412 observed in soils from spring-harvested miscanthus and windrowed switchgrass (Table 2).
413 Increased N return via senescent leaf loss in PG plots over winter contributes to the soil organic
414 matter pool (Heaton et al., 2009), and may have contributed to elevated total (16S rRNA)
415 bacterial populations in these soils, concomitantly increasing *nirS* abundances (Huang et al.,
416 2011).

417 **4.3 Temporal Changes in Bacterial Gene Abundances**

418 Total soil bacterial communities (16S rRNA) followed a seasonal trend, with elevated 16S rRNA
419 gene copies at fall (October) compared to spring (May) sampling dates, possibly due to an
420 increase in the availability and diversity of resources for microbial metabolism and growth over
421 the growing season (Habekost et al., 2008). Denitrifying abundances changed differently than
422 the total bacterial community, suggesting denitrifiers were influenced by different proximal
423 regulators than the total bacterial community (Fig. 3 and 4). Seasonal dynamics of N-cycling
424 microbial communities have been previously characterized (Boyer et al., 2006; Nemeth et al.,
425 2014: Wolsing and Priemé, 2004; Dandie et al., 2008; Bremer et al., 2007), and are tightly
426 coupled with seasonal changes in labile C and N pools, temperature and soil H_2O (Butterbach-
427 Bahl et al. 2013; Rasche et al., 2011), indicating that local edaphic drivers may often take
428 precedence over crop-specific drivers (Mao et al., 2013).

429 **5.0 Conclusions**

430 Miscanthus consistently produced large yields and supported larger *nosZ*-bearing communities
431 than the corn-soybean rotation, emphasizing its influence on soil N cycling and its potential to
432 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.

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705

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 ($p< 0.05$).

718 Table 2. Mean gene abundance responses to crop and harvest management, averaged over nitrogen application rate and time at the Elora Research
719 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 x 10^9b	1.95 x 10^6b	2.63 x 10^5b	7.42
Miscanthus	Fall Harvest	1.38 x 10^9b	2.30 x 10^6ab	4.47 x 10^5a	5.15
Miscanthus	Spring Harvest	1.91 x 10^9a	3.02 x 10^6a	5.25 x 10^5a	5.75
Switchgrass	Fall Harvest	1.41 x 10^9b	2.19 x 10^6b	3.55 x 10^5ab	6.17
Switchgrass	Spring Windrow	1.48 x 10^9ab	2.46 x 10^6ab	3.98 x 10^5ab	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

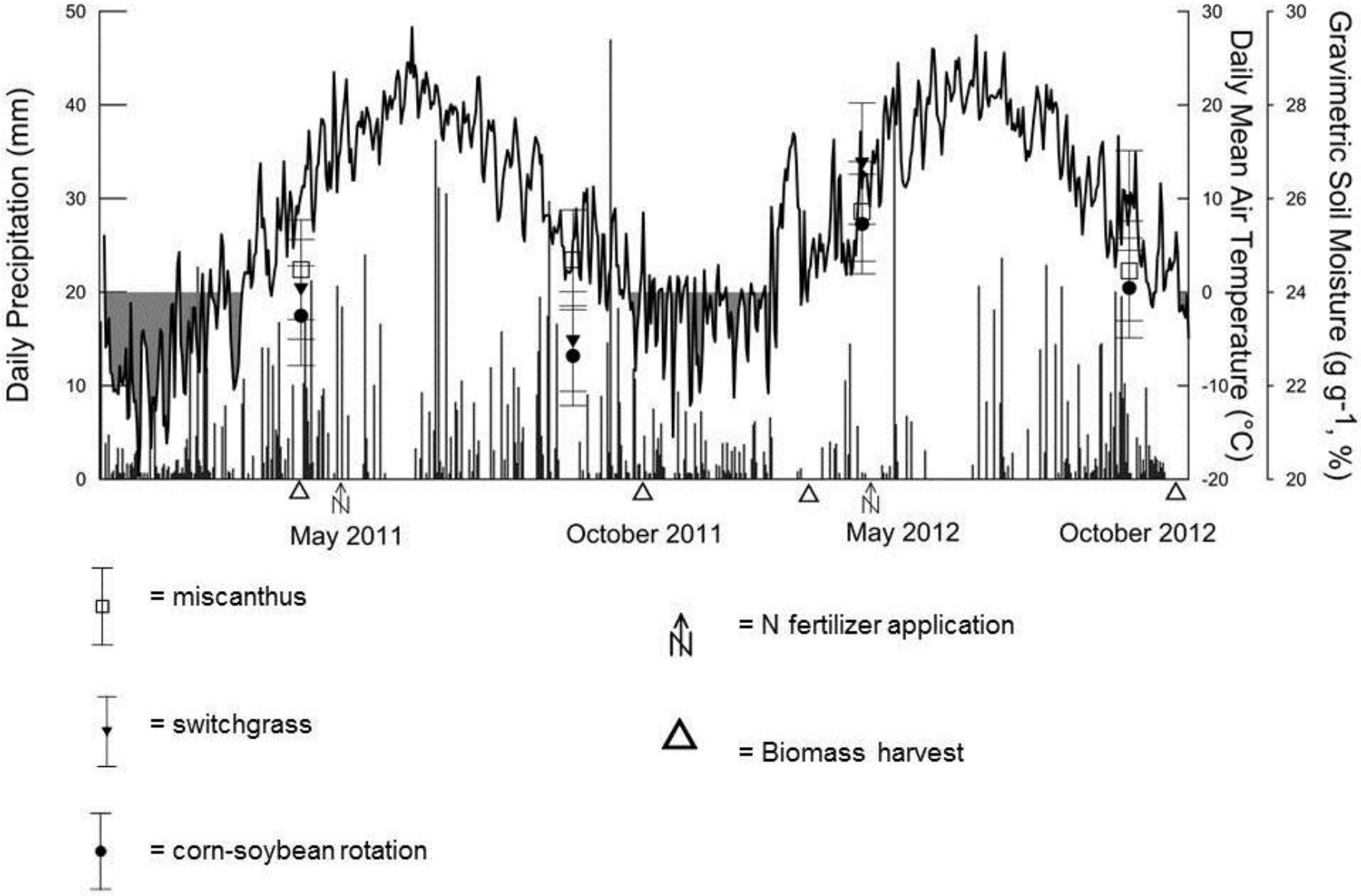
Figure 1. Mean daily air temperature (°C) and daily precipitation (mm) at the Elora Research Station from January 2011 to November 2012. Soil gravimetric H_2O was measured on a per-sample basis and is shown as crop means (±SE) for each sampling date (May 9th, 2011; October 30th, 2011; May 2nd, 2012 and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



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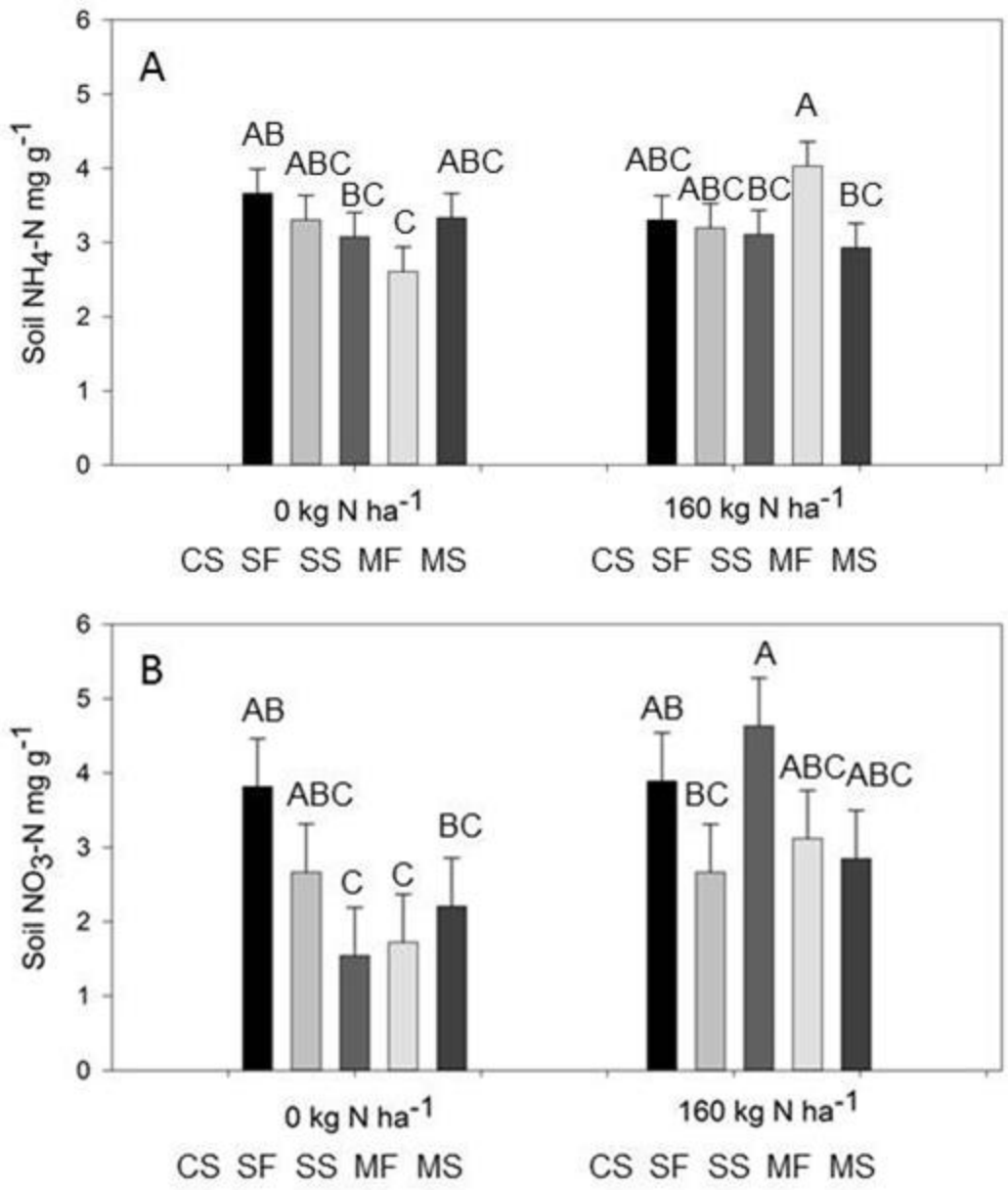
738 Figure 2.



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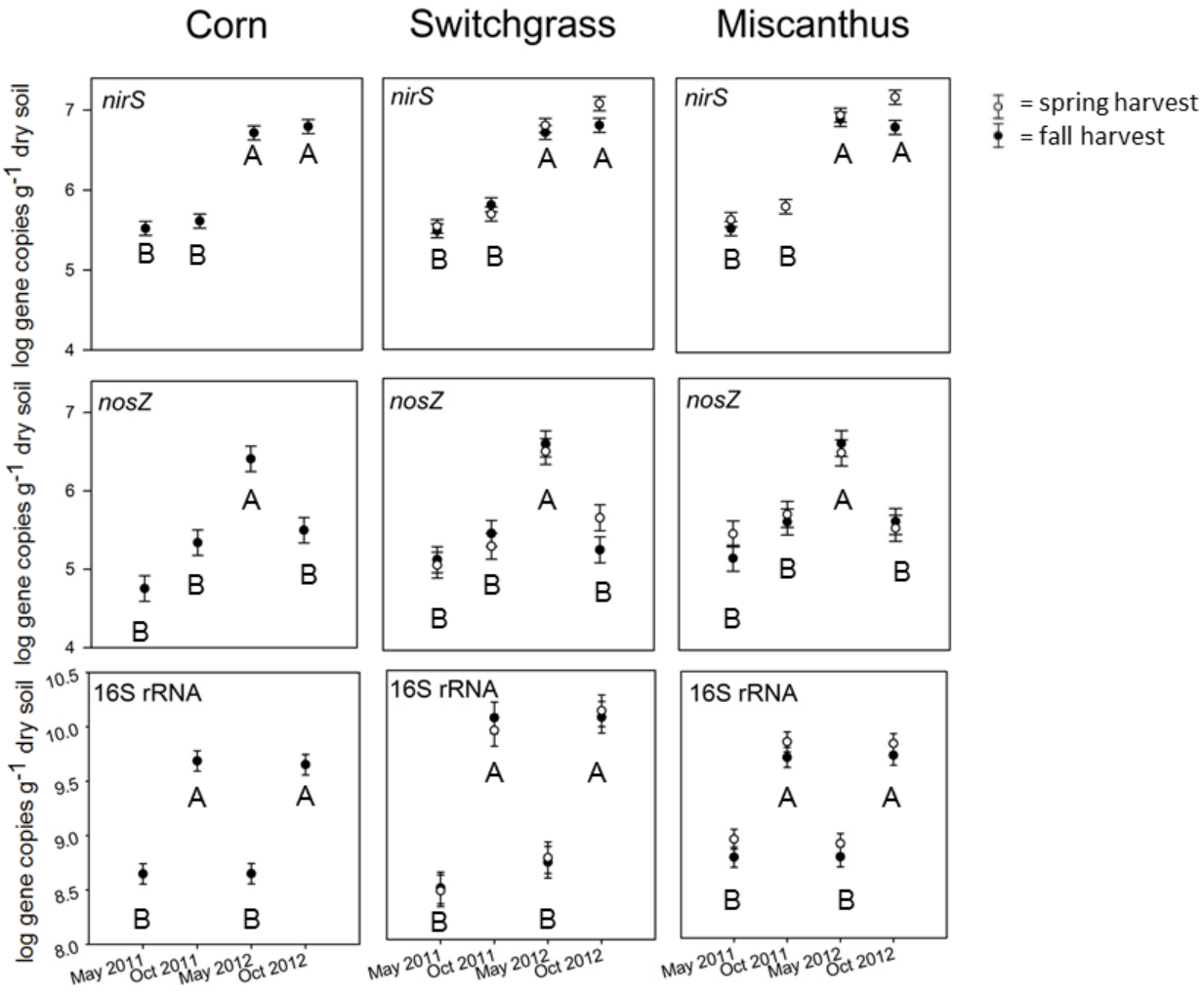
740 Figure 3.



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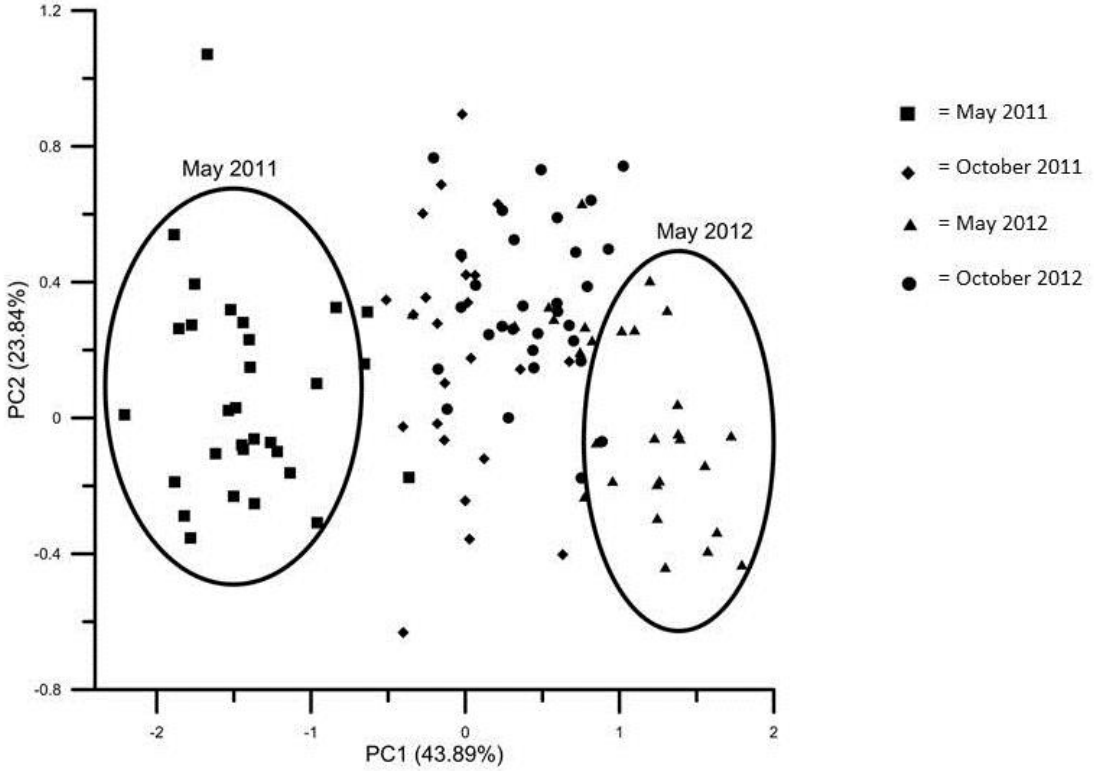
742 Figure 4a.



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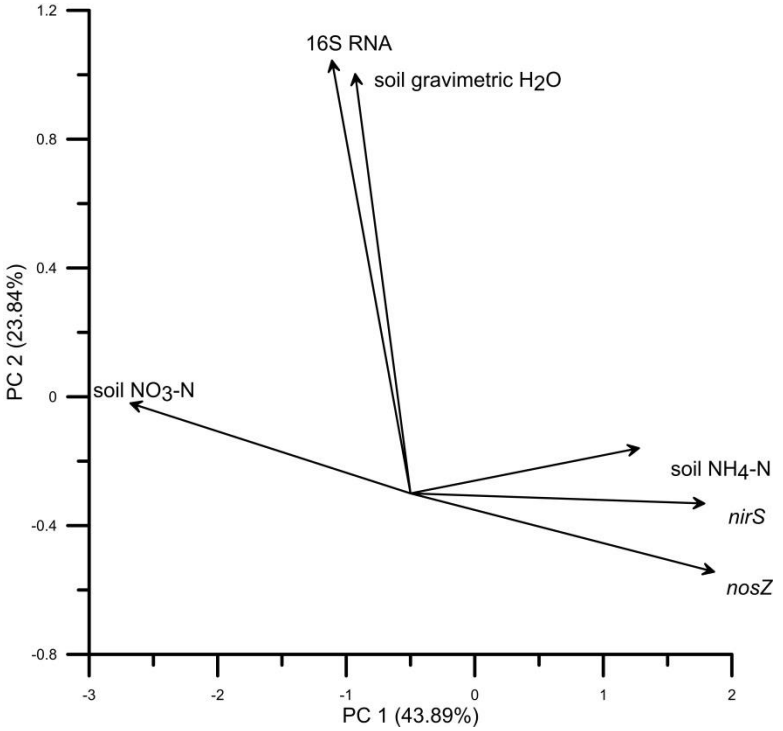
744 Figure 4b.



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