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Seasonal changes in the abundance and activity of bacterial and fungal denitrifying communities associated with different compost amendments

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Abstract (200 words)

Composts can be efficient organic amendments in potato culture as they can supply carbon and nutrients to the soil. However, more information is required to the effects of composts on denitrification and nitrous oxide emissions (N_2O) and the emission-producing denitrifying communities. The effect of three compost amendments (municipal source separated organic waste compost (SSOC), forestry waste mixed with poultry manure compost (FPMC), and forestry residues compost (FRC)) on fungal and bacterial denitrifying communities and activity was examined in an agricultural field cropped to potatoes in during the fall, spring and summer seasons. The denitrification enzyme activity (DEA), N_2O emissions and respiration were measured in parallel. N_2O emission rates were greater in FRC-amended soils in the fall and summer, while soil respiration was highest in SSOC-amended soil in the fall. A large number of *nirK* denitrifying fungal transcripts was detected in the fall, coinciding with compost application while the greatest *nirK* bacterial transcripts were measured in the summer when plants were actively growing. Denitrifying community and transcript levels were poor predictors of DEA, N_2O emissions or respiration rates in compost-amended soil. Overall, the sampling date was driving the population and activity levels of the three denitrifying communities under study.

Keywords

Denitrification; gene expression; *nirS* denitrifying bacteria; *nirK* denitrifying bacteria; *nirK* denitrifying fungi; primers

Introduction

Denitrification is a facultative anaerobic process that consists of the stepwise reduction of nitrate (NO_3^-) to dinitrogen (N_2) and is performed primarily by heterotrophic microbes that are ubiquitous in soils (Beauchamp and Bergstrom 1993). Incomplete denitrification can occur and lead to the emission of nitrous oxide (N_2O) in the environment. Nitrous oxide is the most potent greenhouse gas produced by agriculture with a global warming potential 298 times greater than CO_2 (IPCC, 2007). The majority of anthropogenic N_2O emissions are thought to be emitted from agricultural soils, primarily by microbial nitrification and denitrification (Butterbach-Bahl et al. 2003).

Potatoes are the largest vegetable crop in Canada, and particularly in the Atlantic Canada region. Atlantic Canada was responsible for over 39% of the total potato production in Canada in 2019 (Anonymous, 2020). High fertilizer N inputs are commonly applied to the potato crop to achieve the tuber yield and size distribution demanded by the potato processing industry (Zebarth and Rosen 2007). These high N inputs raise concerns as to the potential for increased N_2O emissions (Burton et al. 2012). While N_2O emissions due to agricultural practices are commonly investigated during the growing season, the soil during pre- and post-harvest may also contribute to N_2O emissions. This may be of particular importance for crops like potato which commonly have high residual soil NO_3^- after harvest (Zebarth and Milburn 2003).

The incorporation of organic amendments is commonly used to increase soil nutrient availability, increase soil organic matter content and improve soil physical structure (Chantigny et al. 2002; Tatti et al. 2017; Senbayram et al. 2018). Carbon availability, one of the primary controllers of N_2O emissions (Granli and Bøckman 1994), is increased by such organic amendments which lead to greater microbial activity. The increased microbial activity commonly increases O_2 consumption through aerobic respiration, leading to the creation of anoxic sites within soil, and thereby stimulating production and emission of N_2O (Hojberg

et al. 1994). Additionally, the quality of added C can have a significant influence on denitrification and N_2O emissions (Burton et al. 2012). Plant residues with a low C:N ratio commonly have more labile C increasing soil microbial activities such as N mineralization increasing NO_3^- supply, which may increase N_2O emissions as compared to high C:N ratio plant residues (Huang et al. 2004).

For over a century, denitrification was thought to be exclusively performed by bacteria, however increasing number of studies have shown that fungi are also able to perform this function (Bollag and Tung 1972; Bleakley and Tiedje 1982; Shoun and Tanimoto 1991; Yanai et al. 2010; Novinscak et al. 2016). Most denitrifying fungi lack the enzyme responsible for converting N_2O to N_2 and thus release N_2O as their gaseous end product (Shoun and Tanimoto 1991; Shoun et al. 2012). The use of antibiotics to inhibit bacterial and/or fungal N_2O production in the field has shown that fungi may be larger contributors to N_2O production than previously thought and may even be larger contributors than bacteria in some environments (Laughlin and Stevens 2002; Crenshaw et al. 2007; Yanai et al. 2010). The N_2O flux could be reduced by over 80% when inhibiting fungal activity in grasslands and arable peat soil, while inhibiting bacterial activity only reduced the N_2O flux by less than 30% (Laughlin and Stevens 2002). A study in upland paddy soil showed that N_2O flux rates were positively correlated with soil fungal biomass rather than denitrifying bacteria or soil chemical properties (Zhaorigetu et al. 2008).

The objectives of this study were to examine the effects of three contrasting compost amendments on the seasonal variation in denitrifying soil community size and denitrification gene expression. Composts were selected to encompass a wide range in C:N ratio, which primarily reflected the proportion of the wood wastes present in the compost feed stocks. We hypothesized that: 1) fungal and bacterial denitrifier populations and activity will differ among soils amended with contrasting composts; 2) fungal denitrifiers will be favored over bacterial denitrifiers in soil amended with compost containing a greater proportion of

wood waste residues; and 3) denitrifying microbial abundance and activity will vary seasonally. This study was part of a larger project to assess the effects of compost products on different aspects of potato production (Wilson et al. 2018a; Wilson et al. 2018b; Wilson et al. 2019).

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Material and Methods

The activity and abundance of the functional bacterial genes *nirS* and *nirK*, and the fungal gene *nirK*, as well as denitrification enzyme activity (DEA), N₂O emissions and respiration rates were measured in soil collected from an agricultural field during the fall, spring and summer seasons.

Experimental site

This study used samples collected from a subset of treatments in an existing field experiment, referred to as Experiment 1 by Wilson et al. (2018a). The experiment was established in the fall of 2014 at the Fredericton Research and Development Centre of Agriculture and Agri-Food Canada, Fredericton, New Brunswick, Canada (Lat. 45°55'N; Long. 66°36'W). Soils at the site were developed in coarse loamy till and are classified as podzols based on Food and Agriculture Organization of the United Nations (Rees and Fahmy 1984). The soil textural class (pipette method following organic matter removal, (Kroetsch and Wang 2007)), was a loam with 381 g kg⁻¹ sand, 493 g kg⁻¹ silt and 126 g kg⁻¹ clay at the 0-15 cm depth. The site was cropped to spring barley (*Hordeum vulgare* L.) in 2014 and to potatoes (*Solanum tuberosum* L.) in 2015.

Experimental design and soil sampling

For the purpose of this study, the experiment used a randomized complete block design with three compost treatments and one non-amended control and four replicates. Treatments included: 1) no compost control; 2) municipal source separated organic waste compost (SSOC; negligible wood waste residues; C/N ratio 10:1); 3) forestry waste and poultry manure compost (FPMC; medium wood waste residues; C/N ratio 27:1); and 4) forestry residues compost (FRC; high wood waste residues; C/N ratio 63:1) (Table 1). Compost treatments were applied by hand at a rate of 45 Mg ha⁻¹ on a dry weight basis from October 9-14, 2014. All plots were cultivated to a depth of 15 cm to incorporate the compost products on October

15, 2014. Individual plots were 8 m long by six potato rows (5.46 m) wide. Potatoes were planted on June 5, 2015. Fertilizer was banded at planting at a rate of 1060 kg ha⁻¹ of granular fertilizer (analysis N:P:K 17:17:17) to reflect commercial grower practices. Tillage operations and the control of insects and diseases followed accepted industry standard practices. No irrigation was applied as is common under rain-fed potato production in New Brunswick.

Composite soil samples were collected from each plot on three sampling dates: 1) October 27, 2014, twelve days after compost incorporation (fall season); 2) May 27, 2015, prior to planting the potato crop (spring season); and 3) July 13, 2015, during the potato growing season after ridges were formed (summer season). Each composite sample was comprised of six cores collected using a random sampling strategy with a 2.54 cm diameter soil probe, except for the July 13, 2015 sampling when cores were collected in the ridge close to the potato plants. Soil was thoroughly mixed, and ~10 g of soil was subsampled, placed in a 15 ml falcon tube, and stored at -80°C. Samples were subsequently lyophilized in a freeze dryer and stored at -80°C for nucleic acid isolations.

Nucleic acid isolations

DNA and RNA were isolated from all samples using the nucleic acid co-extraction protocol developed by Griffiths et al. (2008). At the end of the extraction, 100 µl of nucleic acids were obtained and split into two 50 µl aliquots. One was used as-is for DNA amplifications while the other was subjected to three consecutive DNase treatments, as described elsewhere (Paulin et al. 2009) to obtain total RNA. The absence of co-extracted contaminating DNA was confirmed by quantitative real-time PCR (qPCR) on non-retro-transcribed RNA (data not shown).

PCR amplifications

Primers targeting the fungal *nirK* gene currently exist in the literature (Long et al. 2015; Maeda et al. 2015; Wei et al. 2015), however, these primers were not efficient for qPCR under our conditions, and attempts were made to design a new primer pair targeting a conserved region of the fungal *nirK* gene. To do this, amplicons of the fungal *nirK* gene were obtained from denitrifying fungal strains isolated from soil in a previous study (Novinscak et al. 2016) and were aligned using the ClustalW function of the BioEdit sequence alignment editor v7.0.4.1 (Hall 1999). Highly conserved regions were targeted as potential primer sites. These sites were verified in the Primer Express v3.0 software (Thermo Scientific, Waltham, USA) to assure that the melting temperatures of the primers were similar, and that little non-specific amplification of the primers would occur. The most promising primer pair was synthesized (IDT DNA, Coralville, USA; *nirK2F* 5'-CCA AYG GYA TGT AYG GVC-3' and *nirK2R* 5'-GGT ARA ACT CGC TYT GCA-3') and tested on the DNA extracted from 27 denitrifying fungal cultures (Novinscak et al. 2016) as well as the soil samples obtained in this study. Fragments of correct size (97 bp) were obtained for all samples and the fragments obtained for the soil samples were cloned into the 2976 bp pKRX plasmid (National Institute of Genetics, Mishima, Japan). Each clone was sequenced using a 3130xl genetic analyzer (Applied Biosystems, Foster City, USA) and the Big Dye Terminator v3.1 Sequencing Reaction kit. All sequenced fragments were shown to match with *nirK* fungal fragments in the GenBank database.

Transcripts of *nirK* denitrifying fungi, *nirK* denitrifying bacteria, and *nirS* denitrifying bacteria were reverse transcribed using primers *nirKF2* (developed in this study), *nirKH1R* (Henry et al. 2004), and *nirSR3cd* (Kandeler et al. 2006), respectively, using the TaqMan Reverse Transcription Reagents Kit (Thermo Scientific) according to the manufacturer's supplied protocol. RT reactions were carried out as recommended by the manufacturer in a CFX Connect PCR machine (Bio-Rad, Hercules, USA). The *nirS* and *nirK* amplicons were amplified using the iTaq Universal SYBR Green supermix (Bio-Rad). Twenty microliter reactions were prepared as described by the manufacturer containing 0.5 μ M of each primer.

Quantitative PCR was performed in triplicate using the cycling protocol recommended by the manufacturer in a Real Time PCR machine. The annealing temperatures were adjusted for each target gene and consisted of 56°C for primer pair *nirKH1F-nirKH1R* (targeting *nirK* bacterial gene) (Henry et al. 2004), 53°C for primer pair *nirKff2-nirKfr2* (targeting *nirK* fungal gene) and 55°C for primer pair *nirSCd3aF-nirSR3cd* (targeting *nirS* bacterial gene) (Kandeler et al. 2006). Each reaction was performed for 50 amplification cycles and negative controls were included in each plate.

Absolute copy numbers were measured by including a five-fold standard curve for each target gene. The standards were obtained by amplifying the target region directly from soil samples using the developed qPCR protocol and the corresponding primers. The fragments were verified for specificity and size by migration on a 1.5% agarose gel. Corresponding fragments of the correct size were purified using the MinElute PCR purification kit (Qiagen, Germantown, USA). Purified fragments were cloned into the pKRX vector. Vectors containing the correct insert were verified by gel electrophoresis and were subsequently sequenced as mentioned previously. Following the sequence analysis of the fragments, the plasmids containing the correct fragments were transformed into *Escherichia coli* strains and subsequently extracted and serially diluted in a 10 mM Tris solution and used as real-time PCR standards. The efficiencies ranged from 98-106% for bacterial *nirK* standard, 97-112% for bacterial *nirS* standard and 95-105% for fungal *nirK* standard.

Biochemical and analytical methods

Sample were processed on the day that soils were sampled. The field moist soil samples were passed through a 4.75 mm sieve. A subsample of approximately 20 g was oven dried at 105°C to determine gravimetric water content (GWC).

Soil NO_3^- , ammonium (NH_4^+) and extractable organic carbon (EOC) were extracted by shaking 25 g moist soil for 30 min with 50 ml 0.5 M K_2SO_4 , and the extracts frozen at -20°C until analyses. NO_3^- , NH_4^+ and EOC concentrations of the filtered aqueous extract were measured using a Technicon Auto Analyzer II system, following Technicon Industrial Method #100-70W, Technicon Industrial Method #98-70W and Technicon Industrial Method #455-76W/A, respectively. Blank solution samples were also filtered to estimate contamination.

Soil respiration rate and N_2O emissions were determined as described by Miller et al. (2008). Briefly, field moist compost-amended soil (and only soil for control samples) (50 g) was incubated in 500 mL Mason jars at 25°C . Headspace gas samples (20 mL) were collected at approximately 0, 30 and 60 minutes using a syringe, and were injected into previously evacuated Exetainers. Headspace gas samples were analyzed for N_2O and CO_2 by a Varian Star 3800 Gas Chromatograph (Varian, Walnut Creek, CA) fitted with an electron capture detector (ECD), thermal conductivity detector (TCD) and a Combi-PAL Autosampler (CTC Analytics, Zwingen, Switzerland). Respiration rate and N_2O emissions were calculated as the rate of CO_2 -C and N_2O -N accumulation in the headspace over time per unit oven dry soil as described by Miller et al. (2008). These measurements were taken with samples from the fall, summer and spring seasons.

Denitrifying enzyme activity (DEA) was determined using a modified anaerobic slurry technique (Beauchamp and Bergstrom 1993). The assay was performed at each time point (fall, summer and spring season) using 25 g of field moist compost-amended soil (and non-amended soil for control) as described by Miller et al. (2008) except that no chloramphenicol was used.

The remaining soil was dried at 30°C for 72 hours and passed through a 2 mm sieve. Soil pH was determined in soil water suspension (1:1) (Hendershot et al. 2008).

Statistical analyses

The effect of compost amendment and sampling date on all measured variables was determined using ANOVA with treatment and sampling time as fixed factors. The model used was: Measured variable = effect of compost amendment + effect of sampling date + interaction of compost amendment and sampling date. DEA and N₂O results were log-transformed while all other variables were rank transformed to obtain normality of the data. Significant differences among compost amendments and sampling dates were determined using Tukey's honestly significant difference (HSD) *a posteriori* analysis at 0.05 level of significance. Principal component analysis (PCA) was used to determine the relationship between soil biochemical measurements and the size and gene expression of denitrifying bacteria and fungi communities. All statistical analyses were performed using SAS Studio v3.8 (SAS Institute Inc., Cary, USA), except for the PCA analysis which was performed using R v3.6.3 using the PCA function and the FactoMineR and factoextra packages.

Results

nirK denitrifying fungi primer development

Previously developed PCR primers were used to target the *nirS* and *nirK* denitrifying bacterial genes (Henry et al. 2004; Kandeler et al. 2006). New primers targeting the *nirK* denitrifying fungal gene were developed. The *nirK* denitrifying fungal primer set was tested on previously identified denitrifying and non-denitrifying fungal strains (Novinscak et al. 2016) as well as on the soil samples obtained in this study. The new primer pair was able to amplify the *nirK* gene in all denitrifying fungal pure cultures and did not yield amplicons in non-denitrifying strains (results not shown). When testing the primers on the soil samples, *nirK* denitrifying fungal fragments were amplified from all samples and the identity of the sequences obtained following sequencing and comparison to the GenBank database were to *nirK* denitrifying gene fragments from fungal isolates (results not shown).

nirK and *nirS* denitrifying bacteria and *nirK* denitrifying fungi populations

The sampling date was found to be a significant factor influencing the abundance of all three denitrifier communities under study. A decrease in *nirK* denitrifying bacteria from fall, to spring, then summer coincided with an increase in *nirK* denitrifying fungi from fall, to spring and summer and an increase in *nirS* denitrifying bacteria from spring to summer (Figure 1). The population levels for *nirK* denitrifying bacteria was found to decrease over time from approximately 1×10^9 to 2×10^7 gene copies g^{-1} soil. Contrary to this, *nirS* denitrifying bacteria and *nirK* denitrifying fungi were found to increase over time from approximately 3×10^6 to 4×10^7 and 1×10^6 to 4×10^7 gene copies g^{-1} soil, respectively. When comparing the amounts of each denitrifying community under study, it was observed that *nirK* denitrifying bacteria were found to be the dominant denitrifying community in the compost-amended soils during the fall and spring sampling. During the summer sampling, the amounts of *nirS* denitrifying bacteria and *nirK* denitrifying fungi increased to levels similar to the *nirK* denitrifying bacteria (Figure 1).

Compost amendments only had an effect on the abundance of *nirS*-type bacterial denitrifiers. The highest population values were observed in the FPMC and the SSOC treatments, while the CTRL and FRC treatments were found to be significantly lower (Figure 1b).

nirK and nirS denitrifying bacteria and nirK denitrifying fungi activity levels

When examining the activity levels for each denitrifying community, it was observed that only a small proportion of the denitrifier population was actively expressing the *nir* gene transcripts. Only sampling date and not compost amendment, showed a significant effect on the expression levels of the bacterial *nirK* and *nirS* gene transcripts. No effect, either of sampling date or compost amendment, significantly influenced the transcript levels of fungal *nirK* gene. The expression levels did not follow the same trends as the population levels for any of the three communities under study (Figure 2). For the *nirK* denitrifying bacteria, the transcript levels were found to be greatest in the fall and the summer, with significantly less transcripts detected in the spring. The *nirS* denitrifying bacterial transcripts were found to significantly increase from fall to spring, then significantly decrease in the summer to levels below the fall values. Finally, the activity levels of *nirK* denitrifying fungi were not found to be affected by the sampling date and the values remained constant throughout all three seasons. Interestingly, the highest transcript levels were measured for the *nirK* denitrifying fungi (average of 3.44×10^4 transcripts g^{-1} soil), while the lowest were measured for the *nirS* denitrifying bacteria (average of 2.08×10^2 transcripts g^{-1} soil), and the *nirK* denitrifying bacteria transcript levels were similar to those measured for the *nirK* denitrifying fungi (average of 1.96×10^4 transcripts g^{-1} soil). When examining the gene expression level of each denitrifying community, the major contributor was found to be the *nirK* denitrifying fungi in the fall and spring, while in the summer the number of transcripts produced by *nirK* denitrifying bacteria increased, and in some cases surpassed those produced by *nirK* denitrifying fungi (Figure 2).

The ratio of activity levels and population abundance for each denitrifying community was also determined, i.e. RNA/DNA ratios (Figure 3). The ratios were used to assess if the community was more active independently from an increase or decrease in abundance, thus providing an indication of cellular activity in the environment as reported previously in Tatti et al. (2015). The RNA/DNA ratio of *nirK* denitrifying bacteria indicated that the activity levels significantly increased from the fall to the spring and the highest values were observed in the summer. As for the RNA/DNA ratio of *nirS* denitrifying bacteria, the values significantly increased from the fall to the spring and finally significantly decreased in summer to values below those of the fall. Finally, the RNA/DNA ratio of *nirK* denitrifying fungi were significantly higher in the fall, then decreased in the spring and finally reached their lowest values in the summer.

Denitrification enzyme activity (DEA)

Compost amendments did not have any effects on the DEA measurements, while significant changes in DEA was observed over the sampling dates (Figure 4a). The DEA values were found to remain constant through the fall and spring sampling and to significantly decrease in the summer.

N₂O production

The N₂O measurements were significantly influenced by the compost amendment as well as the sampling date (Figure 4b). The highest values were found in the FPMC treatment while the lowest were in the FRC treatment. The greatest N₂O emissions were observed in the fall and the summer while those in the spring were significantly lower.

CO₂ respiration

The carbon respiration values were found to be significantly affected by compost amendment and sampling date (Figure 4c). Values were highest in the SSOC and FPMC treatments, followed by the FRC treatment and finally the CTRL treatment. Values were also higher in the fall, followed by the spring and lower in the summer.

A PCA analysis of all biochemical measurements and *nir* gene population and activity levels was conducted to examine the association between the different measured variables. PCA of the data identified two components with eigenvalues over 1 (4.42 and 2.01) that accounted for over 71% of the data variability (Fig. 5). The following variables (loading values are in brackets) were the main contributing variables to principal component 1, which explained 49% of the variability in the data: *nirS* population (-0.425), DEA (0.417), *nirK* fungal population (-0.404) and *nirK* bacterial activity (-0.343). *nirK* bacterial population (0.570), respiration (0.481), N₂O (0.416), and *nirS* activity (-0.397) were found to be the variables contributing to principal component 2 (loading values in brackets), which explained 22% of the variability in the data. The two axes grouped the data by the sampling dates.

Discussion

Our study showed that compost application to soil resulted in changes in the respiration, i.e. in the availability of the organic carbon in soil. Respiration was highest in the SSOC and FPMC treatments, followed by the FRC treatment, a reflection of the lower C:N ratios of SSOC and FRC compared with FPMC. Increased respiration due to compost application to soil has previously been reported (Chantigny et al. 2002). Respiration was also highest in the fall, twelve day after the compost application, compared to the spring and lowest in the summer indicating decomposition of the compost products in soil over time. However, the increase in respiration in SSOC and FPMC did not result in an increase in the abundance of the three denitrifying communities targeted in this study. It is possible that the increase in soil organic C due to the compost amendments might be negligible compared to soil organic C or that the organic C is too recalcitrant to increase the denitrifying communities.

A distinct seasonal variation in the abundance of all three denitrifying communities was observed in this study. The dominant denitrifying community in all samples in the fall and spring was *nirK* denitrifying bacteria. Many studies have typically shown the dominance of one type of denitrifying community, such as the dominance of *nirK* bacterial denitrifiers in rice paddy field soil (Yoshida et al. 2009; Azziz et al. 2017), in long-term fertilized soils (Hou et al. 2018), and in European mountain grasslands (Szukics et al. 2019). The dominance of one type of denitrifier in specific habitats has led to the idea that there might be niche-based selection of *nir*-type denitrifiers that can be based on different factors, including environmental gradients (Azziz et al. 2017), soil pH (Enwall et al. 2010) and the presence/absence of plants (Hallin et al. 2009; Hou et al. 2018). Our results indicated that in the summer, the abundance of the denitrifying communities shifted and the abundance of all three communities was found to be similar. This increase in *nirS* denitrifying bacteria and *nirK* denitrifying fungi could be due to the increased nitrate concentration, temperature and substrate availability due to the presence of roots from the potato crop in the summer months. These results are somewhat contrary to previous results that indicated that *nirS* and

nirK bacterial denitrifiers were less abundant in summer than other seasons (Coyne 2018). However, seasonal fluctuations in the denitrifying communities have previously been observed in other studies (Wolsing and Prieme 2004; Bremer et al. 2009).

The abundance values obtained for the dominant *nirK* denitrifying bacteria (1×10^9 to 2×10^7 bacteria g^{-1} soil) were found to be similar to the values obtained by Azziz et al. (2017), who examined the abundance of *nirK* denitrifying bacteria in rice paddy fields and contained an average of 5.6×10^8 and 1.1×10^9 copies g^{-1} soil and those obtained by Szukics et al. (2019) in European mountain grasslands where values varied from 1×10^9 to 7×10^9 copies g^{-1} soil. Values for *nirS* denitrifiers (ranging from 3×10^6 to 4×10^7 copies g^{-1} soil) were similar to those obtained by Nemeth et al. (2014) who found that *nirS* denitrifying bacteria were the dominant denitrifiers in agricultural fields with corn-soybean-wheat rotations and that their abundance ranged from 3.4×10^6 to 6.5×10^6 copies g^{-1} soil. Additionally, Deslippe et al. (2014) found that *nirS* denitrifying bacterial populations represented 1×10^6 to 1×10^7 copies g^{-1} soil and Szukics et al. (2019) found *nirS* denitrifying bacterial populations ranging from 5×10^6 to 1.5×10^7 copies g^{-1} soil. As for the *nirK* denitrifying fungi, there are very few studies that have directly measured their abundance in soil, and our values (ranging from 1×10^6 to 4×10^7 copies g^{-1} soil) are lower than those observed by Xu et al. (2019), which varied from 9.9×10^8 to 3.2×10^9 copies g^{-1} soil, but could be due to the use of different primers targeting the fungal *nirK* gene or to different soil properties between the two studies. Other factors can also explain differences in the amount of targets detected per soil, including the nucleic acid extraction efficiency, the presence of inhibitors in the nucleic acids, or the enzyme used to amplify the nucleic acids. Additionally, as primers targeting *nirK* denitrifying fungi have only been recently developed (Long et al. 2015; Maeda et al. 2015; Wei et al. 2015), very few studies have directly measured the abundance of *nirK* denitrifying fungi in soils and the environmental parameters possibly affecting their abundance are not well understood. A study by Xu et al. (2019) found that the ratio of fungal/bacterial

nirK gene abundance was positively correlated to the ratios of fungal/bacterial contributions to N₂O emissions.

When examining the denitrifying transcript levels of the three communities under study, we observed that only a small number of transcripts were measured when comparing these values to the abundance values obtained. If the *nir* gene contained in each bacteria or fungi is thought to be a single copy gene (Braker et al., 2000), our results seem to indicate that only a small fraction of the denitrifier population is involved in actively transcribing denitrification genes. When examining each denitrifying community separately, it was observed that the transcript levels for *nirK* denitrifying fungi remained constant throughout the three seasons. However, when examining the *nirK* denitrifying fungi RNA/DNA ratio, a large production of *nirK* transcripts by the denitrifying fungi was observed in the fall, followed by a decrease in the spring and the summer (Figure 3). The production of *nirK* denitrifying fungi gene transcripts seemed to be stimulated by greater respiration and low nitrate concentrations in the fall as compared to the spring and summer. The *nirS* denitrifying bacterial transcripts and *nirS* RNA/DNA ratio increased from the fall to the spring and decreased to the lowest values in the summer perhaps due to dryer conditions in summer compared to fall and spring. When examining the *nirK*-type bacterial transcript levels, they were significantly higher in the fall and the summer than in the spring. The *nirK* denitrifying bacteria RNA/DNA ratio indicates that the transcript levels were lower in the fall, increasing in the spring and the summer, possibly due to the presence of the potato plants. A study by Nemeth et al. (2014) observed that *nirK* denitrifying bacterial transcripts were below detection levels in agricultural fields with a corn-soybean-wheat rotation, while *nirS* denitrifying bacterial transcripts ranged from 1.2×10^3 to 2.9×10^2 transcripts per g⁻¹ soil, which is similar to the transcript values obtained in our study (Nemeth et al. 2014).

DEA is a measure of the potential denitrification since both organic C and nitrate are supplied and that it represents the pre-existing enzymes in the soil samples and not enzymes that are synthesized during the incubation (Smith and Tiedje 1979). The measured DEA values were found to remain constant through the fall and spring and to significantly decrease in the summer, probably indicating the pre-existing enzymes were low in soil due to the dry soil conditions in July. Results from previous studies tend to vary and have shown no significant correlation between the denitrifier community abundance and DEA (Dandie et al. 2008; Miller et al. 2008; Hallin et al. 2009; Yin et al. 2014; Azziz et al. 2017), while others have shown positive correlation between DEA and the abundance of *nirS* denitrifying bacteria (Enwall et al. 2010; Deslippe et al. 2014) or that DEA was significantly correlated to the *nirK* denitrifying bacteria community structure (Yin et al. 2014).

Several studies have suggested that DEA and/or N₂O emissions may better correlate with the expression of the denitrification genes (mRNA transcripts) as opposed to the abundance of denitrifying organisms because the detection of mRNA suggests that the microorganisms were actively expressing these genes and likely producing the associated enzymes (Yoshida et al. 2009; Maul et al. 2019). Nemeth et al. (2014) have shown that the *nirS* transcript activity and the gene's abundance were negatively correlated to N₂O emissions in two conventionally tilled fields while *nirK* denitrifying bacterial transcripts were below the detection limit. Hou et al. (2018) found that *nirK* denitrifying bacterial abundance was significantly negatively correlated to N₂O production in long-term fertilized soils.

The N₂O emissions were significantly influenced by the compost amendment as well as the sampling date. The highest values were found in the FPMC treatment while the lowest were in the FRC treatment indicating a higher organic C availability in soils treated with FPMC than those treated with FRC. The sampling date showed that the highest N₂O emissions were observed in the fall when soil had a greater

soil moisture content creating anoxic conditions and in the summer when greater soil N_2O concentration due to fertilisation was present compared to spring. Increases in denitrifying communities did not lead to higher denitrifying gene transcripts or higher N_2O values. Previous studies have claimed that how denitrifier communities relate to N_2O emissions is not fully understood (Stres et al. 2008; Szukics et al. 2009; Chen et al. 2014), and as previous studies have shown that fungal denitrifiers can contribute large emissions values of N_2O (Johnson et al. 1996; Laughlin and Stevens 2002; Crenshaw et al. 2007). We attempted to correlate the N_2O emission rates to the abundance or transcript levels of all three populations. However, no significant correlation was observed (results not shown). In contrast, the use of substrate-induced respiration inhibition (SIRIN) tests to attribute N_2O production to bacteria and fungi has previously shown that bacterial and fungal populations were positively correlated to their respective production of N_2O emissions (Xu et al. 2019). Several reasons can explain why there was an absence of correlations between the abundance or denitrifying gene transcripts in this study including an important proportion of emissions being N_2 (Maul et al. 2019) or the loss of deeper N_2O flux from the soil profile since only the top layer was sampled. Additionally, the denitrification pathway may be regulated at the post-transcriptional level (Burton et al. 2012; Maul et al. 2019).

In conclusion, we were able to detect and quantify the population and activity levels of *nirK* and *nirS* denitrifying bacteria and *nirK* denitrifying fungi in compost amended soils. Unexpectedly, the compost amendments only displayed an effect on the population of *nirS* denitrifying bacteria. The main factor driving the population and activity levels of the three communities under study was the sampling date. The population levels and denitrifying transcript levels do not seem like good predictors of DEA, N_2O rates or respiration rates in compost-amended soil. Additional studies under different field conditions and geographical locations will be required to better understand the complex interactions existing between these variables.

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Table 1. Characteristics of compost products before their application to the soil (with the exception of dry matter and pH, all values are expressed on dry matter basis).

Characteristic	FRC*	SSOC	FPMC
Dry matter (%)	42.6 ± 1.8 [†]	48.6 ± 1.4	53.6 ± 2.0
pH	6.97 ± 0.06	7.70 ± 0.10	7.57 ± 0.06
C:N	62.8 ± 0.8	10.0 ± 0.4	27.4 ± 1.2
Ash (g kg ⁻¹)	517 ± 41	591 ± 30	700 ± 25
C (g kg ⁻¹)	239 ± 17	221 ± 26	172 ± 15
N (g kg ⁻¹)	3.8 ± 0.3	22.0 ± 1.9	6.3 ± 0.5
P (g kg ⁻¹)	0.49 ± 0.02	8.78 ± 2.88	2.86 ± 0.10
K (g kg ⁻¹)	1.32 ± 0.23	4.40 ± 0.04	4.28 ± 0.79
Ca (g kg ⁻¹)	7.9 ± 0.7	38.4 ± 5.6	36.7 ± 1.0
Mg (g kg ⁻¹)	3.15 ± 0.47	3.80 ± 0.09	7.35 ± 1.91
Zn (mg kg ⁻¹)	54 ± 3.0	189 ± 11.5	277 ± 46.3
B (mg kg ⁻¹)	4.4 ± 0.2	22.0 ± 0.8	26.1 ± 3.0
Mn (mg kg ⁻¹)	543 ± 13	624 ± 44	2599 ± 240
NO ₃ -N (mg kg ⁻¹)	<5	603 ± 55	184 ± 67
NH ₄ -N (mg kg ⁻¹)	35.5 ± 1.3	27.7 ± 5.7	40.0 ± 6.0

*FRC, forestry residues compost; SSOC, municipal source separated organic waste compost;

FPMC, forestry waste and poultry manure compost.

[†]Mean ± standard deviation

Figure Legends

Figure 1. Abundance of *nirK* denitrifying bacteria (A), *nirS* denitrifying bacteria (B), and *nirK* denitrifying fungi (C) communities detected by quantitative PCR at three sampling dates. CTRL indicates control samples, SSOC : municipal source separated organic waste compost derived from organic curb-side waste; FPMC: manure compost with paper produced by composting of paper mill residue, poultry manure and wood ash; FRC: forestry residues compost product derived from wood-waste feedstock including bark, paper mill residue and wood-ash with approximately 5% manure. Letters indicate statistical differences observed by Tukey's HSD multi-level test at 5% significance.

Figure 2. Gene expression of *nirK* denitrifying bacteria (A), *nirS* denitrifying bacteria (B), and *nirK* denitrifying fungi (C) detected by reverse transcription followed by quantitative PCR and expressed g^{-1} soil at three sampling dates. CTRL indicates control samples, SSOC : municipal source separated organic waste compost derived from organic curb-side waste; FPMC: manure compost with paper produced by composting of paper mill residue, poultry manure and wood ash; FRC: forestry residues compost product derived from wood-waste feedstock including bark, paper mill residue and wood-ash with approximately 5% manure. Letters indicate statistical differences observed by Tukey's HSD test at 5% significance.

Figure 3. Ratio of RNA/DNA for *nirK* denitrifying bacteria (A), *nirS* denitrifying bacteria (B), and *nirK* denitrifying fungi (C) at three sampling. The ratio of RNA/DNA was obtained by dividing the number of transcripts for each denitrifying community by their respective gene copy numbers g^{-1} of soil. CTRL indicates control samples, SSOC : municipal source separated organic waste compost derived from organic curb-side waste; FPMC: manure compost with paper produced by composting of paper mill residue, poultry manure and wood ash; FRC: forestry residues compost product derived from wood-waste

feedstock including bark, paper mill residue and wood-ash with approximately 5% manure. Letters indicate statistical differences observed by Tukey's HSD test at 5% significance.

Figure 4. Denitrification enzyme activity (A), N₂O emissions (B) and respiration rates (C) measured at three sampling dates. CTRL indicates control samples, SSOC : municipal source separated organic waste compost derived from organic curb-side waste; FPMC: manure compost with paper produced by composting of paper mill residue, poultry manure and wood ash; FRC: forestry residues compost product derived from wood-waste feedstock including bark, paper mill residue and wood-ash with approximately 5% manure. Letters indicate statistical differences observed by Tukey's HSD test at 5% significance.

Figure 5. Principal component analysis examining the relationship between soil biochemical measurements and abundance and gene expression of denitrifying bacteria and fungi.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.