Earthworm - denitrifier interactions determine increased nitrous oxide emissions from soil mesocosms amended with crop residue.

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ABSTRACT

Earthworm activity is known to increase emissions of nitrous oxide (N_2O) from arable soils. Earthworm casts and burrows have exhibited higher denitrification activity, implicating priming of denitrifying organisms as a possible mechanism for this effect. Furthermore, earthworm feeding strategy may be a driving factor, as it determines access to fresh organic carbon 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 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 qPCR on days 9, 21 and 90.

L. rubellus increased denitrification enzyme activity and N_2O emissions on the last two dates (day 21: P=0.034, P=0.002; day 90: P=0.001, P=0.007), as well as overall cumulative N_2O emissions (76%; P=0.014). *A. caliginosa* significantly increased N_2O emissions on day 21 (117%; P=0.005) and decreased microbial biomass (-28%; P=0.004). Total *nosZ* abundance was significantly increased (100%; P<0.05) on day 90 in the treatment containing *L. rubellus* alone.

We conclude that *L. rubellus* increased N_2O emissions by affecting denitrifier community activity via incorporation of fresh residue into the soil; the *A. caliginosa* effect appeared to be linked to an increase in general microbial turnover.

INTRODUCTION

Denitrification is a microbial process in which organisms reduce inorganic nitrogenous oxides to nitrous oxide (N_2O) or nitrogen (N_2) gas. Denitrifying organisms comprise up to 5% of the total soil microbial community, and their activity represents an important component of nitrogen (N) losses from soils (31). Furthermore, 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 emission (31). In order to mitigate this trend, considerable research has gone into understanding the environmental drivers that stimulate the denitrifiers to decrease the N_2O:N_2 ratio of emissions from agricultural soils.

Many physical and chemical soil conditions have been linked to increases in N_2O emissions from denitrifiers, including bulk density, water content, pH, and availability of mineral nitrogen (N) and organic carbon (C) (IPCC, 2007). Common biological processes within the soil may indirectly enhance N_2O emissions through altering one or more of these soil parameters. Earthworm activity has been implicated in higher rates of denitrification and N_2O emission rates, both *in vivo* and from soils affected by earthworms (7), (11), (15), (19), (27), (34). Agricultural management has direct consequence for earthworm population activity, -composition, and -size, which can reach several million individuals per hectare. Therefore it is important to understand the driving factors of earthworm enhanced N_2O emissions from agricultural ecosystems.

Earthworms are considered to be ecosystem engineers due to their ability to affect soil structure and organic matter dynamics. Earthworms can facilitate the movement of air and water through the soil by creating macropores and large soil aggregates. Furthermore, earthworms have been known to occlude organic matter into stable microaggregates within macroaggregates, causing long-term storage of soil C (4). So prominent are the earthworm effects on biochemical soil properties, that portions of soil under earthworm influence are classified in a distinct functional domain called the drilosphere, which includes earthworm gut, skin surface, burrow walls, and casts (5).

The influence of earthworms on soil properties depends on their ecological function; (i) *epigeic* earthworms primarily reside in the soil-litter interface, pulling down and ingesting fresh organic matter; (ii) *endogeic* earthworms inhabit deeper soil layers, feeding on lower quality organic matter and soil particles, creating small,

transient burrows; and (iii) *anecic* earthworms create large, permanent vertical burrows, as a result of transporting fresh litter deep into the soil profile. It has been shown that earthworms enhance N_2O emissions by increasing turnover of added nitrogen sources (34), (27), (19). Also, interactions between earthworm functional groups can affect N_2O emissions. Giannopoulos *et al.* (19) found a positive interaction between epigeic and endogeic species resulting in enhanced N_2O emissions.

The mechanism of earthworm mediated N_2O emission is not clearly understood, but is likely to involve interactions with soil denitrifiers within the drilosphere. The process of denitrification requires anaerobic conditions in combination with the availability of nitrate (NO_3^-) (or nitrite (NO_2^-) and electron rich C which are precisely the conditions found in the earthworm gut (22), (15) (23). Indeed increased N_2O emissions have been measured within the earthworm gut, (22), which coincides with an observed 300-fold increase in culturable bacterial denitrifiers in the gut compared to the bulk soil (23). Furthermore, in contrast with the bulk soil, fresh earthworm casts exhibit a higher microbial biomass, microbial activity, and mineral N content, (36), (1), and earthworm burrows contain higher nitrification and denitrification activity due to earthworm excretion of nutrient rich mucus (30). Together, these processes are known as the earthworm priming effect (5).

Despite evidence for an earthworm-induced priming effect of denitrifying organisms in the drilosphere, little is known about the effect of earthworms on the size and composition of denitrifying populations within the entire soil system. Scaling up to bulk soil would help inform earthworm induced N_2O emission from soils on the ecosystem scale. Thanks to advances in molecular techniques such as qPCR, it is possible to quantify different functional groups of soil bacteria. Several PCR primers that are homologous with bacterial genes involved in denitrification have effectively been utilized to assess changes in dentrifying bacterial populations (41), (21); (24). 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 (28). Selective enhancement of the activity and quantity of denitrifiers by earthworms may help explain the mechanisms behind increased soil N_2O emission as a result of earthworm activity.

A time-course mesocosm study was conducted in which earthworm-induced changes in soil chemical and biological properties could be assessed alongside measurements of soil N_2O emissions. We utilized an epigeic (*L. rubellus*) and an

endogeic earthworm species (*A. caliginosa*) to examine the effects of earthworm ecological strategy on soil parameters and 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, in a departure from studies assessing biochemical activity in earthworm gut, casts and burrows, we sought to examine chemical and biological alterations by earthworms on the scale of the entire bulk soil. We hypothesized that (i) earthworm activity would result in higher N_2O emissions from the soil through stimulation of soil denitrifiers by (ii) contributing higher pools of mineral N and labile C in the bulk soil. (iii) These observations would coincide with increased DEA and higher populations of denitrifying bacteria. Furthermore, (iv) we expected that these effects will be most pronounced in epigeic species *L. rubellus* due to its access to fresh organic matter on the soil surface. Finally, (v) interactive effects between both epigeic and endogeic earthworm species would further augment these earthworm effects.

MATERIALS AND METHODS

Experimental setup. We set up a climate-controlled mesocosm experiment to accurately quantify N_2O and CO_2 emission 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 in a randomized block pattern, consisting of 4 blocks that were reconfigured 3 times over the course of the experiment.

Treatments included a pure soil control (S) without addition of residue or earthworms. In the remaining treatments ^{15}N labeled *Zea mays* L. residue was either incorporated into the soil manually (SM), or applied on top (ST). Individuals of the epigeic *L. rubellus* and the endogeic *A. caliginosa* 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, ST_{RC}). Adults and large juveniles of each earthworm species were applied in 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 (14). Mesocosms were 6.1L polyethylene buckets containing 4 kg of loamy sand which was maintained at 17% gravimetric moisture content. The climate room was maintained at 16˚C and 60% humidity. Previous experiments established that these conditions are optimal for

earthworm activity and survival. All mesocosms were covered with a black, air-permeable polyethylene cloth fixed with a rubber band in order to prevent earthworms from escaping.

Sandy topsoil was obtained from the experimental farm "Droevendaal" in Wageningen (51˚59' N, 5˚39'E), Netherlands with 75% sand, 23% silt, 2% clay, 1.3g N kg^{-1}, 14.2 g C kg^{-1}. Soil was air-dried and sieved at 8mm before it was added to the mesocosm. Prior to maize residue and earthworm application, the freshly sieved soil was incubated one week, and determined to have a pH-water of 5.6, hot water extractable org. C content of 575 μg C kg^{-1} and mineral N content (extraction with 1 M KCl) of 8.93 μg N kg^{-1}. The ^{15}N enriched *Zea mayz* L. had been 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%).

Earthworm species *L. rubellus* and *A. caliginosa* were collected 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 (12). Earthworms were then weighed directly before applying to the mesocosms. Wet weights of *L. rubellus* and *A. caliginosa* averaged 0.8 g and 0.6 g per worm respectively.

N_2O and CO_2 flux measurement. 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. On measuring days we measured N_2O and CO_2 using a static closed chamber technique and an Innova 1412 photacoustic infrared gas analyzer and two Teflon tubes as described by Kool et al. (26). Polypropylene lids equipped with two rubber septa were fixed to the mesocosms for 30 and 50 minutes to measure CO_2 and N_2O respectively. A soda-lime filter was used during N_2O measurements in order to minimize interference effects of CO_2 and water vapor (43). Cumulative emissions were calculated assuming a linear change in rate between sampling days. Gas samples for ^{15}N-N_2O concentrations were taken on days 2, 6, 23, and 89, and sent to the UC Davis Stable Isotope Facility for analysis using a PDZ Europa 20-20 isotope ratio mass spectrometer.

Earthworm and residue recovery. On all destructive sampling days, mesocosms were overturned and earthworms were recovered by hand. Earthworms were placed on wet filter paper for 48 hours to void gut contents by the filter paper method (12) and weighed on a basis of species biomass per mesocosm. Earthworms were subsequently freeze-dried, and ball-milled for ^{15}N analysis.

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, and weighed to determine dry weight recovery. The soil was sieved over 4 mm, and all subsamples were taken from a homogeneous fraction and stored at 2˚C until further use. A representative subsample of bulk soil was taken for ^{15}N analysis.

Sieved soil samples and earthworms were oven dried at 105˚C, quantitatively weighed into tin (Sn) capsules, and sent to UC Davis Stable Isotope Facility for determination of total ^{15}N content.

Analysis of soil chemical properties and microbial biomass. All chemical analysis were performed within 48 hours of sampling. Mineral N was extracted using 1M KCl (26). Mineralizable N was determined anaerobically by incubating 16 g of soil in 40 ml distilled water for 7 days at 40˚C (2). Both mineral and mineralizable N fractions were analyzed colorimetrically for NH_4^+ and NO_3^- content. Mineralizable N was quantified as the increased NH_4^+ concentration after 7 days anaerobic incubation.

Microbial biomass nitrogen was determined by fumigation followed by K_2SO_4 extraction (2), where total soluble nitrogen was measured colorimetrically. The labile organic pool of soil carbon was approximated using the hot water extractable C (HWC) method (18). A 3 g soil sample was combined with 30 ml of water at 80˚C for 16 hours to remove the labile pool of carbon. The extracted fraction was analyzed was measured using an SFA (Skalar SK^{12}) by persulfate and tetraborate oxidation under UV light and infrared detection SK^{12} TOC/DOC analyzer. HWC was defined as the hot water extractable organic C, as determined by subtracting inorganic C from total C.

Analysis of ^{15}N content was performed on remaining extractant from the KCl extraction and fumigation extraction. Fumigation extracts were first processed with Kjeldahl digestion in order to chemically mineralize organic nitrogen. Total mineral N was collected from mineral N and Kjeldahl digested fumigation extract samples using the filter paper disk diffusion method (40), and sent to UC Davis Stable Isotope

Facility for determination of ^{15}N. The mineral N extraction from the SM treatment had insufficient mineral N content for accurate ^{15}N analysis, and was discarded.

Direct bacterial and fungal counts. On each destructive sampling day a 20 g representative subsample was taken and processed for microscopic determination of bacteria and fungi as described by de Vries et al. (13). Hyphal lengths of fungi were calculated using the grid intersection method (13). Fungal biomass C was calculated assuming a mean hyphal diameter of 2.5 μm and a specific carbon content of $1.3x10^{-13}$ g C μm^{-3}. Bacterial numbers and cell volumes were measured automatically with a confocal laser-scanning microscope (Leica TCS SP2), combined with Leica Qwin pro image analysis software. Bacterial volumes were converted to biomass C assuming a density of $3.2x10^{-13}$ g C μm^{-3}.

Denitrification Enzyme Activity. A denitrification enzyme activity (DEA) assay was adapted from Luo et al. (28) in order to determine the relative activity of denitrification enzymes upon destructive sampling. Within 24 hours of sampling, 40 g of fresh soil was placed in an air-tight jar of 1.0 L with a lid containing rubber septa. The jars were flushed of ambient air with N_2 and injected with a 40 ml de-gassed solution containing 10 mM KNO_3 and 10 mM glucose in order to provide non-limiting amounts of nitrate and a high-energy carbon source. Finally, all jars were injected with C_2H_2 at approximately 5% (v/v) in order to inhibit the final enzymatic reduction of N_2O to N_2. The jars were placed on a large shaker at a speed of 124 rpm at room temperature (~20˚C) and N_2O emission was measured at 2 and 5 hours with a Innova 1312 photoacoustic gas monitor equipped with an external soda-lime filter to limit CO_2 and water vapor intereference. The rate of N_2O accumulation from 0-5 hours was used as a measure of pre-existing 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 up-regulation of enzymes and bacterial growth (28).

Quantitative PCR assay. On sampling days 21 and 90, a homogeneous subsample of bulk soil was stored at -80˚C up to 6 months before extraction with FastDNA Spin Kit for Soil (qBIOgene, Montreal, Canada). DNA was washed 2 times

using Wizard SV Genomic Purification Kit (Promega) to remove excessive co-extracted humic acids and stored at -20˚C until further use. DNA concentrations and purity were checked using a Thermo Scientific Nanodrop Spectrophotometer. Purified DNA was amplified using primers homologous to DNA sequences that coded for 16S rRNA (17) *nirS* (24), and *nosZ* (nosZ2 primer pair, 21). The genes were quantified using an ABI Prism 7600 thermal cycler (Applied Biosystems, Streetsville, ON, Canada) with SYBR green detection system. Each 25 µl reaction contained 5 ng extracted DNA, 12.5 ml SYBR green PCR master mix (Invitrogen), 40 ng of $T4_{gp}32$, 1.25 µl BSA, and 0.5 µM primer. Data was obtained using ABI 7000 System Detection Software version 1.2.3. Raw delta Rn values were analyzed using LinReg PCR software (33), in order to correct for baseline variation and estimate PCR amplification efficiencies per reaction. All PCR reactions were statistically tested to ensure there were no significant treatment effects on amplification efficiency, after which a common amplification efficiency was assumed for each amplicon per time step, as estimated by LinReg (35). Purity of amplified products was confirmed by identifying distinct bands of the correct size via agarose gel electroporesis.

Rather than using standard DNA for absolute quantification, relative gene copy numbers of 16S rRNA *nirS*, and *nosZ* were represented as arbitrary fluorescence units (AFU), which were analyzed per gram of soil. In addition, *nirS* and *nosZ* were calculated as percentages of 16S rRNA, by comparing the AFU's per ng of extracted DNA and correcting for relative size of the amplified products.

Statistical analysis. Statistical tests were conducted using SPSS version 15.0.1 software. Two distinct ANOVA tests were performed for data on each sampling day. First, we assessed significant differences in treatment means using ANOVA and post-hoc least significant differences (LSD) between means test at 95% confidence. Secondly, effects of earthworm species were assessed only in treatments receiving residue on top via full factorial 2-way ANOVA, in which the factors were defined as presence or absence of *L. rubellus* or *A. caligonosa*. Thus the S and SM treatments primarily served as a control and reference when comparing treatment effects. 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 emissions directly before each destructive sampling. However, due to destructive sampling, treatment replicates decreased from 12 to 8 to 4, complicating statistical analysis. For simplicity, statistical analysis on N_2O 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. The same statistical method was used for daily and cumulative CO_2 emissions.

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). Maize residue N recovery from the bulk soil of ST_R and ST_{RC} treatments averaged 25% and 41%, respectively, while ST and ST_C treatments averaged 17% and 18%, respectively. The SM treatment averaged 37% residue N recovery, which was significantly different from ST treatments without individuals of *L. rubellus* (P< 0.05).

Earthworm mortality by the end of the experiment was greater for *L. rubellus* than *A. caliginosa*. Mean percent mortalities were 56% and 11% respectively (results not shown), which accounts for much of the observed weight loss (Table 1). After 90 days, *L. rubellus* was approximately 7 times more enriched in residue N than in *A. caligonosa* (Table 1). There were no interactions between the species in regards to weight change or total earthworm ^{15}N recovered on any of the sampling days. However, on day 21 *L. rubellus* was significantly less enriched in % residue derived N in the presence of *A. caliginosa*, and on day 90 *A. caliginosa* was significantly more enriched in % residue derived N in the presence of *L. rubellus* (Table 1).

N_2O and CO_2 emissions The highest N_2O emissions for all treatments were observed on the first day (18 hrs) following maize residue application, particularly in the SM treatment, which had 516% higher emissions than the ST treatment, and averaged 4.5 μg N_2O-N h^{-1} kg^{-1} soil (data not shown, P < 0.05). 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, $p<0.05$) and 18 (Table 2), both *L. rubellus* and *A. caliginosa* significantly increased daily N_2O emissions; by the final sampling day, daily N_2O emissions were increased by the presence of *L. rubellus* alone (Table 2).

Cumulative N_2O emissions on day 89 were highest in the ST_{RC} and SM treatments, which were over twice that of the ST treatment (Fig. 1). On day 89, cumulative N_2O emissions were significantly enhanced by the presence of *L. rubellus* (Fig. 1). There were no significant earthworm effects on cumulative N_2O emissions by day 6, but by day 18, both earthworm species accounted for increased cumulative emissions (ANOVA, Fig. 1). There was no interaction effect between the two species on daily or cumulative emissions on any measuring day prior to destructive sampling (Table 2, Fig.1).

On day 2, the percentage of N_2O derived from maize residue as determined by ^{15}N-N_2O was highly variable, ranging from 0.0-9.3%, with no significant differences between treatments (data not shown), before subsiding by day 5 (Fig. 2). Following the same pattern of daily N_2O emissions, the percent 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 in daily CO_2 emissions, there were no significant earthworm effects among ST treatments (Table 2). Cumulative CO_2 emissions were highest in the SM treatment, which averaged 1,111 mg CO_2-C kg^{-1} soil. There were no significant differences in cumulative CO_2 emissions among the four ST treatments, which together averaged 882 mg CO_2-C kg^{-1} soil.

Earthworm and maize residue effects on soil N and C pools. On day 6 following residue amendment, the soil NO_3^- + NO_2^- pool was highest in the control treatment receiving no residue, and lowest within the SM treatment (Table 3). The ST treatments did not significantly differ with respect to earthworm presence. On day 6 there were no significant differences in the NH_4^+ pool (Table 3).

On day 21, within ST treatments, NO_3^- + NO_2^- levels were significantly enhanced by *A. caliginosa*, and each of the earthworm species significantly contributed to higher NH_4^+ levels in the soil (Table 3). While there were no significant species interaction effects on either of these pools separately, the combined mineral N pool

was positively affected by an interaction between earthworm species (data not shown, $P = 0.012$).

Among ST treatments on day 90, the presence of *L. rubellus* accounted for an increase in both NH_4^+ and $NO_3^- + NO_2^-$, and the latter pool was likewise increased in the presence of *A. caliginosa*. The highest concentration of NH_4^+ occurred in the ST_R treatment, while ST_{RC} had the highest concentration of $NO_3^- + NO_2^-$ (0.73 and 26.70 mg N kg^{-1} soil, respectively).

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 (Table 3). While there were no significant earthworm effects on mineralizable N on day 21, the N fraction was significantly enhanced in the presence of *L. rubellus* on day 90.

The ^{15}N content of the combined mineral pool increased throughout the experiment for all ST treatments. Over all sampling days, the presence of both *L. rubellus* and *A. caliginosa* significantly enhanced ^{15}N enrichment of the mineral N fraction, as well as total mineral ^{15}N (Table 3). The percent of mineral N derived from the maize residue was enhanced by a positive species interaction effect on day 21 and significantly redcued in the presence of both species on day 90. On the final sampling day, total mineral ^{15}N was enhanced due to a positive earthworm species interaction effect (Table 3).

Microbial biomass N (MBN) was significantly higher in the SM treatment than all other treatments on all sampling days, reaching a maximum of 16.86 mg N kg^{-1} soil on day 21 (Table 4). The microbial biomass of the SM treatment also contained the most residue derived N (Table 4). 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 (Table 4). The presence of *A. caliginosa* reduced the amount of ^{15}N within the microbial biomass on days 6 and 21, but a significant negative earthworm species interaction effect on these days indicated that this trend was reversed when *A. caliginosa* cohabitated with *L. rubellus*.

The hot water-extractable carbon (HWC) content ranged in all treatments during 90 days from 490 to 673 mg C kg^{-1} soil, reaching a mean maximum on day 21 ($p < 0.05$, 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 was higher than the ST treatment on

days 6 and 89, by 18% and 13%, respectively (data not shown). The ST treatment was not significantly higher than the S control until day 89; ST_C was the only treatment not significantly higher than S on the final day of the experiment.

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

Abundance of 16S rRNA and denitrifier genes *nirS* and *nosZ* per gram of bulk soil were quantified with on days 21 and 90 of the experiment. There were no significant differences in total 16S rRNA or *nirS* copy numbers between the six treatments on either sampling day. Furthermore, there were no earthworm effects upon 16S rRNA nor *nirS* abundance within the bulk soil among ST treatments. On day 90, there was a significant treatment effect on *nosZ* abundance ($P = 0.049$). On this day treatments SM and ST_R were significantly higher than the S treatment in *nosZ* abundance, by 77% and 100% respectively (Fig. 4B).

On days 21 and 90 the percent of *nirS* copy numbers with respect to 16S rRNA copy numbers ranged between 0.4 to 3.6%, while *nosZ* abundances ranged between 1.6 to 8.1% of 16S rRNA. On day 21, the proportion of *nirS* copy numbers were significantly lower in the SM, ST_R, and ST_{RC} treatments than the S and ST treatments (Fig. 4A). Moreover, among ST treatments, the percentage of *nirS* gene abundance was significantly reduced in the presence of *L. rubellus* (Fig. 4A). There were no significant changes in relative abundance of nirS on day 90. Furthermore, no significant treatment nor earthworm effects upon the relative abundance of *nosZ* were observed on day 21 or day 90.

Direct fungal and bacterial counts. On day 6, fungal biomass was reduced in the presence of *L. rubellus* and increased in the presence of *A. caliginosa* (Fig. 5). Furthermore, *L. rubellus* was associated with a 55% decrease in the F:B ratio on day 6 ($P = 0.021$). On day 21, fungal biomass of the SM treatment was 140% higher than the ST treatment, but there were no significant species effects among the ST treatments. No significant differences in fungal biomass were measured on the last sampling day. There were no significant differences in bacterial counts among treatments on any sampling day.

DISCUSSION

N_2O and CO_2 emissions. In agreement with our first hypothesis and other studies (34), 19), N_2O emissions were significantly enhanced by earthworms after application of crop residue. The effect appeared to be transient and proportionately small in the presence of endogeic species *A. caliginosa*, only occurring within the first 3 weeks of the experiment, and not significantly contributing to cumulative N_2O emissions after 90 days. In contrast, epigeic species *L. rubellus* exhibited an early and pronounced effect on N_2O emissions, significantly contributing to cumulative N_2O emissions (Figure 1). Regardless of the species, enhanced N_2O emissions appear to be the result of earthworm mediated decomposition of freshly applied organic matter rather than general earthworm activity, considering that significant fractions of earthworm enhanced N_2O emissions are derived from the crop residue (Figure 2, see also 19). The authors see this point as the primary distinction between these studies, and others that observed no significant earthworm effects on N_2O emission (10), (39)**.**

Compared to other experiments with different types of crop residue (e.g. (19) (34)), cumulative N_2O emissions were low. This is likely due to the high C:N ratio of the applied residue, making it relatively unpalatable for epigeic earthworms. Nevertheless, the percent increase of N_2O caused by *L. rubellus* was consistent with other studies (34), (19).

Contrary to hypothesis (v), there were no significant earthworm interaction effects on daily or cumulative N_2O emissions, although the ST_{RC} treatment was substantially higher in cumulative N_2O emissions by day 90 (Fig. 1). Positive earthworm interaction effects after 90 days were observed in a similar study involving sandy soil and radish residue (Giannopoulos unpublished data). This discrepancy is accredited to the higher relative contribution of *A. caliginosa* to N_2O emissions in this study, as well as the low resolving power of low N_2O emissions in combination with few (N=4) replicates.

There were no significant earthworm effects on daily or cumulative CO_2 emissions despite evidence of increased decomposition of residue by earthworms, as indicated by residue mass lost from the surface. This has been observed in a similar mesocosm study (4) and suggests that the contribution of earthworms to overall respiration was negligible compared to that induced by maize residue addition itself. Earthworms appear to prime anaerobic soil organisms in particular (15, 23), which may offer an

explanation for the observed discrepancy between residue mass loss and cumulative CO_2 emissions.

Soil N and C pools. It is clear that the high C:N ratio of the applied maize residue led to N immobilization by soil microbes, as evidenced by very low mineral N content in the SM treatment throughout the experiment (Table 3). Furthermore, on day 6 the ST treatment had significantly lower mineral N content than the no residue control. These observations coincide with significantly higher microbial biomass N, particularly in the SM treatment. In such a system, soil denitrifiers may be stimulated by earthworm induced mineralization.

In agreement with hypothesis (ii), both earthworm species significantly increased mineral N fractions in the bulk soil. Furthermore, based upon highly significant earthworm effects on ^{15}N enrichment of soil mineral fractions on all sampling days, it is clear that both species persistently recycled the added maize residue through the mineral N pool. Consistent with other studies (6), (32), (27), *L. rubellus* activity resulted in higher NH_4^+ fractions on days 21 and 90, indicating enhanced mineralization rates. Higher NH_4^+ concentrations on day 21 in the presence of *A. caliginosa* suggested mineralization rates were also increased by this species.

An average of 11% of maize residue was incorporated into the soil by *L. rubellus* over 90 days, while there was no apparent incorporation by *A. caliginosa*. Furthermore, earthworm ^{15}N recovery in earthworm tissue illustrated that the epigeic *L. rubellus* consumed higher amounts of fresh maize residue, while the endogeic *A. caliginosa* consumed a higher fraction of N derived from soil organic matter (Table 1). Thus, N mineralization by *L. rubellus* likely came as a result of its incorporation of residue into the soil. Consistent with these observations is an increased pool of mineralizable N on day 89 in the presence of *L. rubellus* (Table 3). Despite its incorporation of fresh crop residue, *L. rubellus* activity did not appear to result in more mineralized N than *A. caliginosa*, which is contrary to what we expected (hypothesis iv),

The source of mineral N from *A. caliginosa* did not appear to come directly from fresh maize residue, but rather from soil organic N (as indicated by mineralizable N) and microbial biomass N. Corroborating this assumption is the fact that linear regression performed on the ST_C treatment across all sampling days yielded significant negative correlations when mineral N was compared to microbial biomass

N (P = 0.005) as well as mineralizable N (and P = 0.029). Indeed, endogeic earthworms have been known to decrease microbial biomass (37), (32), and may compete with soil microorganisms for limited C and N pools (42), (16).

Both earthworm species significantly affected the cycling of residue N through microbial biomass. On day 6 the endogeic species enhanced microbial immobilization of residue N, possibly due to priming of soil microorganisms, after which it reduced the proportion of residue N within the microbial biomass on day 21 (Table 4). The *A. caliginosa* effect was augmented on day 6 and counteracted on day 21 when it cohabitated with *L. rubellus*, possibly due to increased turnover of maize residue egested by *L. rubellus*. Despite the incorporation of residue, microbial biomass ^{15}N was not increased in the presence of *L. rubellus*, which is a notable contrast to the immobilization of residue N that occurred following manual incorporation of residue (SM treatment).

Thus, as the experiment transpired, both *L. rubellus* and *A. caliginosa* appeared to simultaneously maintain high levels of mineral N and prevent it from accumulating in microbial N pools. This trend, combined with earthworm weight loss over 90 days (Table 1), suggests a possible competition for limiting nutrients (16). Species interaction effects may have resulted in higher turnover of residue N within the pools of mineral N and microbial biomass N, but did not significantly affect the overall size of these pools.

The HWC pool is a rough approximation of labile, organic C within the soil, and its carbohydrate content can range from 40-50% (18). It is clear that incorporation of residue (SM) resulted in substantially higher HWC fractions than top application (ST). However, in contrast with our expectations (hypothesis ii), HWC was not significantly altered by either earthworm species over the 90 days of the experiment. This occurred despite the fact that *L. rubellus* incorporated an average of 11% of residue into the soil, and that some endogeic species are known to excrete significant amounts of labile C into the soil in the form of mucus, at a rate of 6% of earthworm C month^{-1} (Scheu 1991). It is possible that the HWC pool may be an inadequate measure of the earthworm contribution to easily available C, as their energy-rich mucus may exert a disproportionately high influence on heterotrophic soil organisms (5), (15).

DEA and changes in the denitrifier population. In accordance with hypothesis (iii) *L. rubellus* activity significantly increased DEA within the bulk soil. This is in

agreement with a field study by Burtelow et al. (7) who found higher DEA in soil modified by an invasive epigeic earthworm species. The presence of *A. caliginosa* had no added effect on bulk soil DEA despite its contribution to N mineralization and to N_2O emissions. The absence of a DEA effect has also been observed in the casts of endogeic species *Pontoscolex corethrurus* (10). These observations suggest that although *A. caliginosa* increases the activity of denitrifiers through gut passage (22), (23), this effect may be negligible when scaling up to the bulk soil. The lack of denitrification activity may have been due to active competition for labile C substrates between the soil feeding earthworm and soil microorganisms (37). Furthermore, *A caliginosa* priming of fungi (Fig. 5) might have reduced the availability of necessary chemical substrate for bacterial denitrifiers (25)

Interestingly, the SM treatment exhibited the highest DEA on every sampling day, but no significant N_2O emissions on these days (Fig. 1). This suggests that the SM treatment contained an actively denitrifying microbial population throughout the experiment, but maintained an exceedingly low N_2O:N_2 ratio. In contrast to SM, the DEA of ST treatments appeared to be more coupled to N_2O emissions, as indicated by a significant positive correlation between DEA and daily N2O emissions on the final sampling day (P = 0.004, R = 0.67). We attribute the increase in DEA in association with *L. rubellus* to its incorporation of fresh organic matter into the soil, supplying a constant pool of mineral N, and activation of denitrifiers through mucosal excretions (15), (30).

Contrary to our expectations (iii), there were no clear linkages between earthworm priming of soil denitrifiers and bacterial denitrifier populations. It is clear that bacterial growth was limited under the conditions of this experiment. Neither bacterial biomass C from direct counts, nor 16S rRNA, rose above S treatment after addition of residue, even when it was manually incorporated. This is possibly result of rapid immobilization of N following application of high C:N maize residue, and has also been observed when incorporating residues with lower C:N such as red clover (29).

Despite the unchanged bacterial biomass, the ST_R and SM treatments resulted in a higher amount of *nosZ* gene copy numbers than the S control on day 90 (Fig. 4) suggesting a selective enhancement of this denitrifier population. Both treatments have in common the incorporation of residue N, indicating denitrifiers with *nosZ* responded positively to maize addition, whether manually incorporated or incorporated by *L. rubellus*. Other studies have also implicated crop addition with

increase *nosZ* copy numbers in the bulk soil (29), (20). However, there is no significant overall *L. rubellus* effect due to the notably low *nosZ* gene copy number in the ST_{RC} treatment. It is possible that the presence of *A. caliginosa* inhibited the growth of denitrifiers in this treatment due to its notable turnover of bacterial biomass. Without a priming effect, a high turnover of bacterial populations may select against bacteria that specialize in denitrifying capabilities, as it is a less energetic process than aerobic respiration.

Interestingly, on day 21 we observed a significant decrease in the proportion of bacteria containing *nirS* in the presence *L. rubellus*, as well as the SM treatment (Fig. 4). The *nirS* gene abundance represents a subset of the bacterial denitrifier population, as it is only one of the two naturally occuring nitrite reductase genes. The other nitrite reductase gene, *nirK*, was unsuccessfully amplified due to excessive humic acid inhibition, so we could not quantify the entire *nir*-containing bacterial population. To our knowledge there is no literature to date demonstrating that the incorporation of crop residue has effects on subpopulations of denitrifiers such as *nirS*. We can only speculate that the differences in *nirS* and *nosZ* gene dynamics suggest a shift in the denitrifier population as a result of either manual or earthworm mediated residue incorporation. An elegant study by Cavigelli and Robertson (8) suggests that N_2O:N_2 ratios can be affected by bacterial denitrifer community composition alone. More research concerning N_2O:N_2 ratios is necessary in order to determine if this is an important process in earthworm mediated N_2O emissions.

Against hypothesis (iii) there are no significant correlations between N_2O emissions and actual denitrifier populations in soils modified by earthworms. Although it appears that residue incorporation 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.

Nevertheless, we find it exceptional that *L. rubellus* has the capacity to alter denitrifier bacterial populations on the scale of the entire bulk soil, especially considering that the denitrification function is a facultative trait observed across many taxonomic bacterial groups (8). These effects provide evidence of a strong selective enrichment as a result of *L. rubellus* activity and deserves further investigation.

Available C hypothesis. Among ST treatments, the NO_3^- and NO_2^- pool increased throughout the experiment, especially in the presence of earthworms, suggesting

mineralization and nitrification rates outpaced denitrification rates. The accumulation and relatively high concentration of this pool strongly suggests that denitrification rates were not limited by the abundance of NO_3^- and NO_2^- electron acceptors. Indeed, DEA tests on days 21 and 89 performed without addition of 10 mM NO_3^- were just as high as those with nonlimiting NO_3^- addition (data not shown). Furthermore, regression analysis across all treatments and time points indicates that DEA is negatively correlated with the concentration of NO_3^- and NO_2^- (P = 0.001), illustrating that denitrification is not limited by this pool, but simply contributes to increased turnover of mineral N.

Despite the likelihood that mineral N is uncoupled to denitrification rates, a substantial NO_3^- pool could have contributed to a higher N_2O:N_2 ratio during denitrification. Miller et al. (29) propose that N_2O will not be reduced unless concentrations of NO_3^- are lower than a threshold of 5-10 mg N kg^{-1} soil. Thus, it is feasible that earthworms inhibited the final N_2O reduction step in denitrification by maintaining inhibitory NO_3^- and NO_2^- concentrations. Such a mechanism could particularly aid in explaining the observation that *A. caliginosa* increased N_2O emissions on day 18 without causing any significant activation of denitrification enzymes measured on the following destructive sampling day.

Higher concentrations of energy rich C result in a higher biological demand of reducing material. If this demand is substantially higher than mineral N supply, N2O is efficiently utilized as a terminal electron acceptor. It is clear that the SM treatment had a large fraction of HWC, thus leading to efficient reduction of N_2O. Although there were no clear earthworm effects on HWC, there was a positive correlation between DEA and HWC among ST treatments across all timepoints (P = 0.001, R = 0.47), suggesting that denitrification was in fact limited by access to labile C. Further implicating the lack of available C is the observation that soils incubated with earthworms exhibited no significant increase in respiration rates. It is evident that more thorough characterizations of labile C pools are needed in order to make more certain conclusions about earthworm induced changes in soil C availability.

Tiunov and Scheu (42) found that carbon availability improves N assimilation efficiency for an endogeic earthworm species. In the presence of these earthworms, low C availability coincided with high mineralization rates. Indeed endogeic earthworms may compete with soil organisms for labile C (37). This process may explain our observation that *A. caliginosa* did not enhance denitrification rates despite

causing high rates of mineralization, and may have inhibited *nosZ* populations in the presence of *L. rubellus*. It is clear that more experiments are needed in order to determine the relationships between earthworms and labile C pools, denitrification enzyme activity, and N_2O:N_2 ratios of denitrified gas. Although beyond the scope of this study, nitrifier denitrification must also be ruled out as a significant source of earthworm mediated N_2O emissions.

Implications for agricultural management. Although we must use precaution when extrapolating from laboratory studies to the field, the authors feel this study presents interesting implications for agricultural management. We used earthworm populations within the range of measured earthworm density in agricultural soils (14). Environmental incubation conditions were optimal for earthworm activity and survival, suggesting a possible overestimate of earthworm contributions to N_2O emissions. However, significant weight loss and mortality over the course of the experiment suggested that the applied residue was not optimal for earthworms.

We believe that this study highlights the importance of soil biological engineers in mediating greenhouse gas emissions from ecosystems. To our knowledge, this is the first study directly linking earthworm priming of soil denitrifiers to an increase in N_2O production from an intact soil column. Thus earthworm populations should be considered as important mediators of the activity and structure of denitrifying organisms and N_2O production within the entire soil system.

Earthworm populations may be managed through different tillage techniques (9). We propose that longer term field scale studies should be performed where selective alterations of earthworm populations are measured alongside DEA and denitrifier abundance.

In conclusion, *L. rubellus* significantly increased cumulative N_2O emissions, and *A. caliginosa* presence caused a transient increase in N_2O. Both species contributed significantly to the mineral N pool, but we were unable to resolve any significant differences in the HWC pool. The epigeic species *L. rubellus* increased DEA and altered denitrifier populations in the bulk soil, whereas the endogeic *A. caliginosa* had no significant effects on these parameters. There were limited species interaction effects in this experiment, but both earthworm species together appeared to increase the turnover of residue N through soil mineral N and microbial biomass N. Due to the prevalence of a non-limiting pool of mineral N throughout the experiment, and

correlations between DEA and HWC, we attribute earthworm priming effects in this experiment to providing soil denitrifiers with access to a yet uncharacterized labile carbon source.

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TABLE 1. Earthworm recovery statistics

Treatment	Day 6		Day 21		Day 90	
	L. rubellus	*A. caliginosa*	*L. rubellus*	*A. caliginosa*	*L. rubellus*	*A. caliginosa*
			Weight change %			
Average (SE)						
ST_R	-6.17 (6.72)a		5.41 (3.74)b		-79.14 (7.04)a	
ST_C		17.31 (1.99)a		8.00 (3.58)b		-24.05 (3.50)b
ST_{RC}	0.53 (5.68)a	14.17 (7.24)a	-19.74 (9.13)a	4.57 (2.21)b	-48.79 (20.89)ab	-18.88 (7.12)b
2-way ANOVA						
L. rubellus		0.732		0.516		0.637
A. caliginosa	0.616		0.116		0.191	
Block	0.972	0.650	0.719	0.648	0.293	0.832
			^{15}N recovery mg			
Average (SE)						
ST_R	0.027 (0.003)b		0.118 (0.007)c		0.043 (0.015)ab	
ST_C		0.011 (0.001)a		0.021 (0.002)a		0.037 (0.003)a
ST_{RC}	0.027 (0.003)b	0.011 (0.002)a	0.077 (0.010)b	0.025 (0.003)a	0.132 (0.059)b	0.044 (0.004)a
2-way ANOVA						
L. rubellus		0.893		0.492		0.214
A. caliginosa	0.949		0.080		0.136	
Block	0.545	0.622	0.901	0.711	0.226	0.320
			Earthworm N derived from residue %			
Average (SE)						
ST_R	1.74 (0.05)b		6.70 (0.21)b		11.16 (4.04)b	
ST_C		0.36 (0.02)a		0.89 (0.07)a		1.96 (0.17)a
ST_{RC}	1.56 (0.07)b	0.44 (0.12)a	5.67 (0.19)b	1.04 (0.09)a	17.86 (1.25)b	2.43 (0.19)a
2-way ANOVA						
L. rubellus		0.671		0.414		0.001**
A. caliginosa	0.147		0.018*		0.280	
Block	0.563	0.476	0.245	0.918	0.643	0.001**

*p < 0.05, ** p < 0.01, **p<0.001

TABLE 2. Daily N_2O and CO_2 emissions

Treatment	Day 5		Day 18		Day 89	
Average (SE)	N_2O^a	CO_2^b	N_2O^a	CO_2^b	N_2O^a	CO_2^b
S	0.067 (0.022)	121 (32) a	0.015 (0.005) a	75 (16) a	0.017 (0.001) a	69 (16) a
SM	0.063 (0.033)	742 (17) c	0.010 (0.005) a	504 (15) c	0.024 (0.005) a	374 (18) c
ST	0.077 (0.022)	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)	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)	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)	597 (26) b	0.101 (0.012) c	339 (21) b	0.050 (0.011) b	282 (8) b
2-way ANOVA						
Residue on top:						
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*	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] μg N_2O-N hour^{-1} kg^{-1} soil
[b] μg CO_2-C hour^{-1} kg^{-1} soil
*p < 0.05, ** p < 0.01, **p<0.001

TABLE 3. Mineral N fractions in mg N kg^{-1} soil

Treatment	Day 6			Day 21			Day 90		
	NH_4^+	NO_3^-+ NO_2^-	Mineralizable N	NH_4^+	NO_3^-+ NO_2^-	Mineralizable N	NH_4^+	NO_3^-+ NO_2^-	Mineralizable N
Average (St. err.)									
S	0.56 (0.06)	10.33 (0.27) c	12.6 (0.4) a	0.03 (0.03) a	12.83 (0.57) c	10.5 (0.4) a	0.00 (0.08) a	22.90 (0.41) d	7.7 (0.9) a
SM	0.28 (0.03)	0.21 (0.09) a	17.3 (1.0) b	0.00 (0.12) a	0.08 (0.06) a	18.5 (0.7) c	0.25 (0.09) b	1.60 (0.09) a	20.2 (1.0) d
ST	0.80 (0.51)	8.39 (0.29) b	14.8 (0.7) ab	0.17 (0.25) ab	10.60 (0.78) bc	13.4 (1.2) b	0.09 (0.03) a	13.05 (0.54) b	12.1 (0.8) bc
ST_R	0.34 (0.18)	9.11 (0.21) b	15.5 (1.0) b	0.18 (0.09) ab	10.13 (0.36) b	13.1 (0.1) b	0.73 (0.28) b	17.05 (0.33) c	14.3 (1.7) c
ST_C	0.61 (0.11)	9.09 (0.49) b	14.6 (1.1) ab	0.43 (0.09) b	12.01 (0.37) c	11.9 (0.4) ab	0.19 (0.04) ab	24.02 (1.21) de	10.7 (1.1) b
ST_{RC}	0.74 (0.05)	9.00 (0.24) b	14.0 (0.6) ab	1.16 (0.19) c	14.15 (0.91) d	12.8 (0.4) b	0.43 (0.23) b	26.70 (2.34) e	13.9 (1.3) c
2-way ANOVA									
Residue on top:									
L. rubellus	0.554	0.334	0.615	0.023*	0.251	0.517	0.023*	0.009**	0.008**
A. caliginosa	0.276	0.408	0.041*	0.001**	0.001**	0.232	0.613	0.000***	0.252
L. rubellus + *A. caliginosa*	0.223	0.262	0.038*	0.054	0.070	0.466	0.232	0.163	0.338
Block	0.098	0.606	0.001**	0.045*	0.196	0.377	0.257	0.630	0.005**

^{15}N Isotope Analysis of Mineral N

	%N derived from residue	^{15}N µg/kg	%N derived from residue	^{15}N µg/kg	%N derived from residue	^{15}N µg/kg
SM	nd[a]	nd	nd	nd	nd	nd
ST	0.40 (0.05) a	1.27 (0.11) a	0.44 (0.06) a	1.49 (0.22) a	1.94 (0.22) a	9.17 (1.27) a
ST_R	0.60 (0.02) b	2.01 (0.08) b	1.79 (0.13) b	5.85 (0.61) b	6.05 (0.22) c	38.37 (2.18) c
ST_C	0.60 (0.02) b	2.06 (0.07) b	1.40 (0.11) b	5.61 (0.52) b	3.59 (0.15) b	31.13 (2.76) b
ST_{RC}	1.04 (0.10) c	3.60 (0.30) c	2.87 (0.31) c	14.14 (0.73) c	7.02 (0.64) c	66.34 (2.96) d
2-way ANOVA						
Residue on top:						
L. rubellus	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***
A. caliginosa	0.000***	0.000***	0.000***	0.000***	0.002**	0.000***
L. rubellus + *A. caliginosa*	0.534	0.300	0.016*	0.212	0.021*	0.008**
Block	0.386	0.161	0.945	0.594	0.748	0.628

[a]There was insufficient mineral N content in SM treatments for determination of the ^{15}N content

*p < 0.05, ** p < 0.01, **p<0.001

TABLE 4. Microbial biomass N

Treatment	Day 6		Day 21		Day 90	
Average (SE)	Microbial biomass N mg kg^{-1}	^{15}N micr. biomass µg kg^{-1}	Microbial biomass N mg kg^{-1}	^{15}N micr. biomass µg kg^{-1}	Microbial biomass N mg kg^{-1}	^{15}N micr. biomass µg kg^{-1}
S	6.02 (0.41) a	-	8.45 (0.77) a	-	0.40 (1.18) a	-
SM	13.89 (0.13) c	87.4 (6.3) c	16.86 (0.25) d	64.3 (6.1) c	16.31 (0.33) c	90.9 (12.3) b
ST	8.09 (1.28) b	12.6 (2.1) a	11.68 (0.43) bc	37.5 (4.4) b	9.17 (1.54) b	28.6 (5.0) a
ST_R	6.29 (1.60) ab	5.6 (2.9) a	11.95 (1.17) c	22.6 (3.2) a	7.25 (1.43) b	26.0 (8.81) a
ST_C	7.33 (0.83) ab	11.5 (3.8) a	7.68 (0.55) a	15.0 (3.7) a	3.29 (0.52) a	22.3 (8.5) a
ST_{RC}	8.23 (0.60) b	32.1 (6.9) b	9.48 (0.61) ab	19.9 (0.4) a	2.40 (1.44) a	17.5 (4.41) a
2-way ANOVA						
Residue on top:						
L. rubellus	0.449	0.123	0.314	0.094	0.134	0.644
A. caliginosa	0.517	0.011*	0.004**	0.002**	0.000***	0.493
L. rubellus + *A. caliginosa*	0.047*	0.009**	0.400	0.014*	0.787	0.812
Block	0.136	0.324	0.053	0.087	0.023*	0.966

*p < 0.05, ** p < 0.01, ***p < 0.001

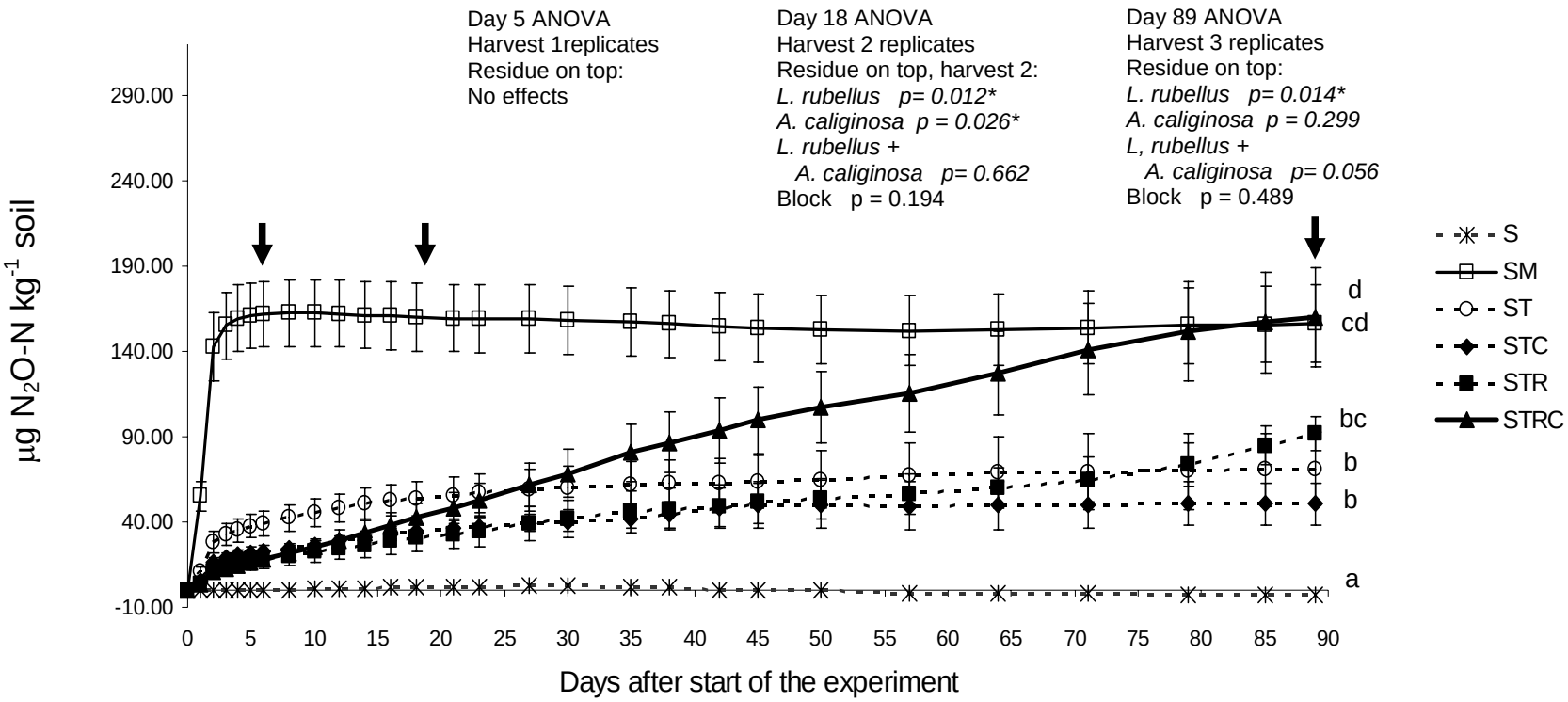

FIG. 1: Cumulative N_2O emissions from the 4 replicate mesocosms that were destructively sampled on the final sampling day. The included 2-Way ANOVA tests were performed on the cumulative N_2O emissions from mesocosms that were directly to be harvested, on the days indicated by the dark arrows. Significant differences in cumulative emissions on day 89 are represented with different letters. *$p < 0.05$, ** $p < 0.01$, ***$p<0.001$

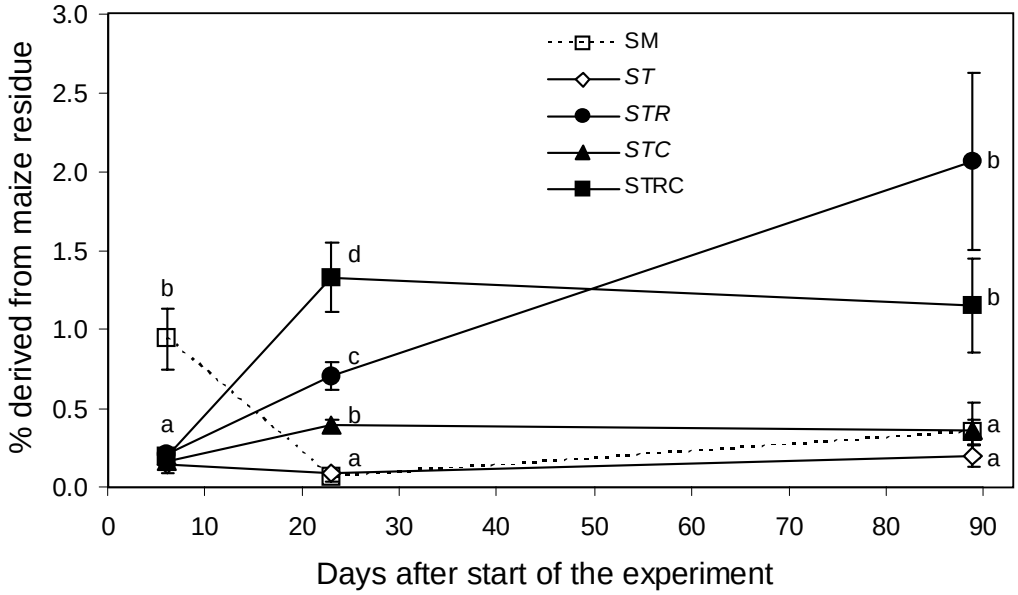

FIG. 2: Percent of N_2O-N derived from maize residue on days 6, 23, and 89 of the experiment. Significant differences on each sampling day are indicated with a different letter.

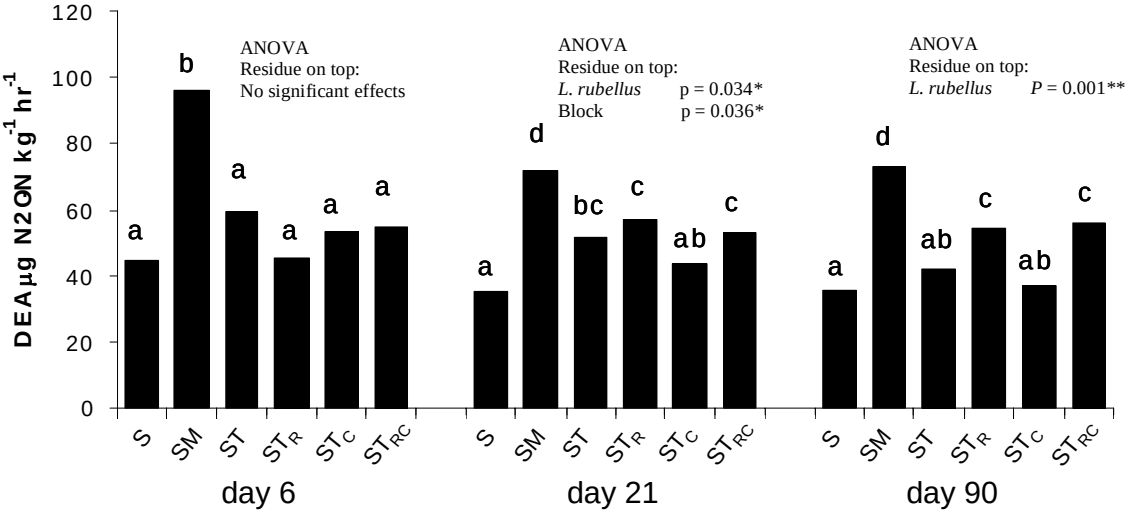

FIG. 3. Denitrification enzyme assay. Significant differences between treatments are indicated with a different letter. Significant 2-way ANOVA effects are listed above the corresponding sampling day. *p < 0.05, ** p < 0.01, ***p<0.001

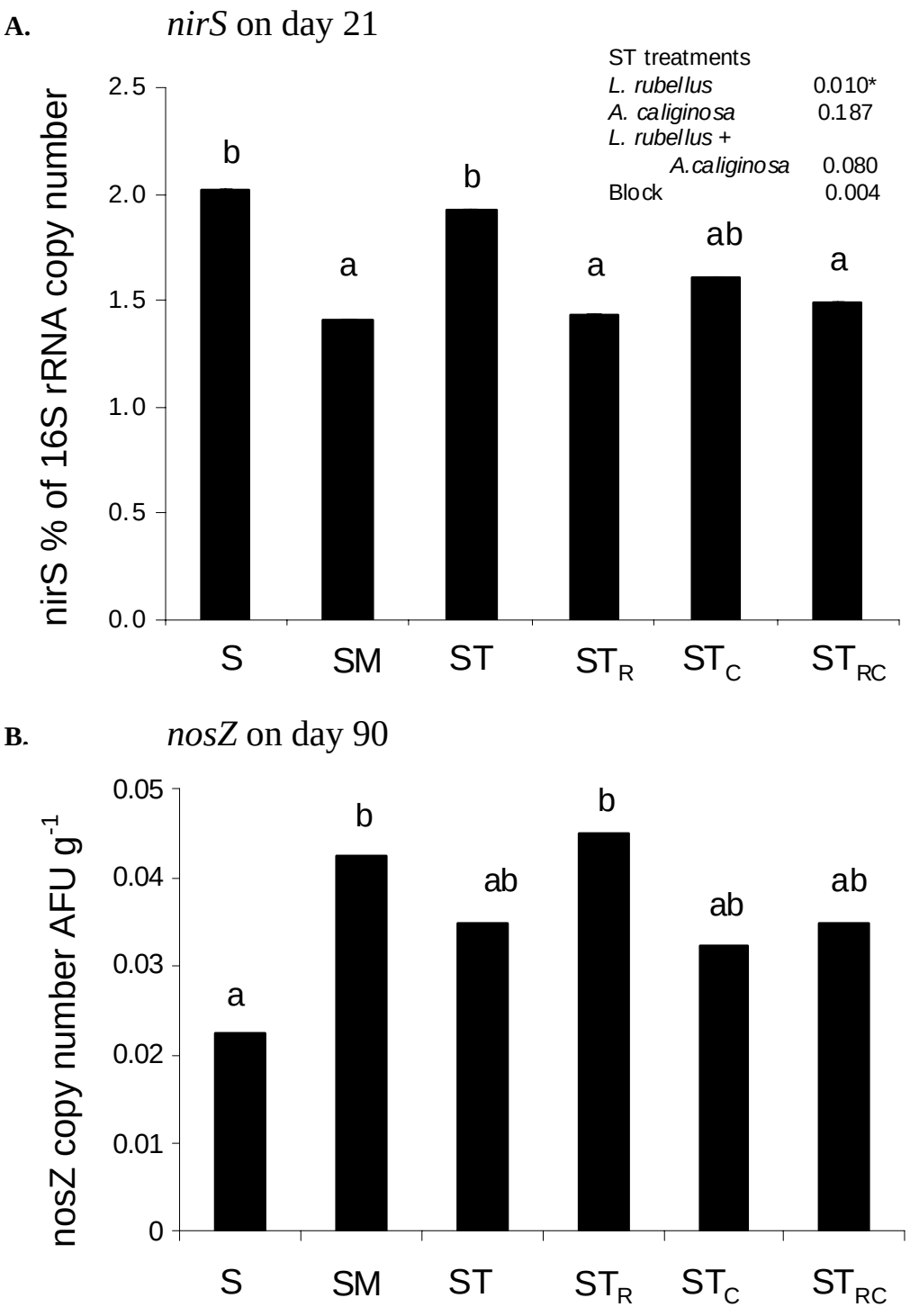

FIG. 4 Summary of significant results for qPCR analysis. Quantification of relative *nirS* gene copy numbers on day 21 (A) and absolute *nosZ* gene copy numbers on day 90 (B). There was a significant treatment effect on the percent of *nirS* with respect to 16S rRNA (A) as well as *nosZ* gene copy numbers (B). Significant differences between treatments are represented by different letters. Furthermore, a 2-way ANOVA on top treatments indicated a significant, negative *L. rubellus* effect on % *nirS* (A).

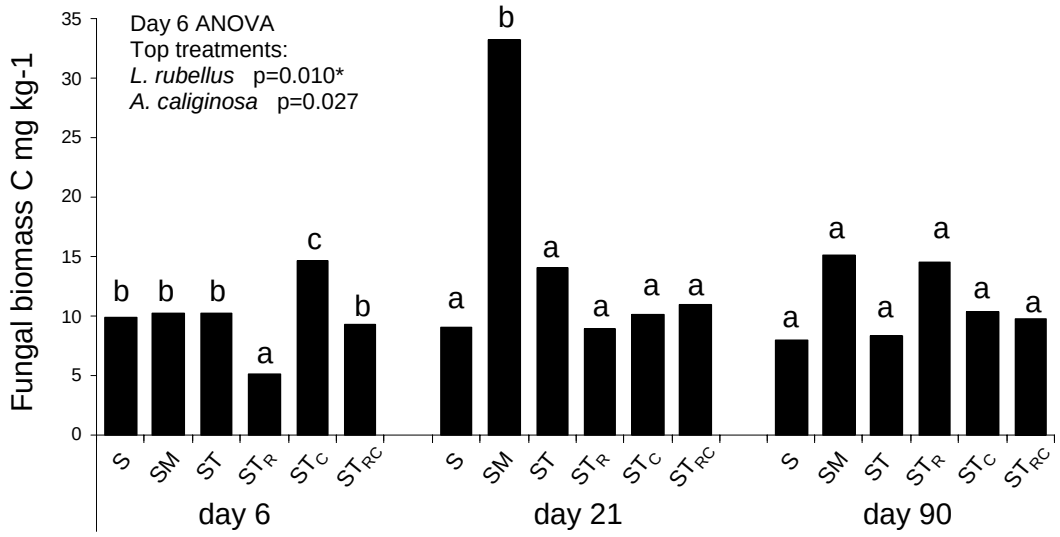

FIG. 5. Fungal biomass. Significant differences as determined by post-hoc LSD, $p < 0.05$ are indicated with different letters. 2-way ANOVA of top treatments indicated a *L. rubellus* effect and *A. caliginosa* effect at day 6. No significant differences on total fungal biomass were observed on Day 90. No significant differences were measured in the active fungal biomass. Error bars are equal to one standard error. *$p < 0.05$, ** $p < 0.01$, ***$p<0.001$