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# Changes in Bacterial Denitrifier Community Abundance over Time in an Agricultural Field and Their Relationship with Denitrification Activity▽

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**This study measured total bacterial and denitrifier community abundances over time in an agricultural soil cropped to potatoes (*Solanum tuberosum* L.) by using quantitative PCR. Samples were collected on 10 dates from spring to autumn and from three spatial locations: in the potato "hill" between plants (H), close to the plant ($H_p$), and in the "furrow" (F). The denitrification rates, $N_2O$ emissions, and environmental parameters were also measured. Changes in denitrifier abundance over time and spatial location were small (1.7- to 2.7-fold for the *nirK*, *nosZ*, and *cnorB*$_B$ guilds), whereas the *cnorB*$_P$ community (*Pseudomonas mandelii* and closely related spp.) showed an ~4.6-fold change. The seasonal patterns of denitrifier gene numbers varied with the specific community: lower *nosZ* gene numbers in April and May than in June and July, higher *cnorB*$_P$ gene numbers in May and June than in March and April and September and November, higher *nirK* gene numbers in early spring than in late autumn, and no change in *cnorB*$_B$ gene numbers. Gene numbers were higher for the $H_p$ than the H location for the *nosZ* and *nirK* communities and for the *cnorB*$_P$ community on individual dates, presumably indicating an effect of the plant on denitrifier abundance. Higher *cnorB*$_P$ gene numbers for the H location than the F location and for *nosZ* and *cnorB*$_B$ on individual dates reflect the effect of spatial location on abundance. Denitrifier abundance changes were not related to any environmental parameter, although a weak relationship exists between *cnorB*$_P$ gene numbers, extractable organic carbon values, and temperature. Denitrification and $N_2O$ emissions were mostly regulated by inorganic nitrogen availability and water-filled pore space but were uncoupled from denitrifier community abundances measured in this system.**

With a global warming potential ~296 times that of carbon dioxide ($CO_2$), nitrous oxide ($N_2O$) is the fourth-most-important contributor to the greenhouse effect after water vapor, carbon dioxide, and methane. $N_2O$ emissions into the atmosphere are increasing at a rate of about 0.3% per year, and this is thought to be associated with increased use of nitrogen fertilizers in agricultural production (18, 23). It has been estimated that agriculture contributes ≥75% of the global anthropogenic $N_2O$ emissions (31). In Canada, $N_2O$ is the main greenhouse gas associated with agricultural production (13).

$N_2O$ is an intermediate product in the soil processes of nitrification and denitrification. In agricultural soils, $N_2O$ emissions are associated primarily with denitrification in soils where the oxygen supply is limited by high water-filled pore space (WFPS) (1) or by high oxygen consumption following inputs of readily available carbon (11).

Soil water content, through its control of soil aeration, carbon, and $NO_3^-$-N availability and soil temperature, has been identified as an important factor controlling denitrification (23, 27, 32). The biogeochemical and soil physical processes influencing denitrification rates and $N_2O$ emissions are generally understood (18); however, the prediction of denitrification rates and $N_2O$ emissions in the field remains problematic. This reflects the high spatial and temporal variability of these controlling factors and their complex interactions in controlling the denitrification process. Field-based research has primarily focused on biogeochemical measures of denitrification. In contrast, the denitrifier bacterial community in situ is one element in this process that has been difficult to study (29, 39) until the recent advent of environmental molecular biology techniques (28).

Molecular tools have been developed to quantify denitrifiers in soil, for the most part based on quantitative real-time PCR techniques. Primer sets using Sybr green detection have been developed for the major genes in the denitrification pathway (*nirS*, *nirK*, and *nosZ*) (14, 15, 19), and primer sets based on the *cnorB* gene targeting *Pseudomonas mandelii* and closely related spp. (*cnorB*$_P$) and *Bosea*/*Bradyrhizobium*/*Ensifer* denitrifier (*cnorB*$_B$) guilds have been developed in our research group to target culturable denitrifiers found in soil from a potato crop system (*Solanum tuberosum* L.) in New Brunswick, Canada (8, 9). Denitrification should be considered a community process, as not all denitrifiers produce the full suite of enzymes to

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TABLE 1. Soil sampling dates, spatial locations sampled, and corresponding soil conditions or crop growth stage, soil temperature, and WFPS value

| Date | Spatial location(s) sampled | Soil condition/ crop stage[a] | Soil temp (°C) | WFPS (% vol/vol)[b] | |
|---|---|---|---|---|---|
| | | | | H | F |
| 30 March | H | Thaw | −0.7 | 68 | NA |
| 20 April | H | Trafficable | 6.4 | 73 | NA |
| 25 May | H/F | Planting | 10.7 | 54 | 61 |
| 20 June | H/F/$H_p$ | Early growth | 19.2 | 50 | 59 |
| 13 July | H/F/$H_p$ | Hilling | 22.4 | 43 | 60 |
| 10 August | H/F/$H_p$ | Tuber bulking | 18.3 | 36 | 55 |
| 6 September | H/F/$H_p$ | Late growth | 18.4 | 46 | 58 |
| 28 September | H/F/$H_p$ | Vine desiccation | 10.8 | 38 | 58 |
| 26 October | H | Postharvest | 4.9 | 60 | NA |
| 23 November | H | First frost | 0.3 | 68 | NA |

[a] Trafficable refers to when fields are able to be driven on by farm vehicles. Vine desiccation refers to the use of diquat to desiccate above-ground vegetation and facilitate tuber maturation prior to harvest.
[b] NA, not applicable.

complete the process (39), and in the environment, it is likely that bacteria of different taxonomic affiliations and differing genetic capabilities contribute together as a sink for the denitrification of N-oxide products. Little research has been performed to study denitrifier communities in situ and the effects of environmental conditions on their population density and activity. It has generally been assumed that denitrifier community composition and abundance have little effect on denitrification rates and $N_2O$ emissions (29). Some studies have linked changes in denitrifier community structure with altered function and sensitivity to environmental change or parameters (5–7, 22), although strong relationships are not always observed (3, 25). A major research focus has been on denitrifier community composition, but there is also a need to quantify abundance in order to link abundance, community composition, and nitrogen emissions in the environment (28).

The primary objective of this study was to investigate the community dynamics of components of the soil denitrifier bacterial community over the course of a field season from spring thaw until autumn freeze in an agricultural field cropped to potatoes. We also aimed to investigate possible relationships between denitrifier community abundance and environmental parameters relating to denitrifier activity. We hypothesized that denitrifier community gene numbers will change over time and spatial location and that changes in denitrifier gene numbers are related to denitrification and $N_2O$ emissions and to environmental parameters. The potato represents the dominant arable crop in Atlantic Canada. Potato cropping systems, which commonly receive high N fertilizer inputs (40), frequently have high $N_2O$ emissions compared with those of other typical arable crops (31, 34). High spatial and temporal variation in $N_2O$ emissions has been documented in potato fields, particularly in relation to the row structure (i.e., the potato "hill" and the "furrow") (4, 31). In this study, denitrifier community abundance, denitrification rates, and $N_2O$ emissions were measured in different spatial locations during the crop growth period and in the bulk soil for the entire monitoring period.

## MATERIALS AND METHODS

**Field plot preparation, soil sampling, and soil characteristics.** Soil samples were collected at the Potato Research Centre, Agriculture and Agri-Food Canada, Fredericton, New Brunswick, Canada (45°52′N, 66°31′W) during 2006. The site has a maritime climate and humid soil moisture regimes. Soils at the experimental site belong to the Research Station soil association, which consists of coarse loamy morainal ablational till over coarse loamy morainal lodgement till, and are classified as orthic humo-ferric podzols (30). The soil properties at the experimental site (0 to 15 cm deep) were sand, 375 g $kg^{-1}$; silt, 503 g $kg^{-1}$; and clay, 121 g $kg^{-1}$ (pipette method with organic matter removal); pH 6.4 (1:1 water); total organic carbon, 31.5 g $kg^{-1}$; and total nitrogen, 1.59 g $kg^{-1}$ (combustion; Leco CNS-1000).

Samples were collected from the potato year of a potato-spring wheat rotation. Sampling dates were chosen to capture changing conditions in the field soil from the time of first thaw (March) until the soil was again frozen (November) (Table 1). Samples were collected from six plots, where each plot consisted of six potato rows 0.91 m apart and 8 m in length. The plots were planted on 15 May to potato cultivar Russet Burbank with 0.51-m within-row spacing. The wide within-row spacing was chosen to allow bulk soil to be collected between plants in the potato hill which would therefore have had limited influence from the potato root systems. Fertilizer was applied in bands about 7.5 cm to each side and 5 cm below the potato seed pieces at planting, according to normal production practices, at 200 kg N $ha^{-1}$ as ammonium nitrate, 150 kg $P_2O_5$ $ha^{-1}$, and 150 kg $K_2O$ $ha^{-1}$. The plots were cultivated on 6 July prior to final hilling on 10 July. Hilling is where a tillage implement is used to remove surface soil from between potato rows to form the furrow and place the soil at the base of the plants to form a hill with the soil physical conditions that promote tuber development. Vine desiccation was performed on 22 September by using the desiccant diquat (1,1′-ethylene-2,2′-bipyridylium dibromide). Standard commercial practices were used for control of diseases, insects, and weeds (2). No irrigation was applied.

**Field soil sampling procedures.** Soil samples were collected on 10 dates (Table 1). During the crop growth period, samples were collected separately from three spatial locations: the bulk soil between plants in the potato hill (H), the soil in the hill in close proximity to the plant roots ($H_p$), and the bulk soil in the furrow (F) (Fig. 1; Table 1). Samples collected from the bulk soil prior to and after the crop growth period were considered comparable to the H location in this study and thus are referred to as "H" because the hill and bulk soil consist of the top soil layer. Soil was sampled in the field using a 2.54-cm-diameter stainless steel sampler, sterilized between samples with 70% (vol/vol) ethanol. For the H and F locations, one composite sample of six cores was randomly sampled from each plot. For $H_p$ samples taken early in the season, 3 to 4 potato plants were uprooted and the soil clinging to the roots was shaken into a sample bag. For $H_p$ samples taken later in the season (10 August and subsequent dates), very little soil adhered to the root system, and therefore, a grab sample of loose soil was

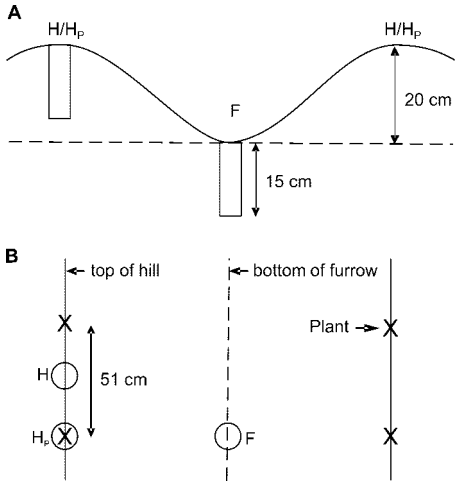


FIG. 1. Schematic representation of potato hill and furrow showing different spatial locations for sampling during the crop growth period. (A) Vertical cross-section of a potato row, showing soil core position and depth for F and H/$H_P$ sample locations. (B) Aerial view of potato row. Spatial sampling locations include H and $H_P$ and F.

obtained from the area from which the potato plant had been uprooted, where root-length density is at a maximum (33). On each sampling date, the soil temperature was taken at a depth of 10 cm. The composite samples were split into subsamples to perform nucleic acid and biochemical analyses as described below.

For nucleic acid analyses, sterile plastic tubes (15 ml) were immediately filled with soil and quick-frozen in liquid nitrogen in the field in a cryogenic vapor shipper (V-900; VWR International, Ville Mont-Royal, Quebec). Liquid-nitrogen-frozen soil tubes were retrieved from the vapor shipper and stored at −80°C until further processing. Soil samples for biochemical analyses were stored in a cooler with ice packs while sampling was completed and immediately transported back to the laboratory for processing. Soil for all analyses (extractable organic carbon [EOC], denitrification enzyme activity [DEA], $NO_3^-$, and $NH_4^+$ determinations) was sieved (<4 mm) and used immediately.

The $CO_2$ flux and $N_2O$ flux were measured on each soil sampling date as described in reference 4 by using nonflowthrough, non-steady-state chambers with a 1.6-liter total volume and covering a soil area of 315 $cm^2$. $CO_2$ flux was used as the measure of respiration. Gas samples were collected at 0, 10, 20, and 30 min. Samples were collected by removing 20 ml of gas from the headspace of the chamber and injecting it into previously evacuated (to 500 millitorr) 12-ml Exetainers (Labcor, United Kingdom), each containing 4 mg of magnesium perchlorate, a desiccant to remove water from the gas sample. One collar was installed in each plot prior to planting, whereas after planting, two collars were installed in each plot, one in the potato hill midway between plants and one in the furrow. Collars were removed to allow cultivation and hilling to be performed and then immediately reinstalled and left in place until the potato harvest. In addition, one collar was installed in each plot following harvest. The collars were constructed from 20.3-cm inner-diameter PVC pipe and were beveled at the bottom to help with insertion into the soil. Two heights of collars were used, an 11.5-cm-tall collar for use in the furrow and a 20-cm-tall collar for use in the hill, to ensure a good seal at the bottom of the collar in the loose soil present in the hill. Collars were installed so as to achieve 5 cm of headspace. Soil respiration (measured as $CO_2$ flux; kg $CO_2$-C $ha^{-1}$ $day^{-1}$) and $N_2O$ flux (g $N_2O$-N $ha^{-1}$ $day^{-1}$) were calculated, as described in reference 4, from the linear regression of the rate of $CO_2$ or $N_2O$ accumulation in the chamber headspace against time, after correction for changes in temperature and pressure according to the ideal gas law.

Soil cores were collected on each sampling date for determination of the denitrification rate by using the acetylene blockage method as described in reference 26. Soil cores were 13 cm long by 6 cm in diameter and were incubated in 1-liter glass mason jars with the addition of 10% $C_2H_2$ over a 24-h period. Gas samples were collected at the start of incubation and after 24 h and stored as described above. Following incubation, soil was removed from the core and used to determine soil bulk density (corrected for coarse fragments), gravimetric water content, and WFPS. The denitrification rate (g N $ha^{-1}$ $day^{-1}$) was calculated based on the accumulation of $N_2O$ over the 24-h incubation period with correction for $N_2O$ dissolved in the soil water and expressed on an area basis assuming a depth of 13 cm and the measured bulk density (range, 0.7 to 1.2 g $cm^{-3}$; average, 1.0 g $cm^{-3}$).

**Biochemical and analytical methods.** The DEA was determined by using an anaerobic slurry technique similar to the phase I assay in reference 35. Soil slurries were prepared by mixing 25 g of field-moist soil in 125-ml Erlenmeyer flasks with 25 ml of room-temperature buffer solution (10 mM glucose, 10 mM $KNO_3$, 50 mM $K_2HPO_4$, and 0.1 g $liter^{-1}$ chloramphenicol to inhibit new enzyme production). The flasks were evacuated three times and flushed with helium gas. After the final flush with He, flasks were brought to atmospheric pressure prior to the addition of 20 ml acetylene. Flasks were shaken for 1 min prior to taking a time-zero gas sample (8 ml). Flasks were shaken for 60 min, with gas samples taken every 15 min. Gas samples were collected by removing 8 ml and storing in previously evacuated 6-ml Exetainers. The gas volume in the flasks was replenished with He (8 ml) after each sampling to maintain pressure.

$NO_3^-$, $NH_4^+$, and EOC were determined from $K_2SO_4$ extracts of nonfumigated samples. Segmented flow analysis (Technicon Industrial Systems, Tarrytown, MA) was used for the colorimetric determination of EOC (Technicon method 455-76 W/A), $NH_4^+$ (EPA method 350.1), and $NO_3^-$ concentrations (Technicon method 100-70W).

Gas analysis was performed by using a Varian Star 3800 gas chromatograph (Varian, Walnut Creek, CA) fitted with an electron capture detector (ECD), a thermal conductivity detector (TCD), and a Combi-PAL autosampler (CTC Analytics, Zwingen, Switzerland). The ECD was operated at 300°C, with 90% Ar, 10% $CH_4$ carrier gas at 20 ml $min^{-1}$, using a Haysep N 80/100-mesh precolumn (0.32 cm in diameter by 50 cm long) and Haysep D 80/100-mesh analytical columns (0.32 cm in diameter by 200 cm long) in a column oven operated at 70°C. The precolumn was used in combination with a four-port valve to remove water from the samples. The TCD was operated at 130°C, with prepurified He carrier gas at 30 ml $min^{-1}$ and a Haysep N 80/100-mesh (0.32 cm in diameter by 50 cm long) precolumn followed by a Porapak QS 80/100-mesh (0.32 cm in diameter by 200 cm long) analytical column maintained at 70°C. $N_2O$ was quantified based on ECD response for concentrations up to 50 μl $N_2O$ $liter^{-1}$ and on TCD response for concentrations of >50 μl $N_2O$ $liter^{-1}$. $CO_2$ concentrations were always measured using TCD.

**Soil DNA extraction and quantitative PCR.** Soil samples in 15-ml tubes were freeze-dried overnight until completely dry prior to nucleic acid extraction. The freeze-dried soil was stored at −80°C. Nucleic acids were extracted from the soil as previously described (9). DNA was quantified with a Picogreen assay (Invitrogen, Burlington, Ontario) using a 96-well-plate fluorometer (Fluoroskan Ascent; Thermo Fisher Scientific, Waltham, MA). Quantitative real-time PCR was performed according to methods previously described to measure the density of the following communities: the 16S rRNA gene (as a measure of total bacterial numbers) (36), $cnorB_P$ and $cnorB_B$ (denitrifier guilds carrying the *cnorB* gene previously isolated from the potato field site) (9), *nosZ* (as a measure of denitrifiers carrying the $N_2O$ reductase gene) (15), and *nirK* (as a measure of denitrifiers carrying the *nirK* nitrite reductase gene) (14). Primers were also tested for the quantification of *nirS* (19); however, technical difficulties with this primer set precluded their inclusion in this study. The plasmids used as quantification standards were as described previously (9) for the $cnorB_P$, $cnorB_B$, and 16S rRNA genes. Plasmid clones were generated for *nirK* and *nosZ* gene quantification standards as described previously (9), using *Achromobacter* sp. strain PD20 and *Pseudomonas brassicacearum* PD5 genomic DNA as templates, respectively. Slight modifications were made to the cycling conditions as described in reference 15 for the quantification of *nosZ*. The reactions were conducted in 2× Applied Biosystems Power Sybr green master mix. The primers were optimized for use at 750 nM (nosZ1F) and 1,000 nM (nosZ1R). T4 Gene 32 Protein (10 mg $ml^{-1}$) was added to the reaction mixtures to reduce inhibition by soil-derived components. The results of standard plasmid DNA quantification with and without the presence of environmental DNA extracts were compared to detect any inhibition by coextracted soil components. The cycling conditions were as follows: 95°C for 15 min (1 cycle), 95°C for 15 s, touchdown of 68 to 62°C (−1°C/cycle) for 1 min, 81.5°C for 30 s (touchdown for 6 cycles), and then the same cycling conditions with a 62°C annealing temperature for the remaining 35 cycles. Slight modifications were also made for the quantification of *nirK* using the primers nirK876 and nirK1040 (14). The extension step at 72°C was removed, the data acquisition step was 30 s (the shortest time allowed on the ABI Prism 7000), and the cycles were repeated 35 times. Dissociation curves were performed for all wells, and the results confirmed the specificity of PCR product formation for all assays. The gene copy numbers reported in this study are per gram of dry soil.

**Statistical analyses.** Statistical analyses were conducted by using the general linear model of SAS (version 8; SAS Institute, Inc., Cary, NC). All parameters were tested for normality, and a natural log transformation was performed if required. Each parameter had a series of analyses of variance (ANOVA) performed. First, ANOVA was performed to test for the effect of sampling date. This ANOVA considered samples collected from the bulk soil before planting and after tuber harvest and considered the H location from planting to harvest. Given that the furrow location includes sampling of subsoil, the hill location, rather than the average across spatial locations (H, $H_p$, and F), was considered to be more comparable to the bulk soil and would provide a more meaningful comparison over time. Second, ANOVA was performed to test for the effect of spatial location across sampling dates. This ANOVA considered only the dates during the crop growth period on which different spatial locations were sampled. Third, ANOVA was performed separately for each sampling date to test for differences among spatial locations within individual sampling dates. In each case, mean values (averaged across samples from replicate plots) were compared using the Student-Newman-Keuls test. Mean values presented in the figures were calculated from untransformed data. The standard errors of the untransformed means are shown in the figures for each treatment mean. The results were considered statistically significant at a $P$ value of <0.05.

A *c*anonical *c*orrespondence *a*nalysis (CCA) using direct gradient analysis was done by using CANOCO version 4.5 (37). Measures of the denitrifier population ($cnorB_P$, $cnorB_B$, *nirK*, *nosZ*, DEA, denitrification, and $N_2O$ flux) were treated as species variables and compared with measures of environmental variables (soil $NO_3^-$, $NH_4^+$, EOC, temperature, pH, moisture content, WFPS, and respiration). Because of the large differences in their magnitudes, the species variables were transformed using a natural log. The statistical significance of the relationship between species and environmental variables was assessed by a Monte Carlo permutation test of both the first ordination axis and the combination of both the

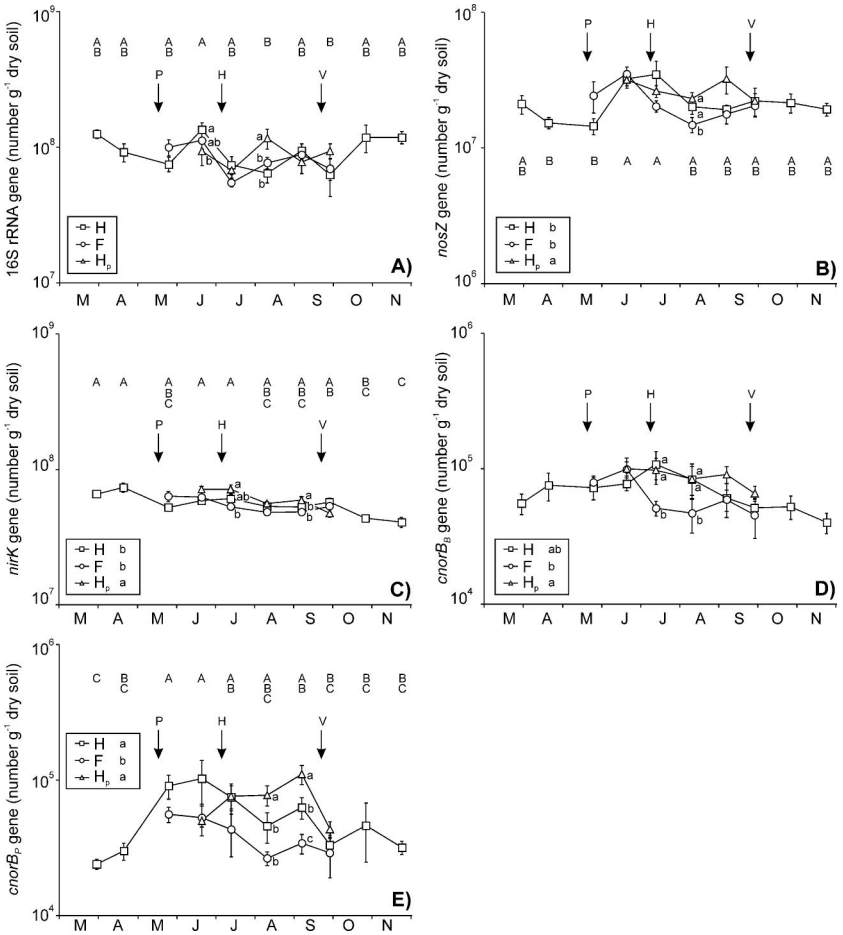


FIG. 2. Changes in gene numbers over time and at different row locations in an agricultural field cropped to potatoes for 16S rRNA genes (A), *nosZ* (B), *nirK* (C), *cnorB*$_B$ (D), and *cnorB*$_P$ (E). Arrows indicate dates of planting (P), hilling (H), and vine desiccation (V); the months during which samples were collected are indicated below each graph. Spatial locations include H, H$_p$, and F. Significant differences among mean values based on the Student-Newman-Keuls test ($P < 0.05$) are represented in the following three ways: (i) differences among sampling dates for the H location only are shown by capital letters across the panels, but only when the main effect of the sampling date is significant; (ii) differences among spatial locations averaged across sampling dates are shown by lowercase letters in the panel keys, but only when the main effect of spatial location is significant; and (iii) differences among spatial locations for individual sampling dates are represented by lowercase letters adjacent to the data points, but only for sampling dates for which significant differences exist among spatial locations. Standard errors of the untransformed means were plotted for each treatment mean.

first and second axes. The ordination plot was generated by using CanoDraw for Windows (37).

## RESULTS

**Quantification of bacterial community abundance.** The mean total bacterial counts, estimated by using 16S rRNA gene real-time quantitative PCR, ranged from $6 \times 10^7$ to $1 \times 10^8$ genes g$^{-1}$ dry soil over all samples (Fig. 2A). Although the 16S rRNA gene numbers showed significant differences over time, the majority of time points did not show significant differences in gene numbers measured among samples from the H location. The effect of spatial location over multiple sampling dates was not significant. Within individual sampling dates, significant differences in the densities of bacterial communities among spatial locations were measured at two time points. The gene numbers for the H location ($1.3 \times 10^8$ genes g$^{-1}$ dry soil) were significantly higher than for the H$_p$ location ($9.4 \times 10^7$ genes g$^{-1}$ dry soil) in June. In August, the gene numbers for the H$_p$ location ($1.2 \times 10^8$ genes g$^{-1}$ dry soil) were significantly higher than for both the H and F locations ($6.4 \times 10^7$ genes g$^{-1}$ and $7.7 \times 10^7$ genes g$^{-1}$ dry soil, respectively).

The mean values for *nosZ* gene numbers ranged from $1.4 \times 10^7$ to $3.5 \times 10^7$ g$^{-1}$ dry soil among all samples, representing a 2.3-fold change in abundance (Fig. 2B). The *nosZ* gene numbers in samples from the H location showed significant differences over time, with lower values for the April and May sampling dates ($1.4 \times 10^7$ to $1.5 \times 10^7$ genes g$^{-1}$ dry soil) than for the June and July sampling dates ($3.2 \times 10^7$ to $3.5 \times 10^7$ genes g$^{-1}$ dry soil). The effect of spatial location on *nosZ* gene numbers was significant, with higher numbers of *nosZ* gene-bearing denitrifiers in the H$_p$ location than in the H and F locations. Among individual sampling dates, there was a significant effect of location only for August, when gene numbers for the H and H$_p$ locations ($2.0 \times 10^7$ to $2.3 \times 10^7$ genes g$^{-1}$ dry soil) were significantly higher than for the F location ($1.5 \times 10^7$ genes g$^{-1}$ dry soil).

The mean values for *nirK* gene numbers ranged from $4.1 \times 10^7$ to $7.3 \times 10^7$ $g^{-1}$ dry soil in all samples (Fig. 2C). Although there was very little change between the maximum and minimum values for denitrifiers bearing the *nirK* gene (1.8-fold), significant differences were obtained over time and among spatial locations. The *nirK* gene numbers in the H location were significantly lower at the end of the monitoring period ($4.1 \times 10^7$ genes $g^{-1}$ dry soil) than at the start of the season ($6.6 \times 10^7$ genes $g^{-1}$ dry soil). The effect of spatial location on *nirK* gene abundance was significant, with higher values obtained for the $H_p$ location than for the H and F locations, similar to the results for *nosZ* gene abundance. Among individual sampling dates, the gene numbers were higher for the $H_p$ location than for the F location in July and on 6 September.

The *cnorB*$_B$ mean gene numbers ranged from $4.0 \times 10^4$ to $1.1 \times 10^5$ $g^{-1}$ dry soil over all samples, representing a 2.7-fold change in abundance (Fig. 2D). There were no significant differences in the numbers of *cnorB*$_B$ gene-bearing denitrifiers over time in the H location. The effect of spatial location for *cnorB*$_B$ gene-bearing denitrifiers was significant, with higher *cnorB*$_B$ gene numbers in the $H_p$ location than in the F location. among individual sampling dates, the gene numbers for the $H_p$ and H locations were significantly higher than for the F location in July and August.

The *cnorB*$_P$ mean gene numbers ranged between $2.4 \times 10^4$ and $1.1 \times 10^5$ $g^{-1}$ dry soil among all samples, showing the largest proportional changes (4.6-fold) in gene numbers of all the communities measured (Fig. 2E). The *cnorB*$_P$ gene numbers changed significantly over time in the H location, with higher values obtained in the May and June samples (average, $9.6 \times 10^4$ genes $g^{-1}$ dry soil) than in samples from March and April and late September and November. The effect of location was significant for *cnorB*$_P$, with gene numbers being significantly lower in the F location than in the H and $H_p$ locations. In August, the gene numbers in the $H_p$ location were significantly higher than in the H and F locations, whereas on 6 September, the gene numbers were highest in the $H_p$ location, lowest in the F location, and intermediate in the H location.

The effect of spatial location on denitrifier community abundance can be examined by comparing values obtained for the H and F locations. When values averaged across sampling dates were considered, only *cnorB*$_P$ showed a significant difference in community abundance between H and F. In contrast, when individual sampling dates were considered, values for the H location were significantly higher than for the F location for *nosZ* (August), *cnorB*$_B$ (July and August), and *cnorB*$_P$ (6 September).

The effect of the potato plant on denitrifier communities may be examined by comparing values obtained in the H and $H_p$ locations. When averaged across sampling dates, the mean values of gene numbers were significantly higher for the $H_p$ location than for the H location for the *nosZ* and *nirK* communities, but not for the two *cnorB* communities. In contrast, the gene numbers on individual sampling dates were significantly higher for the $H_p$ location than for the H location for *nirK* (6 September) and *cnorB*$_P$ (August and 6 September).

**Soil physicochemical properties.** The soil temperature ranged from ~0°C at the start and finish of the monitoring period to 22°C on the 13 July sampling date (Table 1). WFPS was high prior to planting, ranging from 68 to 73% (Table 1). After planting, WFPS declined to values of about 60% or less in the F location and 55% or less in the H location. WFPS was consistently higher in the F location than in the H location during the crop growth period, which primarily reflects lower values of soil bulk density in the hill than in the furrow (data not shown).

Prior to planting, the soil $NO_3^-$ concentrations were low, less than 2 mg N $kg^{-1}$ soil (Fig. 3A). Soil $NO_3^-$ in the H location increased due to fertilizer application at planting (15 May), with soil $NO_3^-$ concentrations remaining high (~60 mg N $kg^{-1}$ soil) in the H location throughout June and then decreasing in July in response to crop N uptake. The soil $NO_3^-$ concentrations in the F location increased slightly over time in response to net soil N mineralization, reaching a maximum value of 10 mg N $kg^{-1}$ soil on 13 July, and then remained low for the remainder of the monitoring period. The effect of spatial location was significant, with $H > H_p > F$.

Soil $NH_4^+$ concentrations were also low, less than 2 mg N $kg^{-1}$ soil, prior to planting and remained low for the duration of the monitoring period in the F location (data not presented). In the H location, the soil $NH_4^+$ concentration reached a maximum of 55 mg N $kg^{-1}$ soil on 25 May in response to fertilizer application at planting, and then decreased to 6 mg N $kg^{-1}$ soil on 20 June in response to nitrification (data not shown).

The soil EOC concentrations ranged from 15 to 124 mg C $kg^{-1}$ soil over all samples (Fig. 3B). Significant differences occurred over time in the H location. EOC increased following soil disturbance (planting) in May, and maximum values of EOC were measured on 6 September when there was visual evidence of crop senescence. The effect of location was not significant. Among individual sampling dates, EOC was significantly higher for the $H_p$ location than for the H location on 6 September.

**DEA, gas fluxes, and denitrification.** The DEA values ranged from 0.08 to 0.44 μg N $g^{-1}$ soil $h^{-1}$ over all samples (Fig. 3C). Significant differences in DEA occurred among sampling dates in the H location. The maximum value in the H location (0.39 μg N $g^{-1}$ soil $h^{-1}$) was measured in August, and this value was significantly higher than the DEA values measured in March, September, October, and November. The effect of spatial location was not significant. Among individual sampling dates, DEA was higher in the H and F locations than in the $H_p$ location on 20 June, whereas DEA was higher for the $H_p$ location than for the F location on 28 September. For the F location, the highest numerical values of DEA were measured on the first two sampling dates after planting and fertilization, prior to the removal of surface soil by the hilling process, and corresponded with the time when maximum denitrification occurred (Fig. 3F).

Soil respiration varied significantly over time in the H location (Fig. 3D). The highest value of respiration, measured on 20 June (14.1 kg C $ha^{-1}$ $day^{-1}$), was significantly higher than the respiration measured in March, April, September, and November. There was no significant effect of location, and there was no effect of location among individual sampling dates.

$N_2O$ emissions for the H location varied significantly over time, with higher values measured for the May and June sampling dates

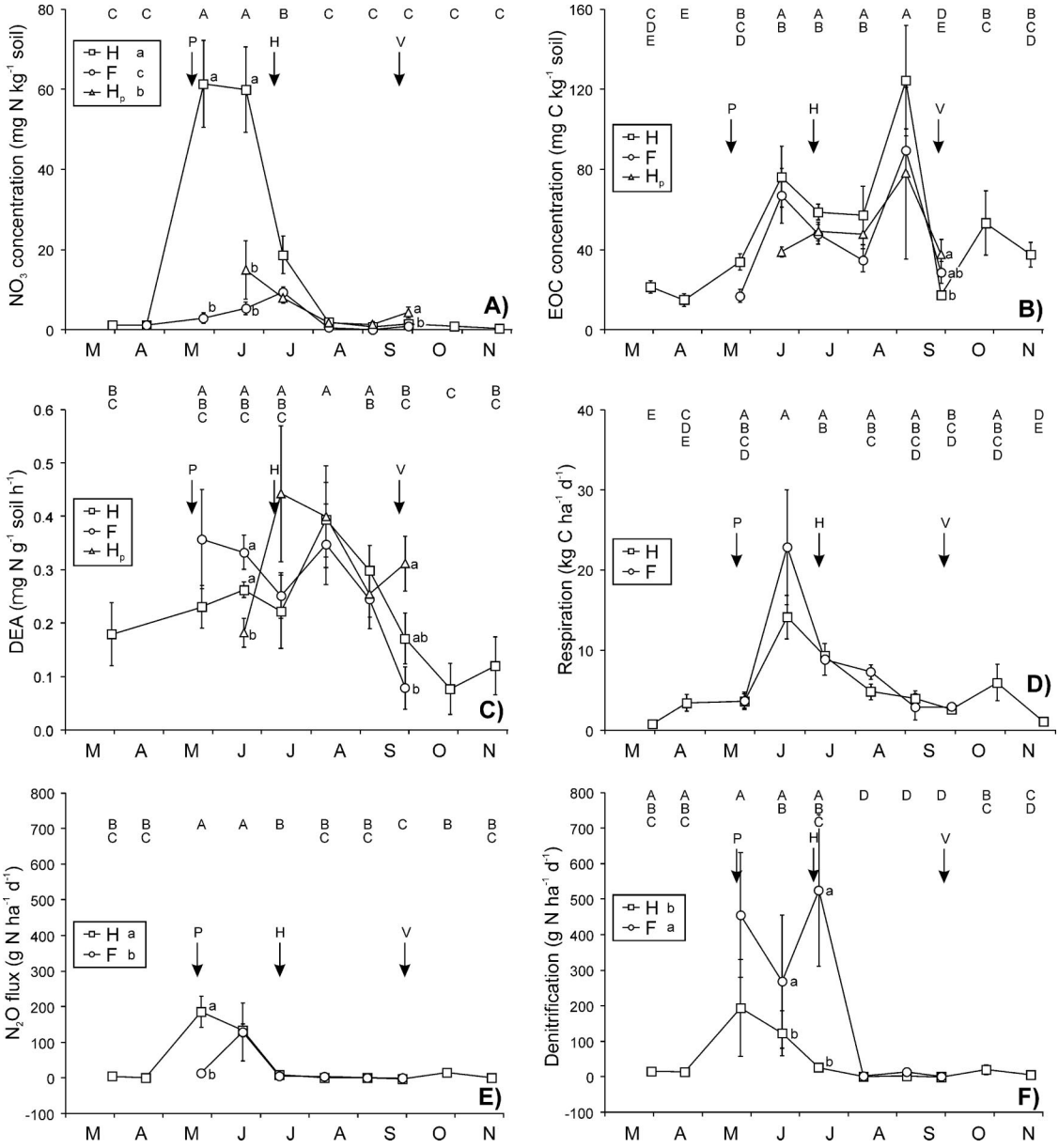


FIG. 3. Changes over time and at different spatial locations in an agricultural field cropped to potatoes for nitrate ($NO_3^-$) (A); EOC (B); DEA (C); soil respiration (i.e., $CO_2$ flux) (D); $N_2O$ flux (E); and denitrification rate (F). Arrows indicate dates of planting (P), hilling (H), and vine desiccation (V); the months during which samples were collected are indicated below each graph. Spatial locations include H, $H_p$, and F. Significant differences among mean values based on the Student-Newman-Keuls test ($P < 0.05$) are represented in the following three ways: (i) differences among sampling dates for the H location only are shown by capital letters across the panels, but only when the main effect of the sampling date is significant; (ii) differences among spatial locations averaged across sampling dates are shown by lowercase letters in the panel keys, but only when the main effect of spatial location is significant; and (iii) differences among spatial locations for individual sampling dates are represented by lowercase letters adjacent to the data points but only for sampling dates for which significant differences exist among spatial locations. Standard errors of the untransformed means were plotted for each treatment mean.

than for all other sampling dates (Fig. 3E). The effect of location was significant; however, this reflected higher $N_2O$ emissions in the H location (186 g N $ha^{-1}$ $day^{-1}$) than in the F location (13 g N $ha^{-1}$ $day^{-1}$) on the 25 May sampling date.

Denitrification rates for the H location varied significantly over time (Fig. 3F). The maximum denitrification rate, measured on 25 May, was significantly higher than the denitrification rates measured from August to November. The effect of spatial location was significant, with a higher denitrification rate measured in the F location than in the H location. Among individual sampling dates, higher denitrification rates were measured in the F location than in the H location on the June and July sampling dates. The cumulative denitrification values for the 0-to-13-cm soil depth over the crop growth period were estimated to be 9.5 ± 4.7 (mean ± 1 standard error) and 31.3 ± 7.1 kg N $ha^{-1}$ for the H and F locations, respectively. In

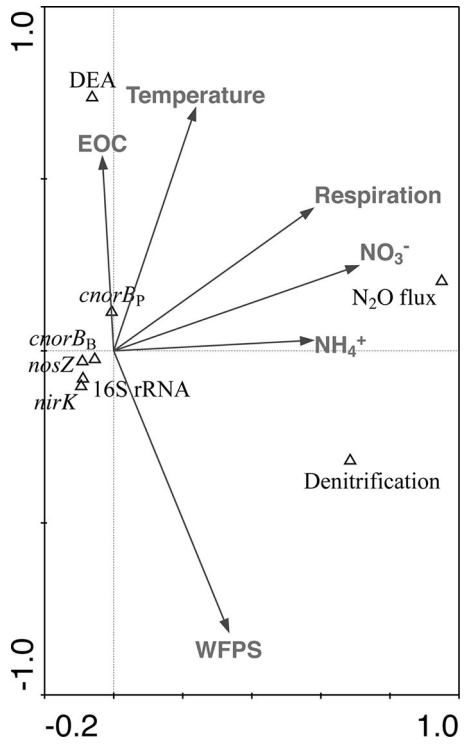


FIG. 4. CCA of measures of the total bacterial and denitrifier gene abundances (plotted as triangles) in relation to soil environment variables (plotted as vectors). Canonical axis 1 (*x* axis) explains 52.0% of the total bacterial and denitrification gene abundance-environment variance in the data and is significant at a *P* value of 0.002. Canonical axis 2 (*y* axis) explains an additional 34.1% of the total bacterial and denitrification gene abundance-environment variance in the data and is significant at a *P* value of 0.002.

comparison, the cumulative $N_2O$ emissions over the crop growth period were estimated to be 9.6 ± 1.6 and 4.3 ± 2.3 kg N $ha^{-1}$ for the H and F locations, respectively.

**CCA.** CCA resulted in the first two canonical axes explaining 19.8% of the variance in the denitrifier gene abundance data and 86.1% of the total denitrifier gene abundance-environment relationship. The first canonical axis explained 52.0% of the total variation, was statistically significant at a *P* value of 0.002, and was dominated by the environmental variables $NO_3^-$ (0.71), $NH_4^+$ (0.58), and respiration (0.58). The second canonical axis explained an additional 34.1% of the total variation, was statistically significant at a *P* value of 0.002, and was dominated by WFPS (0.81), temperature (0.70), and EOC (0.56). $N_2O$ flux was primarily influenced by the first canonical axis, reflecting the effects of $NO_3^-$, $NH_4^+$, and respiration (Fig. 4). Denitrification was influenced by both the first and second canonical axes, reflecting the role of moisture and WFPS, in addition to $NO_3^-$ and respiration. The amount of denitrifier enzyme (measured as DEA) was primarily influenced by the second canonical axis, reflecting the role of EOC and temperature. Gene numbers (*cnorB*$_B$, *cnorB*$_P$, *nosZ*, *nirK*, and 16S rRNA genes) were not strongly influenced by any of the environmental variables on either axis. The number of *cnorB*$_P$ genes was most-strongly influenced by EOC and soil temperature; however, this was not a strong relationship.

## DISCUSSION

The primary objective of this work was to examine spatial and temporal changes in the abundance of soil denitrifier communities. Denitrifier community abundances were measured by using a selection of quantitative PCR primer sets targeting different genes in the denitrification pathway. The *cnorB* primer sets (9) targeted specific components of the denitrifier population known to be present in our field site soil (8). The *nirK* and *nosZ* primer sets (13, 14) targeted larger components of the soil denitrifier population. This study demonstrated the ability to quantify temporal and spatial variation in the abundance of denitrifier communities in the field within an agricultural production system. Although data for *nirS* gene community abundance (18) would have been a valuable addition, we were unable to generate satisfactory results for this gene. It should be noted, however, that even the existing primer sets for components of the denitrifier community (e.g., *nirK*, *nosZ*, and *nirS*) likely characterize only a limited proportion of the total soil bacterial denitrifier community (16). The future development of primer sets to characterize currently unknown components of the soil denitrifier community will be critical for our understanding of the dynamics and function of soil denitrifier communities.

Limited variation in the total bacterial population density was measured over the monitoring period in the bulk soil. Similar results have been obtained for total bacteria in agricultural soil by He et al. (12), who found no significant changes in the density of total bacteria in soil treated with different fertilizers in summer and winter. Okano et al. (24) measured the density of total bacteria over 28 days in response to ammonium nitrate addition and also found no significant changes. The addition to agricultural soil of up to 500 mg C $kg^{-1}$ soil as glucose in laboratory microcosms did not cause a significant change in the density of total bacteria as measured by 16S rRNA quantitative PCR (9).

Temporal variation was detected in the abundance of some denitrifier communities. The magnitude of the variation (1.7- to 4.6-fold) was generally limited and varied with the targeted denitrifier community. *cnorB*$_B$ showed no significant change in community density over time. Although this is the first time we have analyzed the community density of this population in the field, the limited changes observed are not unexpected given the lack of growth response of this community in soil to additions of up to 500 mg $kg^{-1}$ glucose (9). *nosZ* showed similar limited changes in abundance over time for the total bacterial community, with the majority of values not significantly different. The *cnorB*$_P$ relative abundance changed the most over time out of all communities and was similarly more responsive to the addition of carbon in laboratory microcosms (9). *cnorB*$_P$ abundance in this study was shown by CCA analysis to be weakly influenced by EOC and soil temperature. *nirK*-bearing denitrifiers showed a slow but steady decline in abundance during the study period. The decline in the abundance of *nirK* gene numbers over time was a unique response among the denitrifier communities measured in this study. Several possibilities may explain the decline in abundance of the *nirK* community, including competition (with other denitrifiers, i.e., *nirS*-bearing denitrifiers), predation, or seasonal succession.

The presence of the potato plant and root system showed

some effects on denitrifier community abundance, particularly for *nirK* and *nosZ* gene-bearing denitrifiers. Potato plant and root system presence may provide root exudates which can be used as carbon sources for microbial growth. When Henry et al. (16) incubated agricultural soil with the daily addition of artificial root exudates for 4 weeks, only one root exudate composition out of four tested elicited a growth response in *nirK* and *nirS* communities, and *nosZ* community abundance was not stimulated by any of the additions. The composition of root exudates from potato is unknown and likely to change over time during the growing season, so it is possible that this is one aspect responsible for the higher denitrifier community abundances observed in close proximity to the plant in this study. The methodology used in this experiment was not capable of specifically determining the influence of the plant's presence on denitrification and $N_2O$ emissions. The significance of more-abundant denitrifier populations close to the plant in relation to denitrification rates and $N_2O$ emissions in this system therefore cannot be determined from the data set presented.

Limited effects of location on community densities were observed when the H and F locations were compared. The average *cnorB*$_P$ community density was significantly higher in the H than in the F location, and significant differences between mean community densities for the other denitrification genes were not observed. It is interesting to note that the hilling process, which moves most of the organic-rich surface soil from the furrow to the hill, had a limited effect on denitrifier community abundance in this study. Soil depth has been shown to have an effect on the abundance of denitrifier communities (22); however, the difference in depth between hill and furrow (both still in the A horizon) may not be enough to generate significant differences in denitrifier community abundance in this agricultural soil system.

It is currently unclear what other factors may influence denitrifier community abundances in agricultural ecosystems. $NO_3^-$ was not found to correlate with denitrifier abundance in forest soils (20), whereas Wallenstein (38) found that N fertilization decreased denitrifier abundance, based on quantitative PCR functional gene measurements. Organic C was found to correlate with denitrifier abundance in the work reported in reference 17. It is possible that for such a diverse range of denitrifying microorganisms, it will be difficult to ascribe the dominant factors controlling their abundance within a particular environment.

The second major objective of this work was to determine the relationship between denitrifier community abundances and denitrification activity or other environmental variables. Elevated denitrification and $N_2O$ emissions were measured in the field when nitrate, soil respiration, and WFPS were high. During the early and late parts of the growing season, when nitrate, WFPS, and respiration were low, little denitrification or $N_2O$ emission occurred. Different patterns of denitrification and $N_2O$ emissions occurred between the H and F spatial locations. In the H location, the cumulative emissions of $N_2O$ were similar to the measured cumulative denitrification rate, indicating that most gaseous emissions from denitrification occurred as $N_2O$. This is consistent with the results of previous work that led to the conclusion that $N_2O$ is the dominant end product of denitrification where there is an abundant supply of $NO_3^-$ as the terminal electron acceptor for the denitrification process (10, 11). Nitrification of $NH_4^+$ fertilizer may also have contributed to $N_2O$ emissions at this time (18). The results of the CCA showed combined roles of soil respiration (i.e., $CO_2$ flux, representing plant and microbial activities which are acting as a sink for oxygen supply) and $NO_3^-$ and $NH_4^+$ availability in influencing $N_2O$ emissions from denitrification. The relationship between $NH_4^+$ and $N_2O$ emissions may indicate a contribution of nitrification to $N_2O$ emissions (41).

The denitrification rates were higher in the F than in the H location, likely as a result of the higher WFPS resulting from higher soil bulk density, in addition to the accumulation of water due to the elevation differences between the H and F spatial locations. This influence of WFPS on denitrification is demonstrated in the CCA biplot (Fig. 4). In contrast to the H location, $N_2O$ emissions from the F location were low relative to the denitrification rate. The low soil $NO_3^-$ supply in the F location did not appear to limit the denitrification process, but rather, the combination of a low $NO_3^-$/N-oxide supply and restricted diffusion of $N_2O$ increased the reduction of $N_2O$ to $N_2$, resulting in $N_2$ being the dominant end product (11).

We hypothesized that denitrifier community gene numbers would change over time and spatial location, that denitrification/$N_2O$ emissions would be related to changes in denitrifier numbers, and that both are also influenced by environmental parameters. We found that the denitrifier community abundances measured did change over time and spatial location, but denitrifier abundance was not related to denitrification, $N_2O$ emissions, or, for the most part, environmental parameters. We conclude that denitrifier community abundance of the genes tested was not a controlling factor for denitrification activity in this system. Ma et al. (21) also concluded that the density of denitrifiers, measured by using *nosZ* quantitative PCR, was not clearly linked to $N_2O$ emissions. Denitrifier abundance showed seasonal changes, with the highest values generally obtained in early/midsummer, with the exception of the *nirK*-bearing community which declined over the duration of the monitoring period. In this study, $N_2O$ emissions were primarily correlated with soil respiration and $NO_3$ and $NH_4^+$ availability, with some evidence of a role of nitrification, as indicated by the relationship with $NH_4^+$. In addition to the factors influencing $N_2O$ emissions, the denitrification rates reflected the influence of soil aeration, as indicated by WFPS. The most striking observation is that while denitrification and $N_2O$ emissions were most strongly influenced by respiration, inorganic N ($NO_3^-$ and $NH_4^+$) availability, and WFPS, the number of denitrifier gene copies appeared to be only weakly influenced by soil environmental conditions. Consequently, it appears that the rate of denitrification and levels of $N_2O$ emissions are primarily functions of environmental conditions and may be decoupled from the density of the denitrifier communities targeted in this study.

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