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Short-Term Effects of Mineral and Organic Fertilizer on Denitrifiers, Nitrous Oxide Emissions and Denitrification in Long-Term Amended Vineyard Soils

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Short-term effects (i.e., 21 d) of mineral or organic fertilizer application on long-term (i.e., 8 yr of applications) amended soil on denitrifier community abundance, denitrification gene mRNA transcript numbers, denitrification rate, and emissions of N_2O were explored. Soil was collected from a vineyard in Italy receiving annual applications of either mineral fertilizer (conventional management system, CS) or municipal compost (organic management system, OS). Each soil was incubated using three treatments: no amendment, NH_4NO_3, or municipal compost. Microcosms set up with soil treated with compost showed higher *nirS*, *nirK*, and *nosZ* abundance in comparison to conventional fertilization. Short-term compost addition increased *nirK* gene abundance over time in OS and CS soils, whereas *nirS* and *nosZ* gene abundance increased after compost addition only in OS soil. In OS soil, *nosZ* gene mRNA transcript numbers were higher at all time-points for all treatments compared with CS soil. Furthermore, *nosZ* gene mRNA transcript number increased over time after compost addition for both soils, N_2O emissions were higher in both soils after NH_4NO_3 addition compared with no amendment and compost addition. Denitrification was higher in OS than CS soil following NH_4NO_3 treatment. Denitrification rates were much higher than N_2O rates in all cases suggesting most emissions occurred as N_2. Our study demonstrated that long-term urban-waste compost application clearly changed soil denitrifier communities and the response of denitrification and N_2O emissions to different short-term soil amendments.

Abbreviations: CS, conventional management system; OS, organic management system; WFPS, water- filled pore space.

Recent changes in household waste collection and waste management systems in the European Union countries has substantially increased the amount of compost generated from municipal solid wastes (Adhikari et al., 2010). Land application of compost to agricultural fields is desirable because it is a source of stabilized and complex C and N (Hargreaves et al., 2008). Moreover, repeated compost applications have been shown to preserve soil quality and to enhance soil organic matter pools, which play a crucial role in maintaining soil biological activity, soil aggregate stability, and mineral nutrition of crops (Garcia-Gil et al., 2000; Crecchio et al., 2001). Viticulture has been recently shown to cover an approximate area of 7.4 million hectares of plants worldwide (FAOSTAT, http://faostat.fao.org/site/569/default.aspx, accessed 2008), therefore the application of compost into productive vineyards is an interesting approach not only to reduce the amount of waste added to landfill sites but also to maintain soil quality and decrease the quantity of applied artificial fertilizers. However, the environmental implications of this practice (i.e., N_2O production) are not well understood.

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Availability of C and N are two of the major controlling factors in denitrification, a microbial process that involves the stepwise reduction of NO_3^-, via NO_2^- and NO to N_2O and N_2 gas through respiration under oxygen-limited or anoxic conditions (Zumft, 1997). Incomplete soil denitrification can lead to the release of N_2O to the environment, a potent greenhouses gas that contributes to global warming and stratospheric ozone layer destruction (Hofstra and Bouwman, 2005). Moreover, the loss of NO_3^- from the root zone by denitrification represents the loss of an important plant nutrient, which is frequently a yield-limiting factor in many agricultural soils (Tiedje, 1988).

Soil compost application has been shown to reduce N_2O emissions compared with mineral fertilizers because most N is in organic form and converts less readily to NO_3^- form than for mineral fertilizers (Alluvione et al., 2010). In contrast, compost application may enhance total denitrification in the long-term by providing organic matter that can be utilized by microorganisms, thus favoring both the increase of soil NO_3^- concentration through mineralization and nitrification and the creation of anoxic environments through increased oxygen consumption (De Wever et al., 2002).

Addition of organic amendments such as plant residues, manure, or compost to soil can influence soil microbial communities, which in turn influence long-term plant nutrient supply and sustainability of agricultural systems (Kennedy and Smith, 1995). An increase in size or diversity of soil heterotrophic bacterial communities was previously shown in agricultural soils amended with plant residues (Schutter and Dick, 2001), manure (Peacock et al., 2001), or compost (Perez-Piqueres et al., 2006; Calbrix et al., 2007; Carrera et al., 2007). Effects of municipal solid waste compost on size and composition of the soil bacterial community may be rate dependent (Saison et al., 2006) and related to the composition of the organic amendment applied (Calbrix et al., 2007).

Soil bacterial denitrifier community size generally increases after addition of complex C sources such as manure (Miller et al., 2009) or plant residues (Miller et al., 2008; Henderson et al., 2010). There are, however, currently only two studies that have evaluated the effect of compost addition on denitrifiers in soil (Bastida et al., 2009; Dambreville et al., 2006). Bastida et al. (2009) reported that the addition of composted sewage sludge in a semiarid Mediterranean soil increased the abundance of *nirS*-bearing denitrifying bacteria comparing with sewage sludge and nontreated soil. Dambreville and colleagues (2006) showed that long term addition of composted pig manure changed the structure of *nosZ*- and *narG*-bearing denitrifiers and increased denitrification potential in contrast to conventional fertilization. The effect of soil long-term compost additions to soil on denitrifier abundance, denitrification mRNA transcript number, and denitrification rates remains unclear.

The objective of this study was to determine the response of two soils from contrasting long-term management systems (annual applications of mineral fertilizer vs. municipal compost) in a vineyard in Italy to different N amendment treatments (no addition, mineral fertilizer, municipal compost) with respect to N_2O emissions, total denitrification, and denitrifier community abundance (*nirS, nirK*, and *nosZ*) and denitrification gene mRNA transcript number (*nosZ*) using soil microcosms. In addition, possible relationships between environmental and denitrification parameters, and denitrifier abundance and mRNA transcript numbers, were explored.

We hypothesized that long-term addition of compost led to a higher abundance of soil denitrifiers compared with the conventionally fertilized soil. Changes in fertilization practices in the two long-term management systems might affect denitrifiers and denitrification. Therefore, we assumed that mineral fertilizer amendments would result in a more important increase in N_2O emissions, total denitrification rates, and denitrification gene expression in soil that has experienced long term compost application compared to soil under long-term mineral fertilization due to the increased availability of C.

MATERIALS AND METHODS

Soil Characteristics and Collection

Soils were collected from a vineyard located near Marciano della Chiana, Arezzo, Italy (43°19′20″ N lat, 11°47′ 43″ E long) with mean annual air temperature of 14.1°C and mean annual precipitation of 40.5 mm. The experimental site was established in 2001 using field scale plots with two management systems including: (i) a CS using mineral fertilization corresponding to 50 kg N ha^{-1} yr^{-1}, 30 kg P_2O_5 ha^{-1} yr^{-1}, and 70 kg K_2O ha^{-1} yr^{-1} and (ii) an OS using 15 Mg ha^{-1} yr^{-1} dry weight compost generated from urban organic waste as previously reported (Tatti et al., 2012). The CS and OS soils are a silty clay loam Calcaric Cambisols with 169 g kg^{-1} sand, 499 g kg^{-1} silt and 332 g kg^{-1} clay; the organic C concentration was 12.7 and 21.8 g kg^{-1}, and total N concentration was 1.1 and 1.7 g kg^{-1}, respectively (Tatti et al., 2012). The soil pH (1:2.5 soil/water ratio) was 8.1 (Thomas, 1996). Soil was collected at 0- to 15-cm depth from multiple locations within each management site along a 100-m transect to obtain spatially representative composite sample. The soil was then air-dried and shipped to Canada for microcosm experiments. Soils were passed through a 2-mm sieve and water added to achieve a gravimetric water content of 0.3 g g^{-1} soil (approximately 50% water filled pore space, WFPS) then stored in the dark at room temperature for 10 d before use.

Experimental Design

The experiment used two soil management systems (CS or OS), three amendment treatments, and five incubation lengths (0, 2, 7, 14, and 21 d) replicated four times. The experimental unit was a 1-L glass canning jar of approximately 10 cm diameter (Bernardin, Toronto, ON). Amendment treatments included (i) a control with no amendment added (OS_0, CS_0); (ii) amendment with 87 mg N kg^{-1} of dry soil as ammonium nitrate as a solution (OS_F, CS_F); and (iii) amendment with 40 g compost kg^{-1} dry soil (OS_C, CS_C). Each treatment combination had a duplicate set of incubation jars to allow for either addition of 10% (v/v) acetylene (C_2H_2, to inhibit the reduction of N_2O to N_2) to the headspace to quantify total denitrification

or no C_2H_2 addition to quantify N_2O emissions. The rate of compost addition was chosen to supply an equivalent quantity of mineral N as the mineral N treatments based on the compost physiochemical characteristics (Tatti et al., 2012).

For each incubation jar, the required quantity of N amendment and water was added to approximately 290 g air-dried soil to achieve a water content equivalent to 70% WFPS, gently mixed to minimize loss of soil structure, and packed into the jar to a soil bulk density of 1 mg m^{-3}. This resulted in approximately a 5-cm headspace. The jars were sealed with Parafilm (Pechiney Plastic Packaging, Chicago, IL) to reduce water evaporation and pierced with four holes to allow gas exchange. The jars were placed immediately in an incubation chamber at 25°C for 21 d. The weight of the jars was checked periodically and water added as required to maintain constant the chosen water content over time. Jars were destructively sampled at 0, 2, 7, 14, and 21 d.

For jars to be used for quantification of N_2O emissions (with no C_2H_2 addition), two soil cores (approximately 10 g total) were removed from the jar at the end of the incubation period, placed in two 15-mL tubes, flash-frozen using liquid nitrogen, and kept at −80°C for nucleic acid extraction. Jars were then sealed with a screw lid fitted with a rubber septum, and 10 mL of headspace gas were sampled at 0 and 3 h of incubation at 25°C. Gas samples were injected into previously evacuated Exetainer vials (Labco, UK) containing the desiccant magnesium perchlorate. After gas sampling was completed, the jars were opened and 25 g of soil was sampled to perform analytical measurements including NO_3^-, NO_2^-, and NH_4^+ concentrations.

For jars to be used for quantification of total denitrification, jars were sealed at the end of the incubation period with screw lids as described above, C_2H_2 was injected immediately, and 10 mL of headspace gas was sampled at 1 and 4 h of incubation at 25°C. The delay of gas sampling for the jars was to allow time for C_2H_2 diffusion into the soil. Gas samples were collected at time 0 and 3 h for N_2O emissions and time 1 and 4 h for total denitrification according to preliminary experiments that showed gas concentration increased linearly between 0 and 6 h (data not shown).

Gaseous Fluxes and Soil Analytes Measurements

Headspace gas was analyzed for N_2O and CO_2 concentrations using a Varian Star 3800 Gas Chromatograph (Varian, Walnut Creek, CA) fitted with an electron capture detector to measure N_2O, a thermal conductivity detector to measure CO_2, and a Combi-PAL Autosampler (CTC Analytics, Zwingen, Switzerland) as previously described (Henderson et al., 2010). Concentrations of NO_3^-, NO_2^-, and NH_4^+ were analyzed colorimetrically following extraction with 0.5 M K_2SO_4 and then quantified as previously described (Henderson et al., 2010).

Nucleic Acid Extraction

DNA and RNA were co-extracted from 0.7 g of soil, divided using the method of Griffiths et al. (2000) as previously described (Henderson et al., 2010). Before extraction, 50 mg of sterilized skim milk was added to each tube to enhance extraction efficiency (Takada-Hoshino and Matsumoto, 2004). The RNA and DNA were quantified using the fluorescent dyes Ribogreen and Picogreen (Invitrogen, Burlington, ON, Canada), respectively. Average extracted DNA concentration was 1.64 (±1.03) and 1.05 (±0.86) μg g^{-1} dry soil for OS and CS, respectively. Average extracted RNA concentration was 0.43 (±0.31) and 0.14 (±0.14) μg g^{-1} dry soil for OS and CS, respectively. Genomic DNA was also extracted from 1 g of compost as described above.

Quantitative PCR

Gene copy number for *nirS* (Kandeler et al., 2006), *nirK*, and *nosZ* (Henry et al., 2006) was quantified via quantitative (qPCR) using an Applied Biosystems (Streetsville, ON, Canada) ABI PRISM 7000 thermal cycler and SYBR Green PCR Master Mix (Invitrogen) with PCR conditions as previously described (Dandie et al., 2008; Henderson et al., 2010; Dandie et al., 2011).

Primers targeting *nirS* or *nirK* bearing communities from soil previously failed to amplify cDNA in a qRT-PCR reaction even after extensive optimization of qRT-PCR conditions (Henderson et al., 2010). Primers targeting *nosZ* gene were suitable to quantify transcript abundance, as previously described (Henderson et al., 2010). Standard curves were used for absolute quantification of *nirS*, *nirK*, and *nosZ* gene number and transcripts as previously described (Dandie et al., 2008; Henderson et al., 2010; Dandie et al., 2011). Standard curve descriptors and detection level: *nirS* gene copy number: slope: −3.32 to −3.57, R_2 = 0.995–0.997, E = 97–101% y-intercept = 41.1–43.7. *nirK* gene copy number: slope: −3.34 to −3.59, R_2 = 0.997–0.999, E = 89–97% y-intercept = 33.7–34.4. *nosZ* gene copy number: slope: −3.42 to −3.77, R_2 = 0.999, E = 93–99% y-intercept = 37.8–38.8. *nosZ* transcripts number: slope: −3.31 to −3.44, R_2 = 0.998–0.999, E = 83–95% y-intercept = 34.2–36.8. Successful amplification of the desired-size fragment was assessed by agarose gel visualization, and specificity of the amplification was checked by melt curve.

Soil DNA and RNA extracts were tested for the presence of co-extracted inhibitory substances as previously described (Henderson et al., 2010).

Data Analysis

All statistical analyses were conducted using Systat Software 12 (Systat Software Inc., Chicago, IL). Data was tested for normality using the Shapiro–Wilk test and all non-normal data were log transformed. A mixed-model three-way analysis of variance was performed based on a randomized complete block design with management system, amendment, and incubation time as fixed effects. Treatment means in interactions were compared using Tukey adjusted least significant (LS) means and treatment means in simple main effects were compared by performing post hoc Tukey honestly significant difference (HSD) test. Regression analyses were performed to examine possible relationships between NO_3^-, respiration (CO_2), N_2O, or total denitrification, and *nirS*, *nirK*, and *nosZ* gene numbers and *nosZ* gene transcripts at each time point. Treatment means and standard errors were calculated from untransformed data.

RESULTS

Soil NO_3^-, NO_2^-, NH_4^+ Concentrations

There were significant differences in NO_3^- concentrations between management systems and among N amendment treatments ($P < 0.001$) (Fig. 1a). In the unamended soils, NO_3^- concentrations were significantly higher ($P < 0.001$) for soil from the CS than the OS management system during the entire measurement period (average of 25.1 and 0.9 mg NO_3^-–N kg^{-1} dry soil for the CS_0 and OS_0 treatments, respectively) (Fig. 1a). Addition of compost (i.e., OS_C and CS_C treatments) did not significantly increase soil NO_3^- concentration compared with the unamended soil regardless of management system. Addition of mineral fertilizer resulted in the highest NO_3^- concentrations for both management systems (average of 144 and 125 mg NO_3^-–N kg^{-1} dry soil for the CS_F and OS_F treatments, respectively). Where mineral fertilizer was added, soil NO_3^- concentrations differed significantly ($P < 0.001$) between management systems only at 21 d at 174 and 80 mg NO_3^-–N kg^{-1} dry soil for the CS_F and OS_F treatments, respectively.

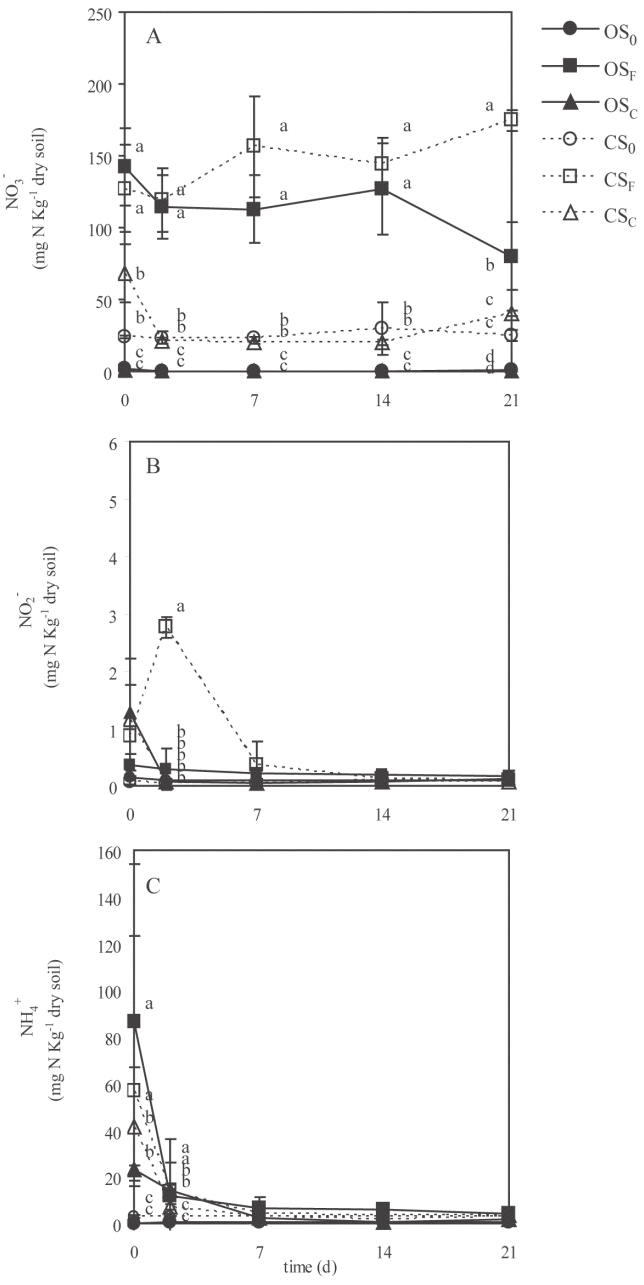

Fig. 1. Soil concentrations of (a) NO_3^-, (b) NO_2^-, and (c) NH_4^+ for organic management system (OS) and conventional management system (CS) soils incubated over 21 d in oxic microcosms. Values are means ± SEM (n = 4). Mean values followed by the same letter on the same sampling date are not significantly different ($P < 0.05$). OS_0, CS_0: control with no amendment added; OS_F, CS_F: amendment with ammonium nitrate; OS_C, CS_C: amendment with compost.

Soil NO_2^- concentrations were very low in almost all cases (Fig. 1b). There was, however, a small transient increase in soil NO_2^- concentration to 2.7 mg NO_2^-–N kg^{-1} dry soil at 2 d for the CS_F treatment.

Soil NH_4^+ concentrations were significantly different among amendment treatments ($P < 0.001$) (Fig. 1c). In unamended soils, soil NH_4^+ concentrations for both management systems were low (<1.6 mg NH_4^+ kg^{-1} dry soil) over the entire measurement period. Compost addition significantly increased NH_4^+ concentrations compared with unamended soils at time zero ($P < 0.001$), with average values of 41.6 and 23.1 mg NH_4^+–N kg^{-1} dry soil for the CS_C and OS_C treatments, respectively. Soil NH_4^+ concentrations in compost amended soils decreased to an average of 9.2 NH_4^+–N kg^{-1} dry soil at 2 d and remained low for the remainder of the incubation. Addition of mineral fertilizer significantly ($P < 0.001$) increased soil NH_4^+ concentrations compared with the other amendment treatments with average values of 71.6 mg NH_4^+–N kg^{-1} dry soil at time zero and average values of 12.3 mg NH_4^+–N kg^{-1} dry soil at Day 2 (Fig. 1c). Soil NH_4^+ concentrations did not differ among amendment treatments between 7 and 21 d.

Nitrous Oxide Emissions, Total Denitrification, and Respiration

In all cases, total denitrification rates (i.e., N_2O evolved in the presence of C_2H_2) were significantly higher than N_2O emissions (i.e., N_2O evolved in the absence of C_2H_2) ($P < 0.001$). The N_2O molar ratio (i.e., ratio of N_2O emissions to total denitrification) was always below 0.21 and mostly below 0.1 indicating that most gaseous N emissions from soils occurred as N_2 (data not shown).

Total denitrification rates responded significantly to amendment treatment, time, and management system ($P < 0.001$) (Fig. 2a). In unamended soils, total denitrification rates were significantly higher for the OS than the CS management at 7 d (16.1 and 1.1 μg N_2O-N kg^{-1} dry soil h^{-1} for OS_0 and CS_0 treatments, respectively) and 14 d (15 and 5.7 μg N_2O-N kg^{-1} dry soil h^{-1} for OS_0 and CS_0 treatments, respectively). For compost amended soils, there was no significant difference in total denitrification between management systems with average value over time of 5.2 μg N_2O-N kg^{-1} dry soil h^{-1}. Moreover, compost application did not increase denitrification rates in both OS and CS compared with unamended soils at any sampling date. Addition of mineral N significantly increased denitrification rates in both OS and CS compared with unamended soils at 2, 7, and 14 d. At 7 d, total denitrification rates significantly decreased compared with Day 2

in both CS_F and OS_F treatments ($P < 0.001$). After 14 and 21 d, total denitrification rates were not significantly different among amendment treatments for the CS soil, whereas denitrification rate for the OS_F treatment was still higher than for the OS_0 and OS_C treatments. The OS_F treatment had higher denitrification rates (average of 156 μg N_2O-N kg^{-1} dry soil h^{-1}) than the CS_F treatment (average of 68 μg N_2O-N kg^{-1} dry soil h^{-1}) (Fig. 2a).

The rate of N_2O emission responded significantly to amendment treatments ($P < 0.001$) but not management systems (Fig. 2b). Unamended and compost amended treatments were not significantly different and were stable over time with an average of 0.035 μg N_2O-N kg^{-1} dry soil h^{-1}. Addition of mineral N significantly increased N_2O emission rates ($P < 0.001$) compared with the unamended and compost amended soils at Day 2 (average of 1.6 μg N_2O-N kg^{-1} dry soil h^{-1}) and at Day 7 (average of 0.37 μg N_2O-N kg^{-1} dry soil h^{-1}) but not at Day 21 (Fig. 2b).

Soil respiration (CO_2 concentration) was measured in this study because it reflects microbial activity and is a measure of carbon availability. Carbon dioxide concentrations did not differ between soils incubated with or without C_2H_2 ($P = 0.217$), suggesting that acetylene was not a significant C source for the soil microorganisms (data not shown). Respiration rates responded significantly to amendment treatment and management system ($P < 0.001$) (Fig. 2c). In unamended soils, CO_2 emissions were significantly higher ($P < 0.001$) for the OS than the CS management system for the entire measurement period (average of 0.44 and 0.14 mg CO_2–C kg^{-1} dry soil h^{-1} for the OS_0 and CS_0 treatments, respectively). Respiration was stable over time after addition of NH_4NO_3, indicating that addition of mineral N did not affect respiration (Fig. 2c). Compost amendment significantly increased respiration rates in both CS and OS soils compared with other amendment treatments ($P < 0.001$), and respiration for the OS_C treatment was significantly higher than for the CS_C treatment at 2, 7, and 14 d (Fig. 2c).

Denitrifier Abundance and *nosZ* Transcript Numbers

The *nirS* gene abundance was about 10 times higher ($P < 0.001$) in OS than CS soil when averaged over time and amendment treatments (Fig. 3a). In unamended soil, *nirS* gene copy number was significantly lower ($P < 0.001$) in the CS_0 treatment compared with the OS_0 at each sampling point (Fig. 3a). Compost and mineral N treatments had significant effects on *nirS* gene copy numbers in both management systems ($P < 0.001$). In soils from the CS management, addition of mineral N significantly increased ($P < 0.001$) *nirS* gene copy numbers compared with unamended soils after 2, 7, and 21 d with average values of 1.8×10^7 and 1×10^7 copies g^{-1} dry soil for the CS_F and CS_0 treatments, respectively (Fig. 3a). In comparison, addition of mineral N in soils from the OS management significantly increased *nirS* gene copy numbers compared with unamended soils only at 21 d (Fig. 3a). Compost addition significantly increased ($P < 0.001$) *nirS* gene copy numbers in soils from both management systems compared with unamended soils. In soils from the CS management, *nirS* gene abundance in the CS_C treatment (average of 1.6×10^8 copies g^{-1} dry soil) was significantly higher ($P < 0.001$) than for the CS_F and CS_0 treatments at each sampling point (Fig. 3a). In soils from the OS management, compost addition increased *nirS* gene copy numbers from 7.2×10^8 copies g^{-1} dry soil at 2 d to 1.7×10^9 copies g^{-1} dry soil at 21 d (Fig. 3a).

The *nirK* gene abundance was significantly higher ($P < 0.005$) in soils from the OS system than the CS system, with average values of 4.1×10^8 and 1.9×10^8 copies g^{-1} dry soil, respectively. The *nirK* gene copy numbers did not change significantly over time for

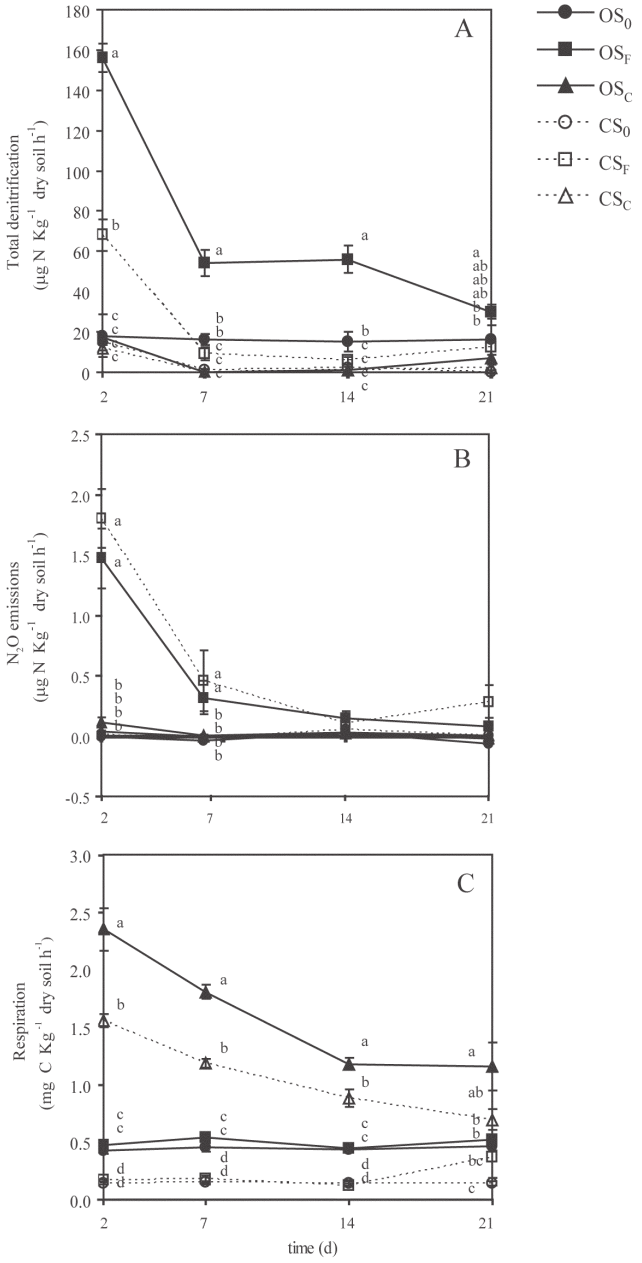

Fig. 2. (a) Total denitrification (i.e., N_2O + N_2) rates, (b) N_2O emission rates, and (c) CO_2 emission rates (i.e., respiration) from organic management system (OS) and conventional management system (CS) soils incubated over 21 d in oxic microcosms. Values are means ± SEM ($n = 4$). Mean values followed by the same letter on the same sampling date are not significantly different ($P < 0.05$). OS_0, CS_0: control with no amendment added; OS_F, CS_F: amendment with ammonium nitrate; OS_C, CS_C: amendment with compost.

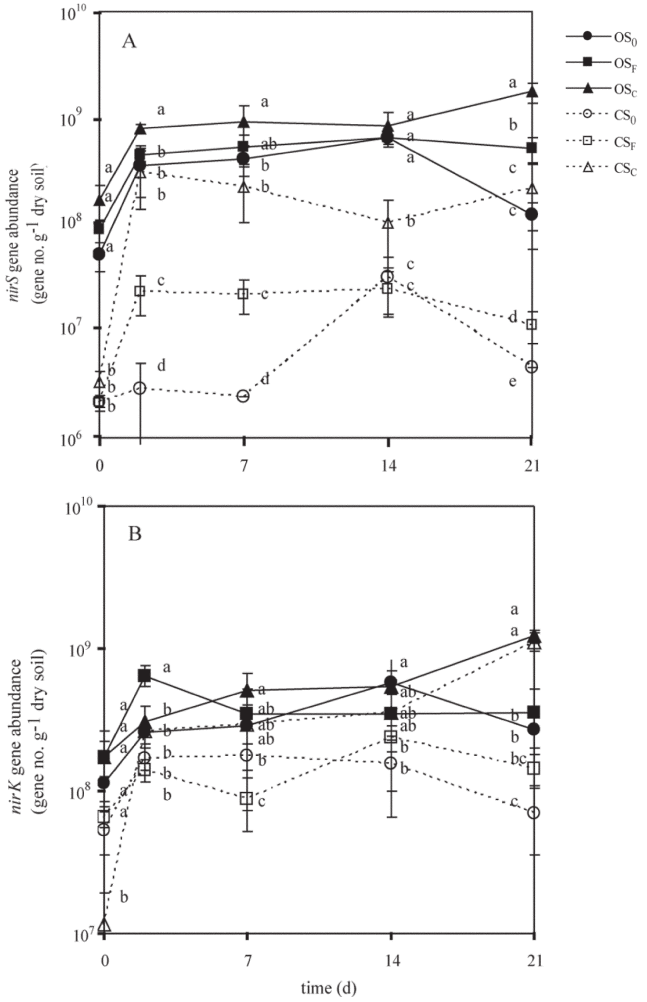

Fig. 3. Quantification of (a) *nirS* and (b) *nirK* gene abundance using quantitative PCR in organic management system (OS) and conventional management system (CS) soils incubated over 21 d in oxic microcosms. Values are means ± SEM (*n* = 4). No templates control reactions were undetectable. Mean values followed by the same letter on the same sampling date are not significantly different (*P* < 0.05). OS_0, CS_0: control with no amendment added; OS_F, CS_F: amendment with ammonium nitrate; OS_C, CS_C: amendment with compost.

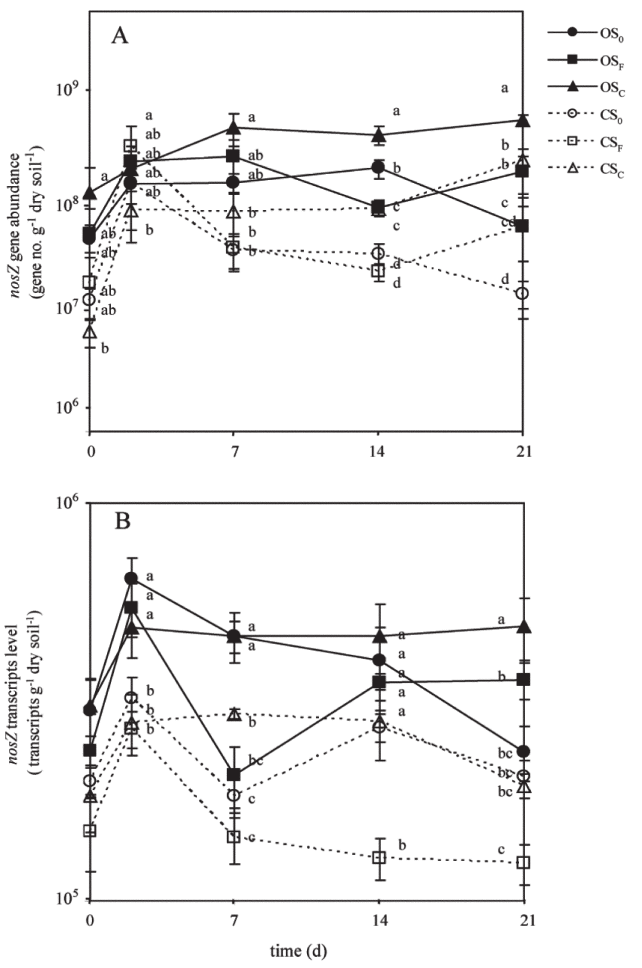

Fig. 4. Quantification of (a) *nosZ* gene abundance using qPCR and (b) *nosZ* mRNA transcript number using RT-qPCR in organic management system (OS) and conventional management system (CS) soils incubated over 21 d in oxic microcosms. Values are means ± SEM (*n* = 4). No templates control reactions were undetectable. Mean values followed by the same letter on the same sampling date are not significantly different (*P* < 0.05). OS_0, CS_0: control with no amendment added; OS_F, CS_F: amendment with ammonium nitrate; OS_C, CS_C: amendment with compost.

the unamended soils, whereas *nirK* gene copy numbers increased significantly in soils amended with compost or mineral N from time zero to Day 2 (Fig. 3b). Compost addition significantly increased ($P < 0.001$) *nirK* gene abundance, with about a 70-fold and 10-fold compared increase at 21 d compared with time zero for the OS_C and CS_C treatments, respectively ($P < 0.001$) (Fig. 3b). In contrast, addition of mineral N did not have a significant effect on *nirK* gene copy numbers compared with unamended soils in both management systems after Day 2 (Fig. 3b).

The *nosZ* gene copy numbers were higher in soils from the OS management (average of 3.1×10^8 copy g^{-1} dry soil over treatment and time) compared with the CS management (average of 1.6×10^8 over time and treatments) (Fig. 4a) There were no significant differences in *nosZ* gene abundances among amendments of OS or CS soils from Day 0 to 7. Addition of mineral N had no significant effect on *nosZ* gene copy numbers for soils from the CS management and had inconsistent effects in soils from the OS management; *nosZ* gene copy numbers were significantly higher for the OS_0 than the OS_F at 14 d, whereas the reverse was true at 21 d. Addition of compost in CS and OS soils increased *nosZ* abundance compared with unamended and mineral N amended soils at 14 and 21 d. The *nosZ* gene copy numbers was higher in the OS_C treatment than the CS_C treatment at 21 d with 9.2×10^8 and 3.9×10^8 copy g^{-1} dry soil, respectively (Fig. 4a).

The abundance of denitrifiers communities was quantified in compost to evaluate the number of gene copies that would be added to soil. qPCR analysis using primers for *nirK* and *nirS* on DNA directly extracted from compost resulted in 7×10^6 and 5×10^6 gene copy numbers g^{-1} dry compost, respectively. Thus, addition of around 10 g of dry compost to 290 g of soil resulted in addition of 2.2×10^6 and 1.4×10^6 of *nirK* and *nirS* gene copy numbers g^{-1} dry soil, respectively. Therefore compost addition contributed at time zero for an average of 2.5 and 2% in *nirS* and *nirK* copy number, respectively. No amplification using *nosZ* primers was achieved in compost extracted DNA.

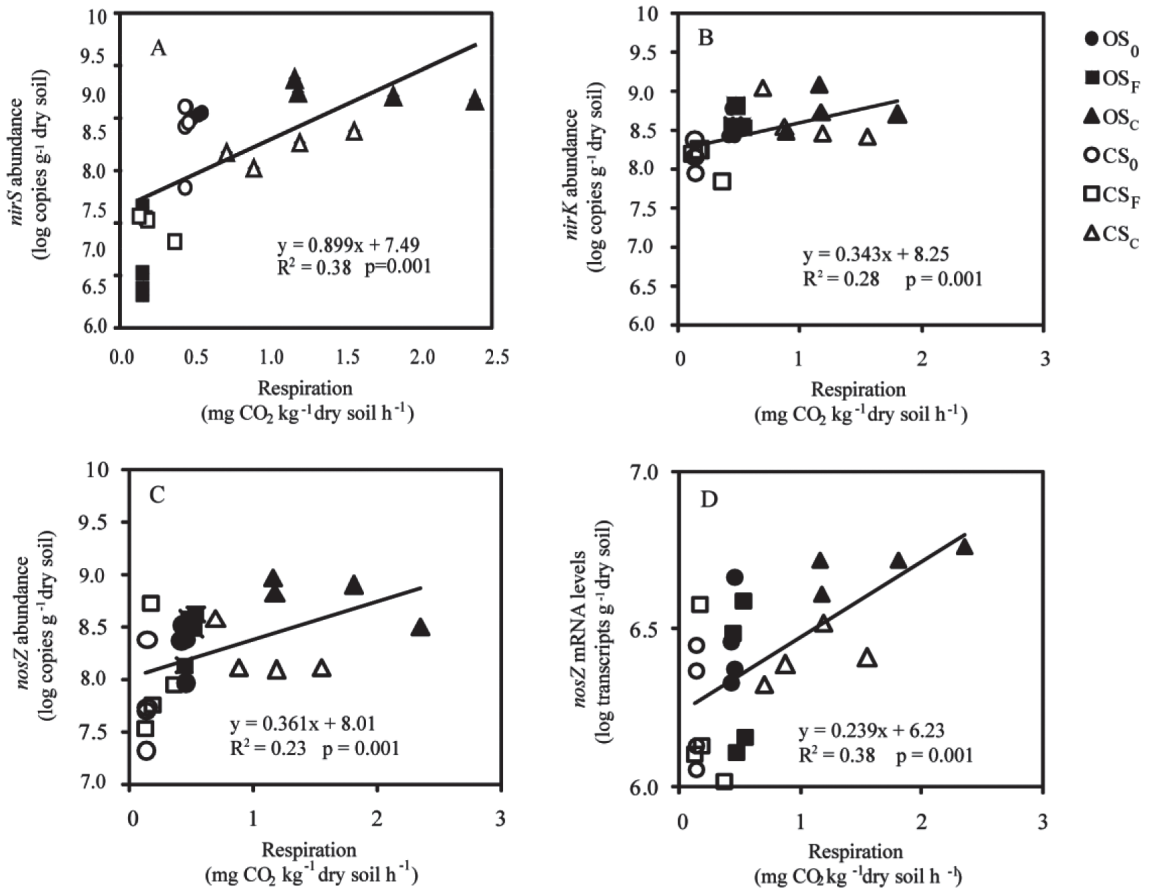

Fig. 5. Relationships between respiration and (a) *nirS*, (b) *nirK*, (c) *nosZ* gene abundance, and (d) *nosZ* mRNA transcript number in organic management system (OS) and conventional management system (CS) soil soils. Line of best fit indicates the linear relationship described by the equations in the figure. Statistical values are given in the figure. OS_0, CS_0: control with no amendment added; OS_F, CS_F: amendment with ammonium nitrate; OS_C, CS_C: amendment compost.

Gene transcript number could only be measured for the *nosZ* gene. *nosZ* gene transcript numbers were significantly higher ($P < 0.001$) in soils from the OS management (5.6×10^6 transcripts g^{-1} dry soil) than from the CS management (2.9×10^6 transcripts g^{-1} dry soil). mRNA transcript numbers of *nosZ* did not change significantly over time in soils from either management system (Fig. 4b). Similarly to *nosZ* gene copies, the *nosZ* gene mRNA transcript numbers increased numerically from time zero to 2 d, but the changes were not significant (Fig. 4b). Compost addition resulted in a significant ($P < 0.005$) increase in *nosZ* mRNA transcript number in both soils on some sampling dates. The *nosZ* transcript numbers in the OS_C treatment were significantly higher ($P < 0.001$) at 21 d compared with the OS_F and OS_0 treatments (4.9×10^6, 3.5×10^6, and 2.3×10^6 transcripts g^{-1} dry soil, respectively) (Fig. 4b). *nosZ* mRNA transcript numbers in the CS_C treatment were significantly higher than for the CS_F and CS_0 treatments at 7 d (Fig. 4b). Conventional management system and OS soils had an RNA/DNA ratio of 0.03 and 0.015, respectively, when averaged over N fertilizer treatments and time (data not shown).

Regression analysis was used to identify possible relationships between analytical measurements, respiration, N_2O emission rates, denitrification rates, and the abundance and *nosZ* mRNA transcript numbers of denitrifier communities. There were significant positive relationships ($P < 0.001$) between respiration rates and *nirK*, *nirS*, *nosZ* gene abundance, and *nosZ* gene mRNA transcript number (Fig. 5). No significant relationships were found between denitrification gene abundance and *nosZ* mRNA transcript numbers and either N_2O emission or total denitrification rates.

DISCUSSION

Effects of Long-Term Compost Application Compared to Long-Term Mineral Application

The long-term (8 yr) application of compost in the investigated Tuscan vineyard soil changed soil chemical properties, leading to an increased C and N content as previously reported (Tatti et al, 2012). In the current study, long-term compost application increased abundance of the *nirS*, *nirK*, and *nosZ* gene-bearing communities 10, 2.2, and two-fold, respectively, compared to CS soils. There are currently no other studies that have reported effects of long-term compost application on abundance of denitrifying bacteria. With respect to other organic amendments, Hallin et al. (2009) reported greater *nirS*, *nirK*, and *nosZ* gene abundance in long-term application of sewage sludge and cattle manure compared with ammonium sulfate. These results suggest that application of organic amendments over the long-term can change not only soil properties but also soil microbial abundance including denitrifiers.

Long-term compost application also changed soil denitrification activity. Total denitrification rate in mineral N amended soil, where nitrate supply was not limited, was significantly higher in soil from the OS management compared with CS management. Increased organic C availability commonly increases denitrification rate in soil incubation studies (Gillam et al., 2008; Miller et al., 2008; Miller et al., 2009), and therefore, the increased denitrification in the OS management soil likely reflects increased C availability. Similarly, Dambreville and colleagues (2006) reported an increase of potential denitrifying activity in soil treated for 7 yr with composted pig manure compared with soil treated with ammonium nitrate, due to a higher total C content and microbial biomass.

Effects of Short-Term Mineral Nitrogen Amendment

Addition of mineral N had no significant effect on the abundance of the *nirK* and *nosZ* gene-bearing communities, while in the CS soil, increased the *nirS* gene copy number up to 2.2-fold compared to the unamended soil. The experimental conditions used in this study created both anoxic and oxic conditions because of the high water content and the presence of large macro pores due to the high clay content of the soil, respectively. Therefore, it is not clear if mineral N availability (i.e., NO_3^-) could have given a competitive advantage to denitrifiers compared to other microbial communities. Some study demonstrated that denitrifier growth rate was better under anoxic conditions (Murray et al., 1992, Philippot et al., 1996) while other studies suggested that denitrifiers were more competitive under oxic conditions (Smith and Tiedje, 1979; Tiedje, 1988). Although addition of mineral fertilizer induced denitrification and N_2O emissions, NO_3^- had no effect on *nosZ* gene mRNA transcript numbers. Given that soil has anoxic sites and denitrification gene induction occurs at low N-oxides concentration (Vollack and Zumft, 2001; Saleh-Lakha et al., 2009), it is possible that the *nosZ* gene expression was already induced in soil.

As expected, mineral N addition had no significant effect on soil respiration, which can be used as an indicator of soil C availability. Mineral N addition increased both N_2O emissions and total denitrification rates. In incubation studies, addition of NO_3^- has resulted either in an increase in denitrification rate (Jordan et al., 1998; Strong and Fillery, 2002) or had no effect on denitrification rate (Myrold and Tiedje, 1985;, De Klein and van Logtestijn, 1996). Gillam et al. (2008) concluded that NO_3^- addition affects denitrification rate only when NO_3^- is limiting the denitrification process, which can occur when the supply of NO_3^- is low or the demand for NO_3^- is high due to limited O_2 supply in combination with high availability of organic C. Nitrate addition increases N_2O emissions in most cases through either an increase in the denitrification rate or through a change in the N_2O molar ratio (Gillam et al., 2008). The N_2O molar ratio is controlled by the relative availability of C and NO_3^- (Firestone et al., 1979; Miller et al., 2008) and is commonly high when there is an abundant supply of NO_3^- in soil (Gillam et al., 2008; Miller et al., 2008). Interestingly, in this study the N_2O molar ratio was low (<0.1 in most cases) even when soil NO_3^- concentration was high. Low N_2O molar ratios in the presence of high NO_3^- concentration have been infrequently reported in the literature (Mahmood et al., 1998; Vallejo et al., 2006). The low N_2O molar ratio in this study may reflect the high (332 g kg^{-1}) clay content of the soils. Most pores within the soil aggregates are small and, consequently, are water-filled at high water content. This would substantially reduce the rate of N_2O diffusion in soil and may result in reduction of N_2O to N_2 before the N_2O can diffuse out of the aggregates (Smith, 1990).

Effects of Short-Term Compost Amendment

A single application of compost increased *nirK* and *nosZ* gene-bearing communities two-fold in soils from both CS and OS management systems. Compost has been shown to stimulate heterotrophs growth in the short term due to C availability (Viti et al., 2010). Moreover, previous studies have reported denitrifier abundance increasing with organic C availability (Miller et al., 2009; Bárta et al., 2010), although others authors showed no effect on denitrifiers (Henry et al., 2008; Miller et al., 2008; Henderson et al., 2010). We hypothesize that the contrasting response of denitrifiers community to C source addition observed in different studies may be due to the type of organic matter (i.e., more readily-available vs. more complex) and differences in experimental conditions used. Compost application increased the *nirS* gene copy number g^{-1} dry soil around 20-fold in soil from the CS system, while the increase in OS system was around two-fold. The *nirS* gene-bearing community in soil from the CS system was more responsive to compost addition than the other denitrifying communities studied here. The contrasting long-term managements may have affected the *nirS* gene-bearing community structure, which then resulted in a differential response to the compost amendment treatment in this study. In contrast to *nirS*, the increase *nirK* gene abundance was evident only after 21 d regardless of the management system, thus indicating a different response to addition of complex organic C. These results, together with the different response to N addition, suggest a difference in ecological niches between *nirK* and *nirS* denitrifier communities, as previously shown in agricultural soils by other authors (Chen et al., 2010; Hallin et al., 2009).

Compost addition significantly increased *nosZ* gene transcripts compared to mineral N addition and unamended soils regardless of management system. *nosZ* gene mRNA transcription is induced by anoxic condition and nitric oxide (Vollack and Zumft, 2001). The higher microbial activity following compost addition, as indicated by increased soil respiration, may have depleted soil O_2 supply and, thereby, favored *nosZ* gene transcription. Very few studies have examined the response of denitrification gene mRNA transcription to complex carbon source addition. Henderson et al. (2010) reported a significant increase of *nosZ* gene mRNA transcript numbers in anoxic microcosms soils amended with plant residues. In this study, the increase in *nosZ* gene mRNA transcript numbers could be due to an increase

in number of transcripts per cell and/or an increase in the number of cells. The *nosZ* mRNA/DNA ratio was two-fold higher in CS soil compared to OS for all treatments: compost addition increased the number of *nosZ* gene bearing denitrifiers; thus, the lower mRNA/DNA ratios observed in compost amended soils compared to other amendments suggested that not all the *nosZ* gene bearing denitrifiers may have been producing transcripts or producing them at the same time.

Compost addition did not increase total denitrification rates and soil mineral N concentrations in both management systems but significantly increased soil microbial activity as indicated by respiration. This may reflect either limited release of N from the added compost or net immobilization following compost addition. The lack of a response of emissions to compost addition can be attributed to a limitation in nitrate supply, which was the main driving factor for this process. Our results were similar to those obtained by Alluvione et al. (2010), who found very low N_2O emission following compost addition compared with urea in field experiments due to less nitrate availability.

Relationships between Denitrifier Abundance and *nosZ* mRNA Transcript Numbers and Soil Processes

Significant correlations were observed between soil respiration and *nirS*, *nirK*, and *nosZ* gene abundance/mRNA transcript numbers. Soil respiration was increased in response to both a long-term compost addition in the OS management and to compost addition as an amendment. The positive correlations are consistent with the key role of organic C in influencing microbial abundance. Miller and coworkers (2009) reported a positive correlation between respiration and nitric oxide reductase gene of *Pseudomonas mandelii* and related species ($cnorB_P$) copy number following liquid manure addition on soil, while other authors did not report such correlation in their analysis after soil addition of plant residues (Miller et al., 2008; Henderson et al., 2010).

No significant relationships were observed between denitrifier abundance or *nosZ* gene mRNA transcript numbers and N_2O emissions or denitrification rates in our study. Similarly, previous studies in anoxic soil microcosms also reported no significant correlation between denitrifier abundance or mRNA transcript numbers and denitrification (Miller et al., 2008; Henderson et al., 2010; Dandie et al., 2011). Although the presence of denitrification gene transcripts suggests their actual involvement in the soil processes, it is known that mRNA may not be directly linked to actual related-enzyme activity, considering all the regulatory steps from gene expression to a fully functional enzyme. Moreover, it could be hypothesized that other players not targeted in the present study (i.e., other bacterial denitrifier groups or denitrifying fungi) might have been involved in denitrification in our system.

In conclusion, our study demonstrated that long-term application of municipal compost increased abundance of soil bacterial denitrifying communities and denitrification gene mRNA transcript numbers. Long-term compost addition also influenced respiration and denitrification. When NH_4NO_3 was added, denitrification rates increased, mainly in the OS soil, where no limitation of both N and C was present. Compost amendment on both OS and CS increased the *nirK, nirS*, and *nosZ* gene abundance and mRNA transcript numbers, and soil respiration, having provided readily utilizable C. In both systems, denitrification and N_2O emission were not affected by compost treatments, which did not bring enough mineral N to soil to fuel these soil processes. Moreover, under the condition tested, we showed that despite the common assumption that N_2O molar ratio is high when nitrate supply is abundant, this may not be the case in clay-rich soil. Obtained result suggests that the use of inorganic fertilizer on long term compost treated soils could lead to a high N loss from the system under conditions favoring denitrification. The results highlighted that urban-waste organic management practices can have a profound effect on soil proprieties, microbial communities, and key soil processes and that these changes can have important implications in terms of soil health and environmental contamination.

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