Applied and Environmental Microbiology, June 2011, p. 4097–4104 Vol. 77, No. 12
0099-2240/11/$12.00 doi:10.1128/AEM.00033-11

Association of Earthworm-Denitrifier Interactions with Increased Emission of Nitrous Oxide from Soil Mesocosms Amended with Crop Residue▽†

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Received 7 January 2011/Accepted 12 April 2011

Earthworm activity is known to increase emissions of nitrous oxide (N_2O) from arable soils. Earthworm gut, casts, and burrows have exhibited higher denitrification activities than the bulk soil, implicating priming of denitrifying organisms as a possible mechanism for this effect. Furthermore, the earthworm feeding strategy may drive N_2O emissions, as it determines access to fresh organic matter for denitrification. Here, we determined whether interactions between earthworm feeding strategy and the soil denitrifier community can predict N_2O emissions from the soil. We set up a 90-day mesocosm experiment in which ^{15}N-labeled maize (*Zea mays* L.) was either mixed in or applied on top of the soil in the presence or absence of the epigeic earthworm *Lumbricus rubellus* and/or the endogeic earthworm *Aporrectodea caliginosa*. We measured N_2O fluxes and tested the bulk soil for denitrification enzyme activity and the abundance of 16S rRNA and denitrifier genes *nirS* and *nosZ* through real-time quantitative PCR. Compared to the control, *L. rubellus* increased denitrification enzyme activity and N_2O emissions on days 21 and 90 (day 21, $P = 0.034$ and $P = 0.002$, respectively; day 90, $P = 0.001$ and $P = 0.007$, respectively), as well as cumulative N_2O emissions (76%; $P = 0.014$). *A. caliginosa* activity led to a transient increase of N_2O emissions on days 8 to 18 of the experiment. Abundance of *nosZ* was significantly increased (100%) on day 90 in the treatment mixture containing *L. rubellus* alone. We conclude that *L. rubellus* increased cumulative N_2O emissions by affecting denitrifier community activity via incorporation of fresh residue into the soil and supplying a steady, labile carbon source.

Denitrification is a microbial process in which organisms reduce inorganic nitrogenous oxides to nitrous oxide (N_2O) or nitrogen (N_2) gas. Denitrifier activity is receiving increasing global attention, as it is a dominant cause of N_2O emissions from agricultural soils, which contribute the majority of anthropogenic N_2O emissions (34). In order to mitigate this trend, considerable research has gone into understanding the environmental drivers that affect the activity of denitrifying organisms.

Earthworms are considered to be ecosystem engineers due to their ability to drive soil ecosystem processes, such as soil structure and organic matter dynamics. They have likewise been implicated in higher rates of denitrification and N_2O emission, both *in vivo* and from soils that they inhabit (7, 12, 16, 21, 37). Agricultural management has direct consequences for earthworm population activity, composition, and size. Therefore, it is important to understand the principle factors behind earthworm-enhanced N_2O emissions from soil.

The mechanism of earthworm-mediated N_2O emission is not clearly understood, but it likely involves interactions with soil denitrifiers within the drilosphere (i.e., earthworm gut, burrows, and casts). The process of denitrification requires anaerobic conditions in combination with the availability of nitrate (NO_3^-) (or nitrite [NO_2^-]) and electron-rich C, precisely the conditions found in the earthworm gut (16, 24, 25). Indeed, increased N_2O emissions have been measured within the earthworm gut (24), which coincides with an observed 300-fold increase in culturable bacterial denitrifiers in the gut compared to the bulk soil (25). Furthermore, in contrast with the bulk soil, fresh earthworm casts exhibit a higher microbial biomass, microbial activity, and mineral N content (1, 40) and earthworm burrows contain higher nitrification and denitrification activities due to earthworm excretion of nutrient-rich mucus (33). Together, these processes are known as the earthworm priming effect (5).

Earthworm priming of denitrifiers may ultimately depend upon the earthworms' ecological feeding strategy, which determines their access to organic matter. For example, epigeic earthworms primarily reside in the soil-litter interface, mixing into the soil and ingesting fresh organic matter, while endogeic earthworms inhabit deeper soil layers, feeding predominantly on soil organic matter. Several studies have concluded that earthworm-mediated N_2O emissions depend largely upon ecological feeding strategy (21, 29, 37).

Despite evidence for an earthworm-induced priming effect of denitrifying organisms in the drilosphere, little is known about emergent effects of earthworms on the size and composition of denitrifying populations on the scale of the entire soil profile. A quantitative PCR (qPCR) assay effectively utilizes soil DNA to assess changes in dentrifying bacterial populations in the soil (23, 26, 44). Furthermore, the denitrification enzyme activity (DEA) assay provides an estimate of the relative activity of denitrification enzymes within these soil denitrifier populations during the time of sampling (30).

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 22 April 2011.

Here, we conducted a time course mesocosm study in which earthworm-induced changes in soil biochemistry and denitrifier populations were measured alongside soil N_2O emissions. Our primary goal was to assess the mechanism of net N_2O emissions from soils, rather than solely N_2O production within the drilosphere. Thus, we sought to examine chemical and biological alterations by earthworms on the scale of the bulk soil. Furthermore, we utilized both an epigeic (*Lumbricus rubellus*) and an endogeic (*Aporrectodea caliginosa*) earthworm species to ascertain the importance of earthworm feeding strategy on the interactions with denitrifiers. We hypothesized that (i) earthworm activity would result in higher N_2O emissions from the soil by increasing the activity and population size of the soil denitrifier community and (ii) the extent of these interactions would depend upon earthworm ecological feeding strategy.

MATERIALS AND METHODS

Experimental setup. We set up a controlled mesocosm experiment for quantification of N_2O and CO_2 emissions over 90 days as a function of earthworm and residue treatment. The study consisted of 12 replicates of 6 treatments, which were destructively sampled in sets of 4 replicates on days 6, 21, and 90. The 72 mesocosms were arranged within a climate room (16°C and 60% humidity) in a randomized block pattern, consisting of 4 blocks that were rearranged 3 times to account for spatial heterogeneity in the room's microclimate.

All mesocosms were 6.1-liter polyethylene buckets containing 4 kg of loamy sand that was maintained at 17% gravimetric moisture content. Loamy sand topsoil was obtained from the experimental farm Droevendaal in Wageningen (51°59′N, 5°39′E), Netherlands. The soil contained 1.3 g total N kg^{-1} and 14.2 g total C kg^{-1}. Soil was air dried and sieved at 8 mm before it was added to the mesocosm. Prior to treatment, we incubated the freshly sieved soil for 1 week to reach steady gas emissions and determined it to have a pH-H_2O of 5.6, hot water extractable organic C content (20) of 575 μg C kg^{-1}, and total mineral N content (extraction with 2 M KCl) of 8.93 mg N kg^{-1}.

Treatments included a control soil (S) without addition of residue or earthworms. In the remaining treatments ^{15}N-labeled *Zea mays* L. residue was either mixed into the soil (SM) or applied on top of the soil (ST). The ^{15}N-enriched maize was harvested from plots containing ^{15}N-labeled fertilizer. Dried roots and shoots were cut in 2-cm pieces, homogenized, and applied at a rate of 20 g per mesocosm with a root:shoot ratio of 0.18 (total C:N ratio of 58; ^{15}N enrichment of 3.557 atom% excess). Individuals of the epigeic *L. rubellus* (R) and the endogeic *A. caliginosa* (C) species were added only to ST treatments in a full factorial pattern, resulting in 4 treatments corresponding to the presence or absence of one or both of the species (ST, ST_R, ST_C, and ST_{RC}). We collected adults and large juveniles of both earthworm species from park areas in Wageningen. Forty-eight hours prior to use, the earthworms were moved to damp filter paper to void gut contents, following the method of Dalby et al. (13), before they were weighed and added to the mesocosms at a density within normal ranges of Dutch soils, resulting in 4 individuals of *L. rubellus* (80 individuals m^{-2}) and 7 individuals of *A. caliginosa* (150 individuals m^{-2}) per mesocosm (15). Mesocosms were covered with a black, air-permeable polyethylene cloth fixed with a rubber band to prevent earthworms from escaping.

N_2O and CO_2 flux measurements. N_2O and CO_2 emissions were measured every day for the first week, every 2 days through week 3, two times per week through week 7, and then weekly until day 90. We measured N_2O and CO_2 by using a static closed chamber technique and an Innova 1412 photo-acoustic infrared gas analyzer (LumaSense Technologies A/S, Ballerup, Denmark) and two Teflon tubes as described by Kool et al. (28). Polypropylene lids equipped with two rubber septa were fixed to the mesocosms for 30 and 50 min to measure CO_2 and N_2O, respectively. We used a soda-lime filter during N_2O measurements to minimize interference effects of CO_2 and water vapor (46). Cumulative emissions were calculated assuming a linear change in rate between sampling days.

Soil and earthworm sampling. On all destructive sampling days, mesocosms were overturned and earthworms were recovered by hand. Earthworms were placed on wet filter paper for 48 h to void gut contents by the filter paper method (13) and weighed on a basis of species biomass per mesocosm. On the final destructive sampling day, remaining crop residue was collected from the top of each mesocosm, washed to remove excess soil particles, oven dried at 50°C, and weighed to determine dry weight recovery.

The soil was sieved over 4 mm, and all subsamples were stored at 2°C until further use. We performed all chemical analyses within 48 h of sampling. Mineral N was extracted using 1 M KCl (28). We determined mineralizable N anaerobically by incubating 16 g of soil in 40 ml distilled water for a standard 7 days at 40°C before 1 M KCl extraction (8). Both extracts ($T = 0$ and $T = 7$ days) were analyzed colorimetrically for NH_4^+ and NO_3^- content. Mineralizable N was quantified as the increased NH_4^+ concentration after 7 days of anaerobic incubation.

Microbial biomass nitrogen (MBN) was determined by chloroform fumigation followed by 0.01 M K_2SO_4 extraction (4), where total soluble nitrogen was measured colorimetrically. The labile organic pool of soil carbon was approximated using the hot water extractable C (HWC) method (20). We combined a 3-g soil sample with 30 ml of water at 80°C for 16 h to extract the labile pool of carbon. The extracted fractions were measured using a segmented flow SK^{12} TOC/DOC autoanalyzer (Breda, Netherlands) through persulfate and tetraborate oxidation under UV light and infrared detection. HWC was determined by subtracting inorganic C from total C in the hot water extract.

^{15}N contents of samples. We analyzed ^{15}N contents from N_2O gas samples, earthworms, bulk soil, mineral N, and MBN. On measuring days 6, 23, and 89, we transferred N_2O gas samples to 12-ml soda glass exetainers (Labco, High Wycombe, United Kingdom). All earthworms were first freeze-dried and ball milled. Air dried, sieved bulk soil samples were also ball milled. Mineral N and MBN was converted to solid form from remaining extractant from the KCl extractions and fumigation extractions, respectively, following the methods of Ros et al. (38). All solid samples were dried at 105°C and quantitatively weighed into tin (Sn) capsules.

Glass exetainers and Sn capsules containing samples were sent to the UC Davis Stable Isotope Facility for determination of ^{15}N content. ^{15}N content of N_2O was determined using a Poroplot Q gas chromatography column (25 m by 0.53 mm; 25°C; helium carrier gas at 1.8 ml/min) interfaced to a ThermoScientific Delta V Plus isotope ratio mass spectrometer (IRMS; Bremen, Germany). ^{15}N contents of solids were determined using a PDZ Europa ANCA-GSL elemental analyzer interfaced with a PDZ Europa 20-20 IRMS (Sercon Ltd., Cheshire, United Kingdom) with a combustion temperature of 1,000°C.

Denitrification enzyme activity assay. The DEA assay was adapted from that described by Luo et al. (30) to determine the relative activities of denitrification enzymes upon destructive sampling. Within 24 h of sampling, we placed 40 g of fresh soil in an air-tight jar of 1.0 liter with a lid containing 2 rubber septa. We flushed the jars using N_2 and injected them with 40 ml of degassed solution containing 10 mM KNO_3 and 10 mM glucose to provide an anaerobic environment with nonlimiting amounts of nitrate and a high-energy carbon source. Finally, all jars were injected with C_2H_2 at approximately 5% (vol/vol) in order to inhibit the final enzymatic reduction of N_2O to N_2. The jars were placed on a large shaker set to 124 rpm at room temperature (~20°C), and N_2O emission was measured after 2 and 5 h using the same N_2O monitoring method described above. The rate of N_2O accumulation from 0 to 5 h was used as a measure of preexisting denitrification enzyme activity, as this is considered to be the maximum amount of incubation time before N_2O accumulation rates are significantly affected by other processes, such as upregulation of enzymes and bacterial growth (30).

Quantitative PCR assay. For qPCR, we stored on sampling days 21 and 90 a 10-g hand-homogenized subsample of bulk soil at −80°C for extraction with a FastDNA spin kit for soil (MP Biomedicals, LLC, Solon, OH). The DNA was purified two sequential times by using a Wizard DNA Clean-Up system (Promega, Madison, WI) to wash away excessive coextracted substances and stored at −20°C. DNA concentrations and purity were checked using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Purified DNA was amplified using primers homologous to DNA sequences that coded for 16S rRNA (18), *nirS* (26), and *nosZ* (nosZ2 primer pair) (23). The genes were quantified using an ABI Prism 7500 thermal cycler (Applied Biosystems, Nieuwerkerk a/d Ijssel, Netherlands) with a SYBR green detection system. Each 25-μl reaction mixture contained 5 ng extracted DNA, 12.5 ml SYBR premix Ex*Taq* (Lucron Bioproducts B.V., Gennep, Netherlands), 0.5 μl ROX dye II (50-fold dilution; Lucron Bioproducts B.V., Gennep, Netherlands), 40 ng of $T4_{gp}32$ (Roche Diagnostics GmbH, Mannheim, Germany) 1.25 μl dimethyl sulfoxide (DMSO; Sigma-Aldrich), 0.5 μM primer (quality Oligogold; Eurogentec, Maastricht, Netherlands), and 5.75 μl distilled sterile water. The thermocycler conditions were a 1-min dissociation step at 95°C, followed by 30 s of each primer's specific annealing temperature condition, and then a 72°C extension step for 1 min for 40 replication cycles. The annealing temperatures for 16S rRNA, *nirS*, and *nosZ* were 53°C, 60°C, and 62°C, respectively, and the *nirS* and *nosZ* an-

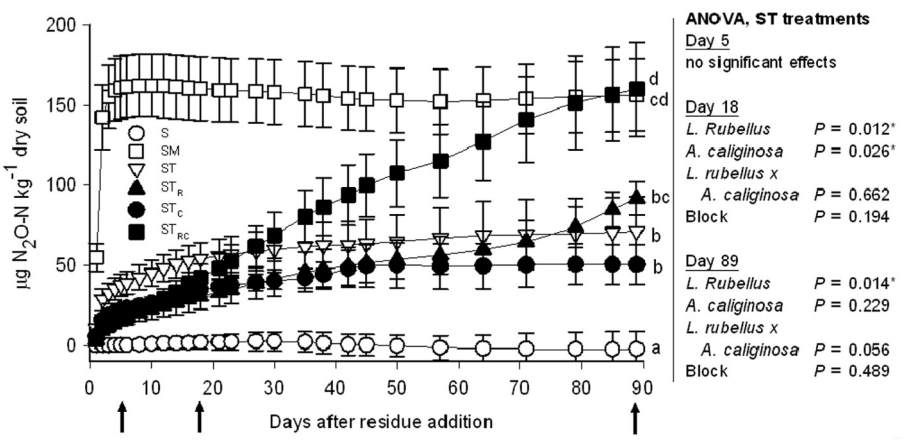

FIG. 1. Cumulative N_2O emissions from the four replicate mesocosms that were destructively sampled on the final sampling day. The included ANOVA tests were performed on the cumulative N_2O emissions from mesocosms measured on days directly before destructive sampling (indicated by dark arrows). Significant differences in cumulative emissions between treatment groups on day 89 are represented with different lowercase letters. Error bars represent the distance of 1 standard error. *, $P < 0.05$.

nealing temperatures were preceded with a 6-cycle touchdown of −1°C per cycle. All DNA samples belonging to the same sampling day were reacted on the same 96-well plate, with 3 replicate reactions per sample. Each reaction was followed by a standard dissociation curve analysis with a temperature ramp from 60 to 95°C in order to test amplicon purity. Specificity of amplified products was confirmed by identifying distinct bands of the correct size via agarose gel electrophoresis.

Fluorescence data from individual DNA samples were directly compared to ascertain treatment effects, rather than being calibrated to an external DNA standard for absolute quantification. To do this, raw fluorescence data were first normalized against ROX Dye II using ABI 7500 system detection software version 1.2.3, and then LinRegPCR software (version 11.5.0.0) was used for comparative analysis (36). LinRegPCR operates under the assumption that the log(fluorescence) increases at a constant rate with PCR cycle when it is first detectable by the instrument. Linear regression analysis was used to estimate baseline fluorescence and doubling efficiency (E) per PCR, and then the line was extrapolated backward to calculate the theoretical fluorescence value of the gene of interest at cycle 0 (N_0). Thus, N_0 is determined by the equation $N_0 = N_T/E^{C_T}$ where C_T is the number of PCR cycles needed to reach threshold fluorescence (N_T). We used a common N_T (optimal at 0.089) for all amplicons to allow for direct comparisons between them (39). A common amplification efficiency was assumed for each amplicon per time step once statistical tests showed individual reaction efficiencies exhibited a normal distribution and were not affected by mesocosm treatment (see reference 39 regarding this assumption). N_0 was reported per g of dry soil as a metric of gene density. In addition, relative abundances of *nirS* and *nosZ* were estimated as percentages of 16S rRNA gene copies, by comparing N_0 per ng of reacted DNA and taking into account variation in N_0 caused by the different sizes of each DNA amplicon.

Statistical analysis. Statistical tests were conducted using SPSS version 15.0.1 software (SPSS Inc., Chicago, IL). Two distinct analysis of variance (ANOVA) tests were performed for data on each sampling day. First, we assessed significant differences in treatment means by using ANOVA and *post hoc* least significant differences (LSD) analysis at 95% confidence. Second, effects of earthworm species were assessed only in treatments receiving residue on top (ST, ST_R, ST_C, and ST_{RC}) via a full factorial 2-way ANOVA, in which the factors were defined as presence or absence of *L. rubellus* or *A. caligonosa*. Where appropriate, data were log transformed to achieve assumptions of normality and homogeneity of variances. Block effects were taken into account in both ANOVA tests.

We were interested in daily and cumulative N_2O and CO_2 emissions directly before each destructive sampling event. However, due to destructive sampling, treatment replicates decreased from 12 to 8 to 4, complicating statistical analysis. Thus, statistical analysis on N_2O and CO_2 emissions was performed only on the 4 replicate mesocosms directly (1 to 3 days) before they were harvested, rather than analyzing all replicates available on that day; results were the same when using all available replicates.

RESULTS

Recovery of maize residue and earthworms. Over the 90 days, top-applied maize residue was visibly incorporated into the soil within ST treatments containing *L. rubellus*, as was confirmed by a significant *L. rubellus* effect on percent residue mass lost ($P = 0.003$) (results not shown). Overall, ST treatments with and without *L. rubellus* lost 50% and 39% of residue mass, respectively. Furthermore, ST treatments with *L. rubellus* significantly increased the percent recovery of maize residue N within the bulk soil as determined by total soil ^{15}N ($P = 0.009$) (results not shown). Thirty-three percent of maize residue N was recovered from the bulk soil in *L. rubellus* treatments, compared to 17% and 18% in the ST and ST_C treatments, respectively. The SM treatment averaged 37% residue N recovery, which was significantly higher than ST treatments without individuals of *L. rubellus* ($P < 0.05$).

Earthworm mortality by the end of the experiment was greater for *L. rubellus* than for *A. caliginosa*. Mean percent mortalities were 56% and 11% (results not shown). Many surviving *A. caliginosa* were found in an inactive diapause state, which occurs during environmental stress or scarcity. After 90 days, *L. rubellus* was approximately 7 times more enriched in ^{15}N than *A. caliginosa* (see Table S1 in the supplemental material), and on day 90 *A. caliginosa* was significantly more enriched in the percent residue derived N when cohabitating with *L. rubellus* (see Table S1).

N_2O and CO_2 emissions. The highest N_2O emissions for all treatments were observed on the first day (18 h) following maize residue application (Fig. 1). Among ST treatments, there were no significant earthworm species effects on daily N_2O emissions before the first destructive sampling on day 6. However, on measuring days 8 though 16 (data not shown) and on day 18 (Tables 1 and 2), both *L. rubellus* and *A. caliginosa* significantly increased daily N_2O emissions. From day 21 through the final sampling day, daily N_2O emissions were generally increased by the presence of *L. rubellus* alone (Fig. 1; Tables 1 and 2).

TABLE 1. Daily N_2O and CO_2 emissions

Treatment	Avg (SE) level[a] on:					
	Day 5		Day 18		Day 89	
	N_2O	CO_2	N_2O	CO_2	N_2O	CO_2
S	0.067 (0.022) A	121 (32) A	0.015 (0.005) A	75 (16) A	0.017 (0.001) A	69 (16) A
SM	0.063 (0.033) A	742 (17) C	0.010 (0.005) A	504 (15) C	0.024 (0.005) A	374 (18) C
ST	0.077 (0.022) A	533 (26) B	0.029 (0.002) A	332 (16) B	0.022 (0.008) A	270 (20) B
ST_R	0.106 (0.036) A	582 (26) B	0.059 (0.012) B	332 (17) B	0.071 (0.016) B	302 (20) B
ST_C	0.059 (0.015) A	568 (39) B	0.055 (0.012) B	303 (13) B	0.017 (0.004) A	292 (6) B
ST_{RC}	0.099 (0.017) A	597 (26) B	0.101 (0.012) C	339 (21) B	0.050 (0.011) B	282 (8) B

[a] N_2O units are reported as N_2O-N hour^{-1} kg of soil^{-1}; CO_2 levels are reported as μg CO_2-C hour^{-1} kg of soil^{-1}. Significant differences within sampling day are represented by different uppercase letters.

Cumulative N_2O emissions on day 89 were highest in the ST_{RC} and SM treatments and were significantly enhanced by the presence of *L. rubellus* (Fig. 1). Interactions between the two earthworm species did not impact daily or cumulative N_2O emissions on any sampling day (Table 2; Fig. 1).

On day 6 there were no significant earthworm effects on the percentage of N_2O derived from maize residue as determined by ^{15}N-N_2O, but SM had significantly higher percent residue-derived N_2O on this day (Fig. 2). The percentage of residue-derived N_2O was enhanced by the presence of *L. rubellus* and *A. caliginosa* on day 23 ($P = 0.000$ and $P = 0.002$, respectively) and by *L. rubellus* alone on day 89 ($P = 0.001$).

While the SM treatment was consistently higher than ST treatments in daily CO_2 emissions (Table 1), there were no significant earthworm effects among ST treatments on any sampling day (Table 2). The same pattern was followed by cumulative CO_2 emissions.

DEA and quantification of 16S rRNA, *nirS*, and *nosZ* genes. Among ST treatments, mesocosms containing *L. rubellus* significantly increased DEA on days 21 and 90 by 15% and 40%, respectively (Fig. 3). Denitrification enzyme activity (DEA) was highest in the SM treatment group on all sampling days (Fig. 3).

According to linear regression of fluorescence data, 16S rRNA gene and denitrifier genes *nirS* and *nosZ* exhibited mean reaction efficiencies of 93%, 81%, and 92%, respectively. Additionally, dilution curves of sample DNA ensured that the reaction efficiency for each amplicon was constant over a 1,000-fold range of concentrations and did not deviate as a result of humic acid inhibition (data not shown). All significant results from the qPCR assay are presented in Fig. 4. There were no significant differences in total 16S rRNA copy numbers between the six treatments on either sampling day. Furthermore, there were no earthworm effects upon 16S rRNA gene density within the bulk soil among ST treatments.

On day 90, there was a significant treatment effect on *nosZ* density (ANOVA $P = 0.049$) (Fig. 4A). On this day treatments SM and ST_R were significantly higher than the S treatment in *nosZ* density, by 77% and 100%, respectively (Fig. 4A). On days 21 and 90 we observed no significant treatment or earthworm effects upon *nosZ* relative abundance.

While the total density of *nirS* was not significantly altered on either day, on day 21 the relative abundance of *nirS* with respect to 16S rRNA gene abundance was significantly lower in the SM, ST_R, and ST_{RC} treatment groups than the S and ST treatment groups (Fig. 4B). Moreover, among ST treatments, the *nirS* gene relative abundance was significantly reduced in the presence of *L. rubellus* (Fig. 4B). There were no significant differences in *nirS* gene density, nor in the percentage of 16S rRNA gene, on day 90.

Soil N and C pools. The NO_3^- plus NO_2^- pool generally increased throughout the experiment in all 6 treatments, averaging 7.7 mg N kg of soil^{-1} on day 6 and rising to 17.6 mg N kg of soil^{-1} on day 90 (see Table S2 in the supplemental material). In contrast, the NH_4^+ pool exhibited no general trends but remained within 1 mg N kg of soil^{-1} throughout the 90 days. On day 6 following residue amendment, there were no significant earthworm effects on either NH_4^+ or NO_3^- plus NO_2^-. Compared to all other treatments, the SM treatment exhibited the lowest NH_4^+ concentrations on days 6 and 21 and the lowest NO_3^- plus NO_2^- concentrations on all sampling days (see Table S2).

Among ST treatments on day 21, NO_3^- plus NO_2^- levels were significantly enhanced by *A. caliginosa* (26%), and pres-

TABLE 2. ANOVA results for daily N_2O and CO_2 emissions[a]

Factor	ANOVA result (P value)[b] on:					
	Day 5		Day 18		Day 89	
	N_2O	CO_2	N_2O	CO_2	N_2O	CO_2
L. rubellus	0.218	0.151	0.002**	0.194	0.007**	0.417
A. caliginosa	0.658	0.323	0.005**	0.448	0.314	0.961
L. rubellus-*A. caliginosa* interaction	0.817	0.704	0.449	0.276	0.520	0.242
Block	0.857	0.071	0.153	0.208	0.892	0.563

[a] N_2O units are reported as N_2O-N hour^{-1} kg of soil^{-1}; CO_2 levels are reported as μg CO_2-C hour^{-1} kg of soil^{-1}.
[b] **, $P < 0.01$.

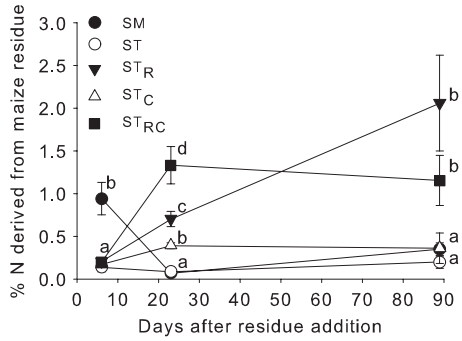

FIG. 2. Percentage of N_2O-N derived from maize residue on days 6, 23, and 89 of the experiment. Significant differences between treatments within each sampling day are indicated with different lowercase letters. Error bars represent the distance of 1 standard error.

ence of both earthworm species significantly increased NH_4^+ levels by 200 to 250% (see Table S2 in the supplemental material). On day 90, the presence of *L. rubellus* accounted for a 320% increase in NH_4^+ and an 18% increase in NO_3^- plus NO_2^-; the latter pool was also increased by 68% in the presence of *A. caliginosa* (see Table S2).

Mineralizable N was negatively affected by the presence of *A. caliginosa* on day 6, particularly when the species was combined with *L. rubellus*, as indicated by a significant species interaction effect on this day (see Table S2 in the supplemental material). While there were no significant earthworm effects on mineralizable N on day 21, this N fraction was significantly enhanced in the presence of *L. rubellus* on day 90.

The ^{15}N content of the combined mineral N pool increased throughout the experiment for all ST treatments. Over all sampling days, the presence of both *L. rubellus* and *A. caliginosa* significantly enhanced the percent enrichment of ^{15}N in the mineral N fraction (see Table S2 in the supplemental material). The percentage of mineral N derived from the maize residue was enhanced by a positive species interaction effect on day 21 and significantly reduced in the presence of both species on day 90.

MBN was significantly higher in the SM treatment group

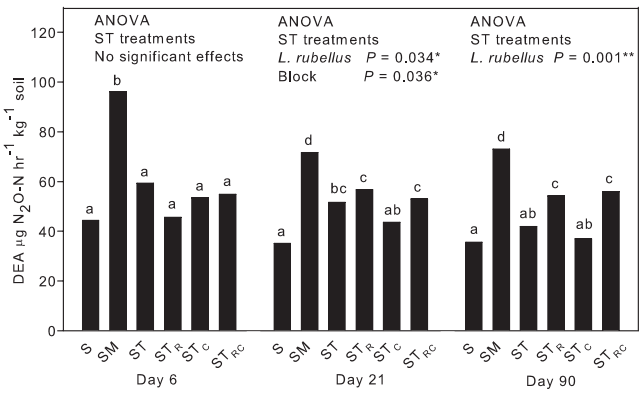

FIG. 3. Denitrification enzyme assay results on days 6, 21, and 90 of the experiment. Significant differences between treatment groups within each sampling day are indicated with a different letter. Significant two-way ANOVA effects are listed above the corresponding sampling day: *, $P < 0.05$; ***, $P < 0.01$.

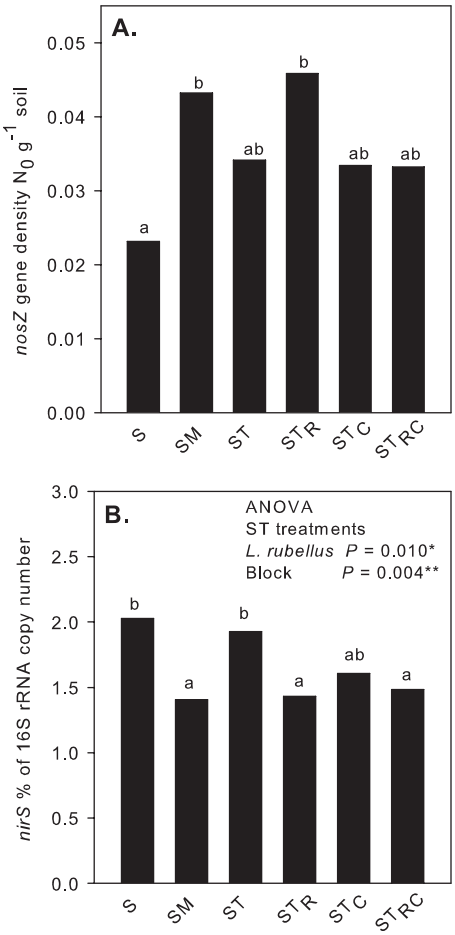

FIG. 4. Summary of significant results for qPCR analysis, including the measure of *nosZ* gene density on day 90 (A) and relative abundance of *nirS* with respect to 16S rRNA on day 21 (B). Note the different *y* axes. Significant differences between treatments are represented by different lowercase letters. Furthermore, significant differences from the two-way ANOVA on ST treatments are depicted above panel B: *, $P < 0.05$; **, $P < 0.01$.

than all other treatments on all sampling days, reaching a maximum of 16.9 mg N kg of soil^{-1} on day 21 (see Table S3 in the supplemental material). The microbial biomass of the SM treatment group also contained the most ^{15}N from the maize residue (see Table S3). Among ST treatments, there were no earthworm effects on MBN on day 6. However, on days 21 and 90 the presence of *A. caliginosa* coincided with significantly lower MBN, by 28% and 68%, respectively (see Table S3). Furthermore, the presence of *A. caliginosa* reduced the amount of ^{15}N within the microbial biomass on days 6 and 21.

Throughout the experiment, HWC content ranged in all treatment groups from 490 to 673 mg C kg of soil^{-1} (data not shown). There were no significant earthworm effects on HWC pools on any sampling day, and no earthworm treatments differed significantly from the ST treatment. On the other hand, the SM treatment HWC was significantly higher than all other treatments on days 6 and 89 and higher than only the ST_C and ST_{RC} treatments on day 21 (data not shown).

DISCUSSION

N_2O and CO_2 emissions. In agreement with other studies (21, 37), earthworms significantly enhanced N_2O emissions after application of crop residue. The effect appeared to be transient and proportionately small in the presence of the endogeic species *A. caliginosa*, only occurring within the second week of the experiment and not significantly contributing to cumulative N_2O emissions after 90 days. In contrast, the epigeic species *L. rubellus* exhibited a persistent effect on N_2O emissions, significantly contributing to cumulative N_2O emissions (Fig. 1). The observed 76% increase of N_2O caused by *L. rubellus* was consistent with other studies (21, 37). Regardless of the species, enhanced N_2O emissions appeared to be the result of earthworm-mediated decomposition of freshly applied organic matter rather than general earthworm activity, given that a significant fraction of earthworm-enhanced N_2O emissions was consistently derived from the crop residue (Fig. 2; see also Giannopoulos et al. [21]). We view these observations as a primary distinction between these studies and others that did not include a fresh organic matter source and did not see significant earthworm effects on N_2O emissions (11, 43).

CO_2 emissions are a general measure of microbial activity, which we assumed would increase in the presence of earthworms. There were no significant earthworm effects on daily or cumulative CO_2 emissions despite evidence of increased decomposition of maize residue by *L. rubellus*. This unanticipated result has been observed in a similar mesocosm study (2) and suggests that the contribution of earthworms to overall respiration is negligible compared to that induced by maize residue addition itself.

DEA and changes in the denitrifier population. In agreement with the first hypothesis and the findings reported by Burtelow et al. (7), *L. rubellus* activity significantly increased DEA within the entire bulk soil. We attribute this to priming of denitrifiers due to incorporation of fresh organic matter into the soil by *L. rubellus* (16, 33). The presence of *A. caliginosa* had no added effect on bulk soil DEA despite its contribution to N_2O emissions on day 21. The absence of a DEA effect has also been observed in the casts of the endogeic species *Pontoscolex corethrurus* (11). These observations suggest that although *A. caliginosa* increases the activity of denitrifiers and N_2O emissions through gut passage (24, 25), this effect may be negligible when considering the entire soil profile. The lack of denitrification activity induced by *A. caliginosa* may have been due to its ecological feeding strategy. *A. caliginosa* did not incorporate any significant maize residue and may have been in active competition with soil microorganisms for organic matter (42).

The SM treatment exhibited the highest DEA on every sampling day but no significant N_2O emissions on any of these days (Fig. 3; Table 2). This suggests that the SM treatment contained an actively denitrifying microbial population throughout the experiment, but the population effectively reduced all measurable N_2O into N_2 gas. The relative increase in nitrous oxide-reducing bacteria over 90 days (i.e., *nosZ* gene density) in this treatment group further corroborates this possibility. In contrast to SM, the DEA of ST treatment groups was more strongly linked to N_2O emissions, as indicated by a significant, positive correlation between DEA and daily N_2O emissions on the final sampling day ($P = 0.004$; $R = 0.67$).

Contrary to expectations, there were no clear linkages between earthworm priming of soil denitrifier activity and dynamics of bacterial denitrifier populations. Bacterial growth was limited under the conditions of this experiment, as 16S rRNA did not significantly rise above the S control treatment group after addition of residue, even when it was manually incorporated. The lack of growth is likely a result of the high C:N ratio of the maize residue, as evidenced by the rapid immobilization of N in the SM treatment group. A low bacterial growth response has also been observed when incorporating residues with lower C:N, such as red clover (31).

Despite the relatively limited bacterial growth, the ST_R and SM treatments resulted in a relatively higher *nosZ* gene density than the S treatment on day 90 (Fig. 4A), indicating a selective enhancement of denitrifiers containing the *nosZ* gene. Notably, the primer pair used for *nosZ* was reported to be selective for Gram-negative bacteria, in particular *Alpha-*, *Beta-*, and *Gammaproteobacteria* (23), suggesting that besides a functional shift, a compositional shift in the denitrifier population may have occurred in these treatment groups. Both treatments have the incorporation of residue N in common, implying that denitrifiers with *nosZ* responded positively to maize addition, whether manually incorporated or incorporated by *L. rubellus*. Indeed, other studies have also implicated crop addition with increased *nosZ* copy numbers in the bulk soil (22, 31). However, there is no significant overall *L. rubellus* effect, as there was a relatively low *nosZ* gene density in the ST_{RC} treatment group (Fig. 4A). While purely speculative, it is possible that the presence of *A. caliginosa* inhibited populations of denitrifiers in this treatment group due to its notable turnover of bacterial biomass N.

On day 21 we observed a significant decrease in the relative abundance of *nirS* with respect to 16S rRNA gene abundance in the presence *L. rubellus*, as well as the SM treatment (Fig. 4A). The *nirS*-containing bacteria represent a subset of the entire bacterial denitrifier population, as it is one of the two naturally occurring nitrite reductase genes. The other nitrite reductase gene, *nirK*, was not analyzed in this study. Regardless, Throbäck et al. (44) reported that this particular *nirS* primer pair appears to amplify a representative cross-section of the denitrifier population.

To our knowledge there is no literature to date demonstrating that either the incorporation of crop residue by hand or that earthworm activity has a negative effect on *nirS*-containing bacteria, although these bacterial populations do appear to respond with respect to agricultural practice (32). We can only speculate that the differences in *nirS* and *nosZ* gene dynamics suggest a shift in the denitrifier population as a result of *L. rubellus* activity. There is evidence of selective activation of certain groups of denitrifiers within the earthworm gut (14) and burrows and casts (19). A study by Cavigelli and Robertson (9) elegantly illustrated that N_2O:N_2 ratios can be affected by bacterial denitrifier community composition alone. It was unclear in our study whether a change in denitrifier community composition affected N_2O emissions. More research concerning N_2O:N_2 ratios is necessary in order to determine whether this is an important process in earthworm-mediated N_2O emissions.

Contrary to our expectations, there were no significant correlations between N_2O emissions and actual denitrifier populations in soils modified by earthworms. Although it appears that residue incorporation and activity of *L. rubellus* may change the composition and size of denitrifier populations, we suggest that DEA is a more accurate indicator of increased N_2O emissions from soils modified by this species.

Earthworm effects on substrates for denitrification. The very low mineral N content in the SM treatment group throughout the experiment (see Table S2 in the supplemental material), coinciding with significantly higher microbial biomass N (see Table S3 in the supplemental material), suggests that the high C:N ratio of the applied maize residue led to N immobilization by soil microbes. Both earthworm species appeared to prevent N immobilization, significantly increasing mineral N pool in the bulk soil on days 21 and 90 but preventing it from accumulating in microbial biomass (see Table S3). Consistent with other studies (6, 29, 35), *L. rubellus* activity resulted in larger NH_4^+ pools on days 21 and 90, indicating enhanced mineralization rates. Nitrogen mineralization by *L. rubellus* likely resulted from incorporation of maize residue into the soil, as evidenced by an increased pool of mineralizable N on day 89 in treatments with *L. rubellus* (see Table S2).

While there was no apparent maize residue incorporation by *A. caliginosa*, its activity resulted in mineral N and mineral ^{15}N levels greater than or equal to those of *L. rubellus*. A positive *A. caliginosa* effect on NH_4^+ concentrations on day 21 suggested that mineralization rates were also increased by this species. The source of mineral N from *A. caliginosa* did not appear to come directly from fresh maize residue but rather indirectly via turnover of microbial biomass N. Indeed, endogeic earthworms have been known to decrease microbial biomass (35, 42) and may compete with soil microorganisms for N and C substrates (17, 45).

The accumulation and relatively high concentration of the mineral N pool strongly suggest that denitrification rates were not limited by the abundance of NO_3^- and NO_2^- electron acceptors. On the other hand, a substantial NO_3^- pool could have contributed to a higher N_2O:N_2 ratio during denitrification, because denitrifiers preferentially reduce NO_3^- over N_2O. Miller et al. (31) proposed that N_2O is effectively reduced only when the concentration of NO_3^- is lower than a threshold of 5 to 10 mg N kg^{-1}, which is significantly below our experimental concentrations.

In addition to mineral N, denitrifying activity is affected by access to labile carbon. The HWC pool represents a rough approximation of labile, organic C within the soil, and its carbohydrate content can range from 40 to 50% (20). We predicted that earthworms would increase availability of labile C to denitrifiers by incorporating maize residue and excreting mucus, which some species are estimated to expel into the soil at a rate of 6% of earthworm C $month^{-1}$ (41). Contrary to our expectations, HWC was not significantly altered by either earthworm species over the 90 days of the experiment. Although there were no clear earthworm effects on HWC, there was a positive correlation between DEA and HWC among ST, ST_R, ST_C, and ST_{RC} treatments across all time points ($P = 0.001$), suggesting that denitrification activity in these treatment groups may have been limited by access to labile C.

While it may have predicted denitrifier activity in this system, HWC may be too coarse a measure to detect the earthworm contribution to easily available C in the soil profile. The HWC pool should be further analyzed for energy-rich compounds commonly provided by earthworms (5, 16).

Implications for agricultural ecosystem management. We believe that this study highlights the importance for soil biological engineers in mediating greenhouse gas emissions from agricultural ecosystems. Earthworm priming of denitrifying bacteria has been well characterized within the gut, burrows, and casts of earthworms (16, 25, 33). However, it is yet unknown how this selective activation of denitrifiers may shift soil bacterial communities over time and, furthermore, how these shifts in bacterial functional groups may lead to changes in N_2O emissions from the soil ecosystem. To our knowledge, this is the first study directly linking earthworm activation of soil denitrifiers to an increase in N_2O emissions from an intact soil column. Although this study addressed denitrifier populations, nitrifier denitrification must also be considered a possible source of earthworm-mediated N_2O emissions and should be further investigated (27).

The sampling methods used here for characterizing denitrifier populations and N_2O flux from the soil surface are practical from an agricultural management standpoint, as they do not require discerning drilosphere soil from bulk soil and simply focus on the net greenhouse gas balance. We propose that long-term field-scale studies should be performed, where selective alterations of earthworm populations are measured alongside N_2O emissions, DEA, and denitrifier abundance and community structure. Earthworm populations of agricultural ecosystems may be actively managed, e.g., through different tillage techniques (10) and plant residue applications (3), which could foreseeably mediate earthworm-induced N_2O emissions on the farm scale.

In conclusion, we sought to understand how earthworm-denitrifier interactions may affect net N_2O emissions from an intact soil column by measuring earthworm effects on denitrifier community activity, size, and N and C substrates on the scale of the bulk soil. We hypothesized that (i) earthworms would cause N_2O emissions through activation of denitrifying populations in the soil and that (ii) the extent of earthworm-denitrifier interactions depended upon earthworm feeding strategy. In agreement with both of our hypotheses, the epigeic *L. rubellus* caused a significant increase in denitrification enzyme activity in the bulk soil, which coincided with a 76% increase in cumulative N_2O emissions over 90 days; endogeic *A. caliginosa* presence caused a transient increase in N_2O but made no significant impact on denitrifier activity or cumulative N_2O emissions. Contrary to expectations, denitrifier bacterial population size was not correlated with the increased activity or N_2O emissions. However, this study provides evidence of a significant alteration in the denitrifying bacteria community as a result of *L. rubellus* activity. Due to the prevalence of a nonlimiting pool of mineral N throughout the experiment and the correlations between DEA and HWC, we attribute earthworm priming effects to providing soil denitrifiers with access to an uncharacterized labile carbon source.

ACKNOWLEDGMENTS

This study was supported by a personal VIDI grant from the Netherlands Organization for Scientific Research/Earth and Life Sciences

(NWO-ALW) to Jan Willem van Groenigen. Additional funding was supplied by the Dutch Ministry of Agriculture, Nature Conservation and Food Quality (project numbers KB-02-001-068, BO-01-002, and KB-01).

We thank An Vos, Eduard Hummelink, Meint Veninga, Eef Velthorst, and Jaap Nelemans for practical assistance. Finally, we thank Julia Rieckmann for help with qPCR and for her comments on a previous version of the manuscript.

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