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Earthworm *Lumbricus terrestris* Contributes Nitrous Oxide Emission from Temperate Agricultural Soil Regardless of Applied Mineral Nitrogen Fertilizer Doses

Mikhail Maslov *, Angelika Astaykina and Lev Pozdnyakov

Soil Science Faculty, Lomonosov Moscow State University, 119991 Moscow, Russia
* Correspondence: maslov.m.n@yandex.ru

Abstract: Agriculture is the main contributor to nitrous oxide (N_2O) emission, but the emission intensity can be controlled by various factors, in particular, the activity of earthworms, one of the most common groups of soil invertebrates. We conducted an incubation experiment to evaluate N_2O emission in earthworm soil samples compared to non-earthworm ones with applications of high (200 kg ha^{-1}) and low (50 kg ha^{-1}) mineral N fertilizer doses. We assessed the cumulative N_2O emission, the dynamics of the soil dissolved organic carbon, and the soil microbial carbon and nitrogen content, as well as the number of *nirK* and *nirS* gene copies in bulk soil samples and in isolates from the earthworms' gut. Our study showed a significant role of the earthworm activity in changing the intensity of N_2O emission after the application of mineral N fertilizers. The main factor leading to an increase in nitrous oxide emission in the presence of earthworms is the stimulation of free-living soil denitrifiers by the organic matter of the earthworms' excretions, as well as the thorough mixing of plant residues and soil. Contrary to our expectations, earthworms did not increase the representation of nitrite reductase genes in soil, although the earthworm's gut can be considered as a refugium for denitrifiers. Our results indicate a possible risk of increased N_2O emission from arable temperate soils with an increase in earthworm populations as the climate warms, even if application rates of mineral fertilizers are reduced.

Keywords: denitrification; soil nitrogen cycling; functional gene abundance; gut microbiota; Albic Retisols

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1. Introduction

Nitrous oxide (N_2O) is an important greenhouse gas, with an effect 298 times greater than that of CO_2 [1], capable of interacting with the stratospheric ozone [2] and having a long duration lifetime in the atmosphere. About $^2/_3$ of all anthropogenic N_2O is generated as a result of agricultural activities [3], mainly due to the use of mineral and organic nitrogen (N) fertilizers [4–6] since agricultural crops are not able to consume all of the applied N. The main part of N_2O is produced in soil as a result of microbiological processes, such as nitrification and denitrification [7–10]. The intensity of soil N_2O production depends on many factors, such as temperature, soil moisture, the availability of nitrogen sources and organic matter [4,11–13] and can be regulated not only by the composition and activity of the soil microbiota, but also, for example, by the activity of soil animals.

One of the most common groups of soil invertebrates is earthworms [14], in particular, the widespread species *Lumbricus terrestris*. Previous studies have shown that earthworms may change many properties, such as moisture, organic matter content, and the composition of microbial communities in agricultural soils [15]. The bioturbation, burrowing, and casting activities of earthworms directly or indirectly affect soil N cycling processes, especially the intensity of nitrification and denitrification [16–19]. Thus, a meta-analysis based on more than 50 experiments in micro- and mesocosms showed that the presence of earthworms increases the N_2O emission from the soil by 42% [20]. An increase in nitrous

oxide emission in earthworm areas compared to non-earthworm ones has also been proved in some field experiments [21–23]. Although recent studies have shown that earthworms themselves can be a source of nitrous oxide [24], an important way that they influence the increase in N_2O emission from the soil is by stimulating the activity of free-living soil-denitrifying microorganisms [25], as well as soil inoculation with earthworm gut denitrifiers [26–28], in which populations of culturable denitrifiers can be up to 300 times higher than in the surrounding soil [29]. In addition, the earthworms' burrowing activity contributes to the mixing of soil and fresh plant litter and changes the structure and air permeability of the soil, which also affects the intensity of nitrous oxide production [30,31].

At the same time, the role of earthworms in regulating nitrous oxide emission from agricultural soils at different levels of mineral nitrogen fertilizers application is still unclear. We hypothesized that (1) earthworms can have a significant stimulating effect on the cumulative N_2O emission from the soil, regardless of the mineral nitrogen fertilizer applied dose; (2) stimulation of N_2O emission can occur both due to the activation of free-living soil denitrifiers, and due to soil inoculation with denitrifiers from the earthworms' gut. To test these hypotheses, we conducted a 30-day laboratory incubation experiment to evaluate nitrous oxide emission in earthworm soil samples compared to non-earthworm ones with applications of high (200 kg ha^{-1}) and low (50 kg ha^{-1}) doses of mineral nitrogen fertilizers.

2. Materials and Methods

2.1. Soil Samples and Earthworms Collection

Samples of Albic Retisols [32] with clay loam texture from the Experimental Station (55°59′21″ N, 37°24′17″ E) of the Lomonosov Moscow State University (near Chashnikovo village, Moscow region, Russia) were used in the experiment. Soil samples were collected from an agriculture site that has been used to grow potatoes for the past decades. The main properties of the soil are presented in Table 1. Fresh soil samples were sifted through a sieve with a mesh size of 2 mm to remove stones, large plant debris, and soil fauna.

Table 1. Main soil properties.

Parameter	Value *
C_{tot}, %	2.0 ± 0.2
N_{tot}, %	0.3 ± 0.1
C:N	7.8 ± 0.5
pH_{H2O}	6.0 ± 0.2
Density, g cm^{-3}	1.15 ± 0.05

*—mean ± one standard error.

Mature earthworms *Lumbricus terrestris* were manually collected from a natural population in the soil of a birch forest near the soil sampling site. Before the start of the incubation experiment, earthworms were kept for a week in a vessel with soil at a constant temperature of +16 °C (the optimal temperature for earthworms) for acclimatization.

2.2. The Experiment Design

The laboratory incubation experiment was carried out for 30 days in standard microcosms (1 L jars with inner diameter of 12 cm). Before the start of the experiment, each microcosm was filled with prepared fresh soil samples (500 g), which were moistened to 65% of the WHC. The following variants of the experiment were set up (Figure 1): control without earthworms (N_0), in which only deionized water was added to the soil; an earthworm-free variant with a low application rate of mineral N fertilizers (N_{50}), in which a KNO_3 solution was added at an equivalent of 50 kg N ha^{-1}; and an earthworm-free variant with a high nitrogen fertilization rate (N_{200}), in which a KNO_3 solution was added at an equivalent of 200 kg N ha^{-1}, as well as identical variants with earthworms (EN_0, EN_{50}, and EN_{200}, respectively). Each experiment series included 4 replications without earthworms and 4 replications with earthworms (3–4 mature individuals of *L. terrestris* at the rate of

10–12 g of wet biomass per microcosm). The earthworm population density in microcosms was equivalent to ≈250 g of wet biomass · m^{-2} or ≈70 individuals per square meter, which is about 2 times higher than the natural average earthworm population density in soils of the region. We used a high earthworm population density in this experiment because it can compensate for the short-duration incubation [28]. However, we understand that high earthworm population density could enhance the influence of earthworms on soil and stimulate N_2O emissions, which could be detected as being above the expected range, which varies naturally due to soil and environmental factors. The soil samples were thoroughly mixed to evenly distribute the solutions throughout the soil volume. Before the start of incubation, as well as after 14 days of incubation, 1.5 g of crushed dry *Acer platanoides* leaf litter (C:N ratio of 52.1) was applied to the soil surface to provide earthworms with food sources during the experiment. An equivalent amount of litter was also added to non-earthworm microcosms. The microcosms were covered with Parafilm to prevent moisture loss. Soil moisture was monitored by the weight method every 2 days. The microcosms were incubated at a constant temperature of +16 °C (the optimal temperature for earthworms [33–35]) in darkness. Nitrous oxide emission from the soil surface was assessed daily. To do this, the microcosm was hermetically sealed with a rubber cap with a septum and incubated for 4 h, after which a gas aliquot was taken for analysis. The concentration of N_2O emitted by the microcosms was registered using a gas chromatograph with an electron capture detector (column length: 1 m; internal diameter: 3 mm; adsorbent: Porapak N 80/100; column thermostat temperature: 60 °C; detector temperature: 200 °C, evaporator temperature: 100 °C, carrier gas (N_2) flow rate: 90 mL min^{-1}).

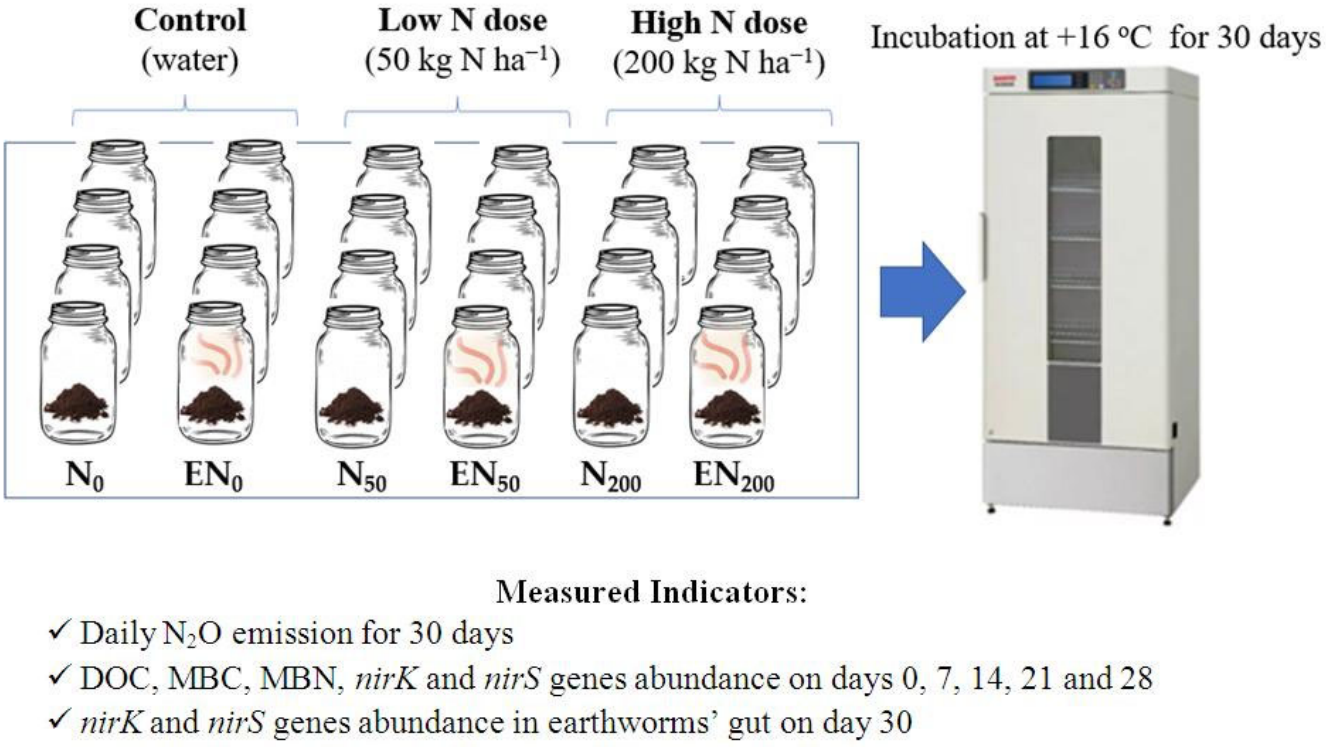


Figure 1. The experiment design scheme.

Before the start of incubation, as well as on the 7th, 14th, 21st and 28th days of the experiment, the content of dissolved organic carbon (DOC), the content of microbial biomass carbon (MBC) and nitrogen (MNB), as well as the abundance of the *nirK* and *nirS* genes (which encode nitrite reductase, one of the key enzymes of the denitrification process) were measured. Soil samples for these analyses were taken from microcosms using a metal drill. At the end of the incubation experiment (on day 30), earthworms were collected and dissected to isolate the contents of their guts, in which the abundance of

the *nirK* and *nirS* genes was assessed. In this study, we only considered the abundance of genes encoding enzymes involved in denitrification and did not consider nitrification as a significant pathway for N_2O production since it was previously shown that the application of KNO_3 minimizes nitrification-related nitrous oxide in soil [36].

2.3. Soil Analyses

Dissolved organic carbon was extracted with deionized water from soil samples at room temperature at a soil-to-water ratio of 1:2.5 *w/v*. After extraction, the solutions were centrifuged at 4500 rpm for 20 min and filtered through a membrane cellulose acetate filter (pore size 0.45 µm). The DOC concentration was quantified using an automated TOC analyzer (LiquiTOC, Elementar, Langenselbold, Germany).

The microbial biomass carbon and nitrogen contents were measured in homogenized soil samples by the chloroform fumigation–extraction method [37,38] according to the previously published recommendations [39–41]. The concentrations of C and N in the extracts were quantified using an automated TOC analyzer (LiquiTOC, Elementar, Langenselbold, Germany). The content of MBC and MBN was calculated as the difference in C or N concentration between fumigated and unfumigated soil samples, then divided by a standard conversion factor of 0.45 for MBC and 0.54 for MBN.

2.4. Earthworms Gut Isolation

Gut isolation was performed according to a previously published protocol [42]. We dissected the minimum required number of earthworms to obtain reliable results, and also tried to prevent animal suffering. All procedures were performed according to the ethical guidelines and regulations approved by Lomonosov Moscow State University. Briefly, an earthworm was anesthetized with boiling water, sterilized with 70% ethanol solution, frozen on a Peltier element to −16 °C, and dissected with a sterile scalpel. For a *q*PCR analysis, the earthworm digestive tract (a section from the clitellum to the anus) was cut out, placed in an Eppendorf tube containing 1 mL of sterile water, and centrifuged at 10,000 g for 10 min. The supernatant was removed, and the obtained isolate was stored at −80 °C until the total DNA extraction.

2.5. DNA Extraction and Quantitative PCR Analysis of Genes Abundance

DNA samples were extracted from soil samples and the earthworms' gut using FastDNA™ Spin Kit for Soil reagent kits (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's recommendations. Samples (0.5 g of soil and 0.25 g for the gut) were homogenized using a Precellus 24 homogenizer (Bertin Technologies, Fontaine, France) for 30 s at 6500 rpm. DNA isolates were stored until further analysis at −80 °C.

The number of copies of the *nirK* and *nirS* genes encoding nitrite reductase was estimated by quantitative PCR. All reactions were carried out in a C1000 CFX96 Real Time thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The reaction mixture contained 10 µL of BioMaster HS-*q*PCR SYBR Blue mixture (Biolabmix, Moscow, Russia), 0.8 µL of each primer, and 1 µL of extracted DNA in a total volume of 20 µL. The reaction was carried out according to the protocol and using the primer sets described in Table 2. The abundance of functional genes in the samples was calculated using the CFX Manager software. To assess the specificity of the *q*PCR product, an analysis of the melting curve was performed (from 65 °C to 95 °C in 0.5 °C increments). The standards were obtained by purifying PCR products and quantifying the concentration using a Qubit fluorometer 2 (Thermo Fisher Scientific, Waltham, MA, USA). The efficiency of quantitative PCR was 90%, and the coefficients of determination were $R^2 > 0.90$ for all standard curves.

Table 2. Conditions for assessing abundance of *nirK* and *nirS* genes by *q*PCR method.

Primer Name	Primer Sequence	Thermal Conditions	Reference
nirK876 nirK1040	ATY GGC GGV CAY GGC GA GCC TCG ATC AGR TTR TGG TT	95 °C, 15 min, 1 cycle 95 °C for 15 s, 63 to 58 °C for 30 s (−1 °C by cycle), 72 °C for 30 s, 80 °C for 15 s, 6 cycles 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, 80 °C for 15 s, 40 cycles, 95 °C for 15 s, 60 to 95 °C, 1 cycle	[43]
nirSCd3aFm nirSR3cdm	AAC GYS AAG GAR ACS GG GAS TTC GGR TGS GTC TTS AYG AA	95 °C, 15 min, 1 cycle 95 °C for 15 s, 65 to 60 °C for 30 s (−1 °C by cycle), 72 °C for 30 s, 80 °C for 15 s, 6 cycles 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, 80 °C for 15 s, 40 cycles 95 °C for 15 s, 60 to 95 °C, 1 cycle	[44]

2.6. Statistical Analyses

All the measured variables were examined for normality (Kolmogorov–Smirnov test) and homogeneity (Levene's test) of variance. The results were expressed on a dry weight basis and presented as the mean ± one standard error. Factorial ANOVA models were used to investigate the effects of nitrogen rate application (three levels) and earthworm presence (two levels) on total N_2O emission, as well as DOC, MBC and MBN content, and functional genes abundance. Turkey's HSD post-hoc test was used to identify significant differences among the treatments. The relationships among physicochemical parameters, functional genes abundance, and N_2O emission were studied using Pearson's correlation analysis. The results were considered statistically significant at $p < 0.05$. All statistical analyses were conducted using STATISTICA 10.0 (StatSoft, Inc., Tulsa, OK, USA, 2011).

3. Results

3.1. Earthworms Survival and Growth

In none of the variants of the experiment was the death of earthworms during incubation noted. At the same time, in the variant of the experiment with the application of mineral nitrogen fertilizer at an equivalent of 200 kg N ha^{-1}, a significant ($p < 0.05$) decrease in the wet weight of earthworms by 19% was found by the end of the experiment. In the control variant of the experiment, as well as when mineral nitrogen fertilizer was applied to the soil at the equivalent of 50 kg N ha^{-1}, there was no statistically significant change in the wet weight of earthworms.

3.2. Soil Nitrous Oxide Emission

The applied dose of mineral nitrogen fertilizer, as well as the presence of earthworms in soil, had a significant ($p < 0.05$) effect on the cumulative N_2O emission. In the experimental variants without earthworms, the N_2O emission by the soil increased three times when nitrogen fertilizer was applied at a rate of 50 kg N ha^{-1} and eight times when fertilizer was applied at a rate of 200 kg N ha^{-1} compared with the control samples (Figure 2). The presence of earthworms in the soil significantly ($p < 0.05$) increased the N_2O emission, but the effect depended on the dose of mineral nitrogen fertilizer applied: the most significant increase (by 55 times compared to the experiment without earthworms) in nitrous oxide emission was recorded in the control variant without fertilization, and the smallest increase (by 12 times compared to the similar variant of the experiment, but without earthworms)—when making the maximum rate of mineral fertilizer (Figure 2).

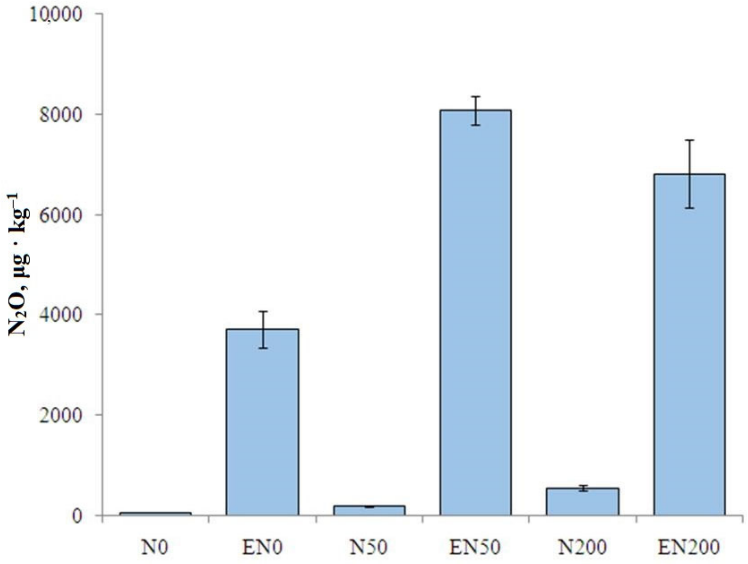


Figure 2. Cumulative 30-day emission of N_2O (µg kg^{-1}).

The presence of earthworms in the soil changed the nitrous oxide release dynamics (Figure 3). The control variant without the application of mineral nitrogen fertilizer and earthworms had an intense rate of N_2O emission in the first few incubation days, followed by a sharp decrease and smooth attenuation by the end of the experiment. An initial increase in the rate of N_2O emission was also noted when mineral nitrogen fertilizer was applied to the soil; however, in these variants of the experiment, an abrupt decrease in the intensity of N_2O emission was not observed by the end of the experiment. The presence of earthworms in the soil led to a gradual increase in N_2O emission during the first weeks of the incubation, followed by fluctuations until the end of the experiment (Figure 3).

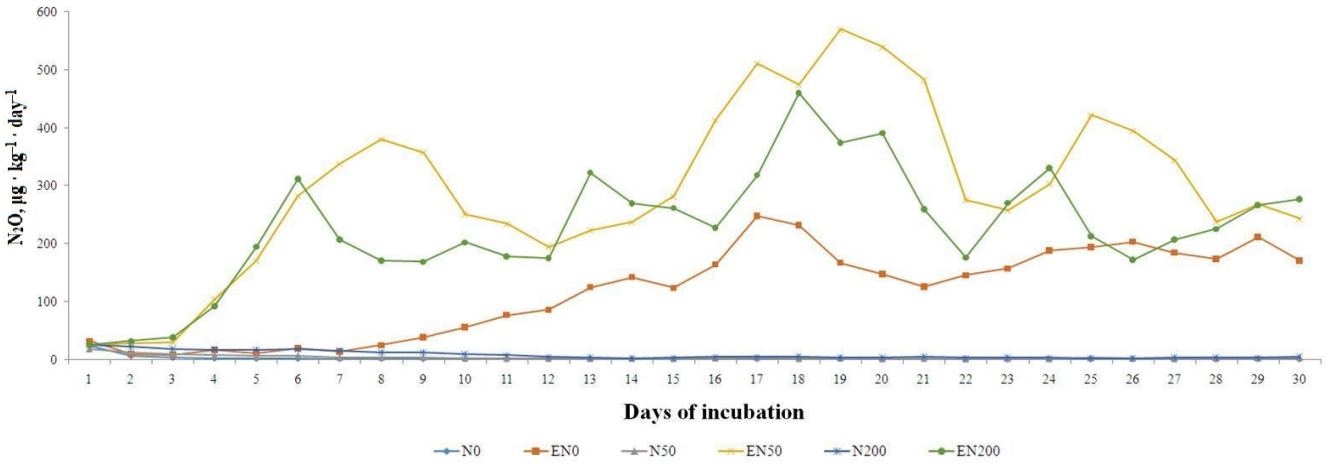


Figure 3. Daily dynamics of N_2O emission (µg kg^{-1} day^{-1}).

3.3. The Content of DOC, MBC and MBN in Soil

The content of soil dissolved organic carbon in microcosms without earthworms practically did not change during incubation (Figure 4). On the contrary, the presence of earthworms in the soil significantly ($p < 0.05$) increased the content of DOC compared to non-earthworm soil samples, especially during the late incubation periods. Nitrous oxide emission closely correlated with the soil DOC content (r = 0.89; $p < 0.05$).

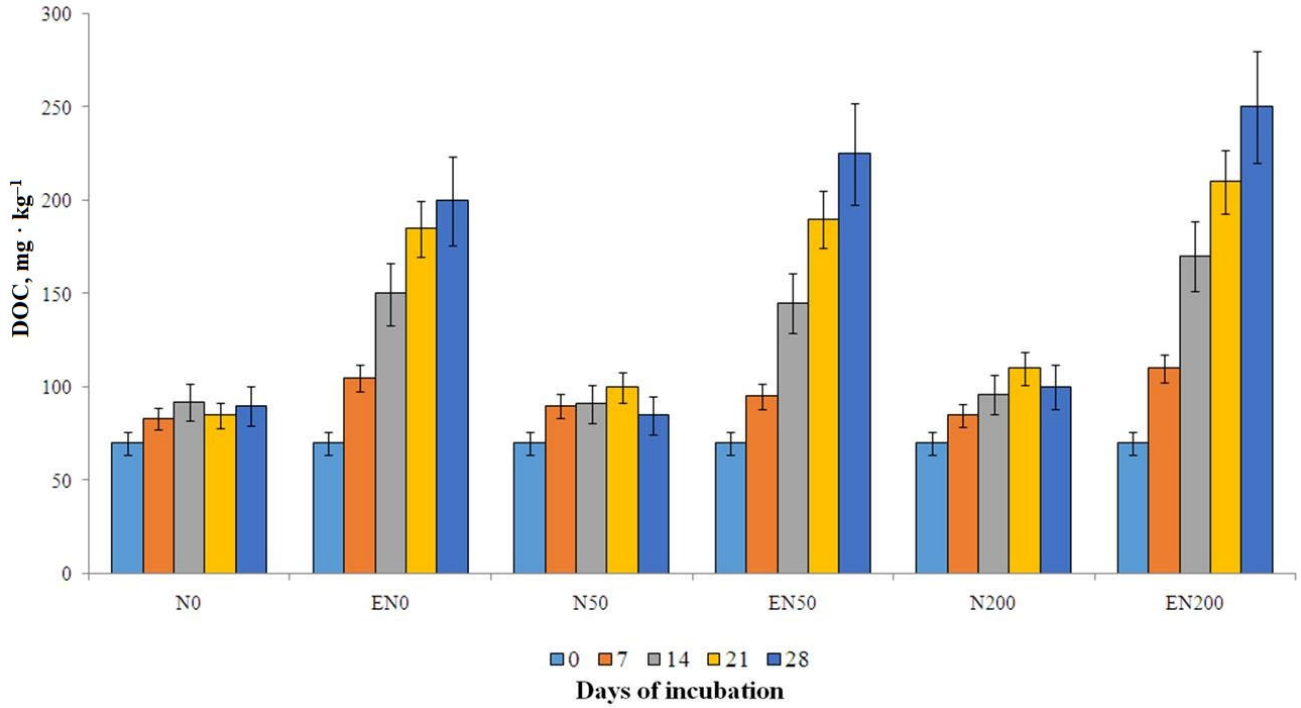


Figure 4. Dissolved organic carbon content in soil (mg kg^{-1}).

The mineral N fertilizer application did not lead to a significant increase in MBC (Figure 5a). An increase in MBC was detected only toward the end of incubation. On the contrary, the presence of earthworms significantly ($p < 0.05$) increased MBC. Nitrous oxide emission correlated with the MBC content (r = 0.80, $p < 0.05$). The effect of mineral N fertilizer and earthworms on the MBN content was similar to MBC (Figure 5b); however, we did not find a significant correlation between N_2O emission and MBN (r = 0.64, $p > 0.05$).

The mineral N fertilizer application, as well as the presence of earthworms in the soil, led to a decrease in the MBC:MBN ratio, i.e., enrichment of soil microbial biomass with nitrogen; however, no statistically significant correlation between this factor and N_2O emission was found (r = −0.75, $p > 0.05$).

3.4. Abundance of nirK and nirS Genes in Soil and Earthworm Gut

The number *nirK* gene copies in the soil was higher than that of the *nirS* gene (Figure 6). During the incubation, the number of *nirK* and *nirS* gene copies remained relatively constant (in the control without earthworms) or gradually increased (in the control with earthworms). The application of mineral N fertilizer resulted in an increase in the number of both nitrite reductase gene copies in the soil; however, the presence of earthworms in the fertilized soil did not have a statistically significant effect on the number of *nirK* and *nirS* gene copies.

At the same time, the number of *nirK* and *nirS* gene copies in the *L. terrestris* gut was significantly higher than in the soil (Figure 7). As in the surrounding soil, the number of the *nirK* gene copies in the earthworms' gut prevailed. Nonetheless, the mineral N fertilizer application had not significantly changed the number of *nirK* and *nirS* gene copies in the earthworms' gut.

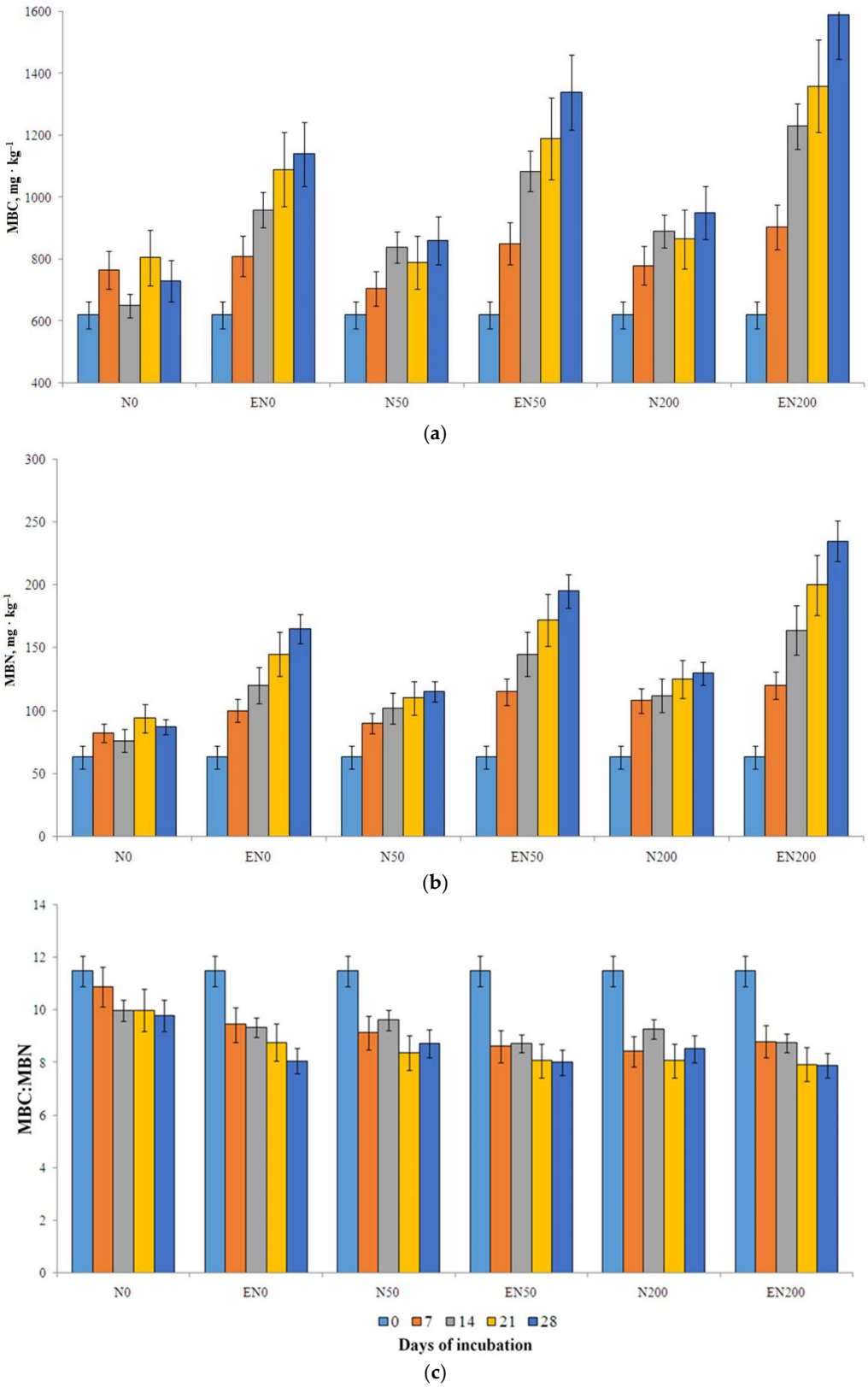


Figure 5. Dynamics of soil microbial biomass carbon (**a**), soil microbial biomass nitrogen (**b**) and MBC:MBN ratio (**c**).

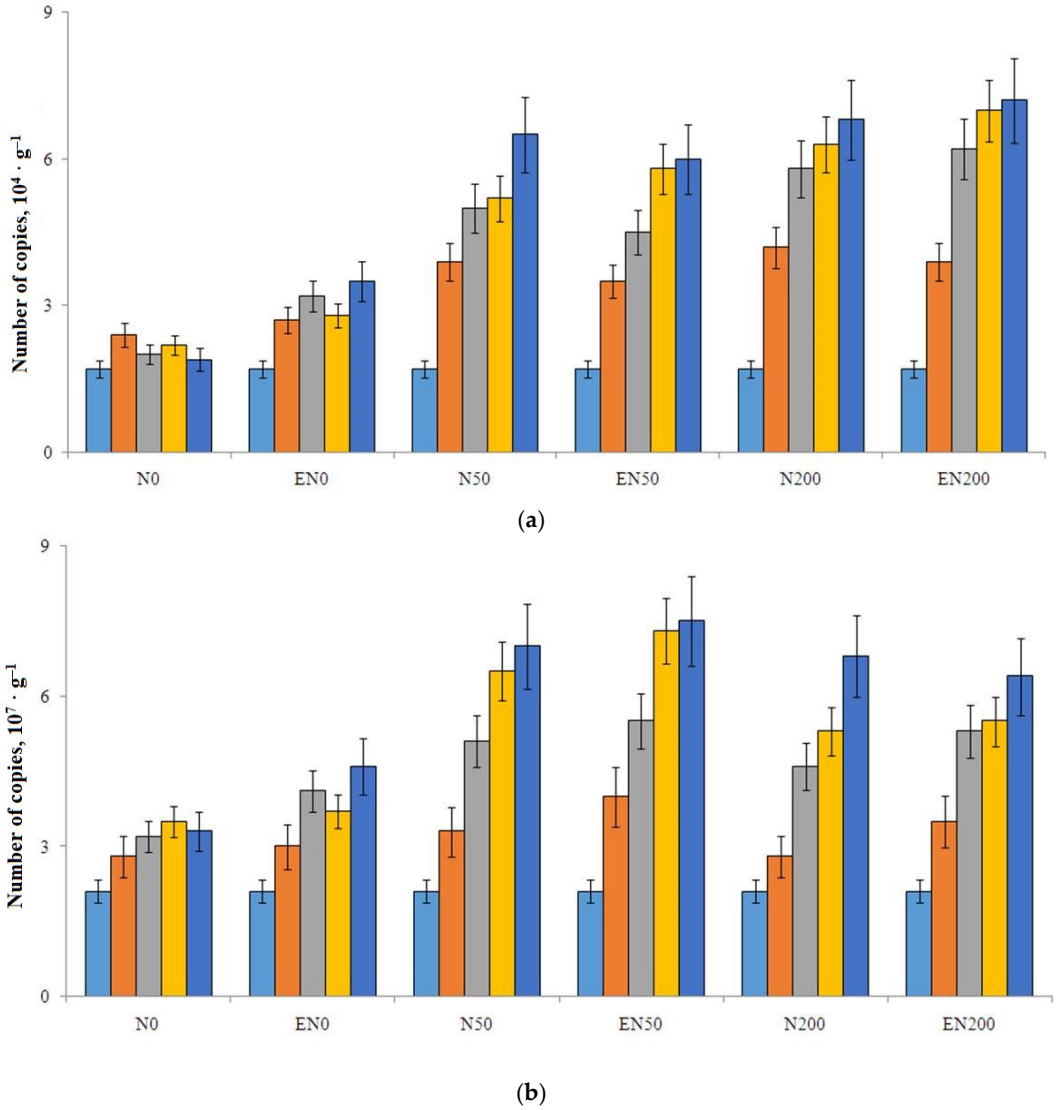
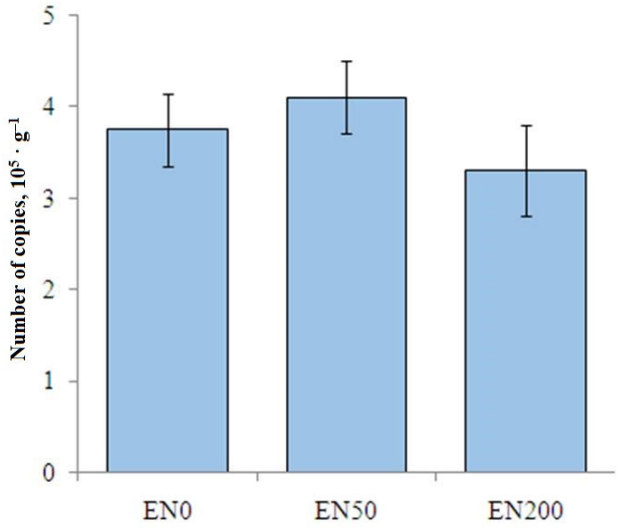
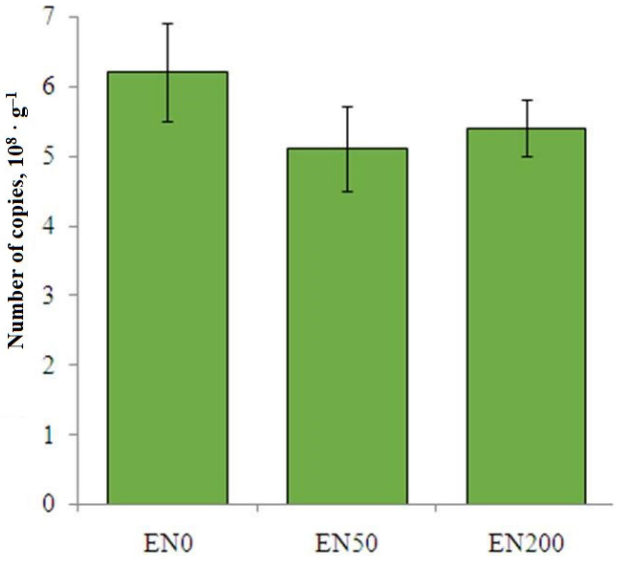


Figure 6. Dynamics of *nirS* (**a**) and *nirK* (**b**) gene copies number in soil.



(**a**)



(**b**)

Figure 7. Number of *nirS* (**a**) and *nirK* (**b**) genes copies in earthworms' gut.

4. Discussion

4.1. Influence of Earthworms on Nitrous Oxide Emission from Soil

Earthworms *L. terrestris* increased the soil N_2O emission by 12–55 times compared to the soil without earthworms (Figure 2). These data correspond with some previously published studies [20,45–48], in most cases confirming the stimulating effect of earthworms of various species and environmental groups on nitrous oxide emission from the soil. For example, Giannopoulos et al. [49] found that epigeic (living in forest floor and feeding on fresh plant litter) earthworms *Lumbricus rubellus* increased N_2O emission from sandy soil by 4 times, while endogeic (feeding on soil organic matter and forming intermittent predominantly horizontal burrow) earthworms *Aporrectodea caliginosa* did not affect the N_2O emission under similar conditions. At the same time, in experiments with loamy soil, *A. caliginosa* increased nitrous oxide emission only if plant residues were manually mixed with soil samples [30]. However, Nebert et al. [31] indicated that the epigeic earthworms *L. rubellus* increased N_2O emission by only 76% compared to non-earthworms' soil, while species similar in lifestyle, *Eisenia fetida*, increased nitrous oxide emission by 4.2 times [50]. Significant stimulation of soil N_2O emission also occurred in the presence of anecic (feeding

on partially decomposed plant litter and forming permanent deep vertical burrows in the soil) earthworm species *L. terrestris* [51] and *Apporectodea longa* [52,53]. At the same time, some studies have not found a significant effect of earthworms on soil nitrous oxide emission [16,54], which may be partly due to these experiments' design because plant residues, which are necessary for earthworms feeding, were not added into the soil. In addition, the increased earthworm population density in our experiment (about 2 times higher than the average for the soils of this region) also may have increased the stimulating effect.

The essential increase in soil N_2O emission in the presence of earthworms, which significantly exceeds previously published estimates, may be due to the fact that mineral nitrogen fertilizer was applied to the soil. Thus, it was previously shown that epi-endogeic earthworm *Amynthas gracilis* and endogeic *Pontoscolex corethrurus* in unfertilized meadow soil only slightly increased nitrous oxide emission [55]. In addition, no increase in N_2O emission in the presence of both the anecic species *L. terrestris* and the endogeic species *A. caliginosa* was previously found in unfertilized soil of the corn agroecosystem in Canada [56]. At the same time, when nitrogen-rich plant litter was introduced into the soil, *A. caliginosa* activity increased soil N_2O emission by 70–90% [57].

4.2. How Earthworms Affect the Soil N_2O Emission

Earthworms can increase nitrous oxide emission by inoculating soil with gut-stimulated denitrifying bacteria during the excretion process as well as by stimulating the activity of free-living denitrifiers (not passing through the earthworm's gut). Although some previously published studies have linked the presence of earthworms in soil with an increase in bacterial functional genes related to denitrification compared to non-earthworm soil samples [16,31,50,58], this effect was not found in our experiment. The increase in the number of copies of the *nirK* and *nirS* genes in the soil was influenced by the application of mineral nitrogen fertilizer (regardless of the dose), and not by the presence or absence of earthworms (Figure 6). At the same time, the number of copies of the *nirK* and *nirS* genes in the *L. terrestris* gut was significantly higher than in the surrounding soil (Figures 6 and 7), which allows us to suggest that earthworms are a refugium or living "Noah's Ark" for denitrifiers. However, studies focusing on earthworm gut microbes associated with the nitrogen cycle are still limited, and we know nothing about whether the high denitrifying activity results from the activation of soil bacteria in the earthworm gut or whether there is a community of denitrifiers that are adapted to living inside the gut. Nevertheless, it must be emphasized that the earthworm gut is an ideal site for the existence of denitrifiers [59] since it is an anaerobic locus with high availability of organic matter and nitrates [26,27,60].

Based on our data on the stimulating effect of earthworms on the dissolved organic carbon content in the soil (Figure 4), the MBC and MBN content (Figure 5), as well as altering the dynamics of N_2O emission during incubation (Figure 3), we suggested that the increase in nitrous oxide emission in the presence of earthworms is related to the activation of free-living soil denitrifiers. The mechanism of this phenomenon may be associated with the enrichment of soil with organic matter due to earthworms' excretions [16,23,61–63], including casts [27,51,55,64]. Thus, it is commonly believed that the drillosphere (soil surrounding earthworm burrows) is more enriched in organic matter and soluble nitrogen compounds compared to the bulk soil [25] and is a 'hot spot' for the activity of free-living soil denitrifiers [26,65,66]. The increased availability of organic matter observed in the soil of earthworm microcosms could stimulate N_2O emission by suppressing the final stages of denitrification, in which nitrous oxide is reduced to molecular nitrogen [62,67]. In addition, an increase in the availability of organic matter in the soil may be the result of the mixing of soil and plant litter, which is carried out by earthworms. Thus, in our experiment, *A. platanoides* leaf litter was placed on the soil surface and then gradually pulled in by earthworms and redistributed throughout the soil volume. Previously, Rizhia et al. [53] concluded that N_2O emission in the presence of earthworms did not increase when crop residues were thoroughly mixed with soil prior to earthworm invasion (as opposed to

the case where crop residues were placed on the soil surface), which may indicate that a significant contribution of earthworms to the regulation of nitrous oxide emission is to drag plant residues into the soil.

Furthermore, it has previously been shown that earthworms are able to significantly increase soil CO_2 emission [16,23,68], which may be associated both directly with the respiration of earthworms and with the respiration of the earthworm-stimulated soil microbial biomass. In turn, the high intensity of soil respiration combined with the high availability of nitrogen sources can also stimulate N_2O emission since denitrification is an anaerobic process. Finally, the anecic earthworm species *L. terrestris* forms permanent vertical burrows in the soil, which could contribute to a freer release of nitrous oxide, which is formed in deep soil layers, into the atmosphere.

4.3. Influence of the Mineral N Fertilizer Dose on the Soil N_2O Emission

The application of mineral N fertilizer to the soil led to an increase in nitrous oxide emission (Figure 1), which may be due to an increase in the availability of nitrates for denitrifiers [36]. At the same time, we did not find a close correlation between the representation of the *nirK* and *nirS* genes in the soil and the intensity of N_2O emission, as, for instance, Linton et al. [69], although such a correlation was previously reported [70,71]. However, denitrification is a modular pathway; consequently, different genes may be considered molecular targets [36,72–74]. Nonetheless, in all variants of our experiment, the representation of the *nirK* gene in the soil was significantly higher than that of *nirS*. These genes encode structurally different types of nitrite reductase (*nirK*—copper-containing (Cu-Nir), and *nirS*—containing heme c and heme d1 (cd1-Nir)) and do not occur in the same cell [75]; therefore, in Albic Retisosl, microorganisms with the copper-containing nitrite reductase gene have an advantage. Dominance of the *nirK* gene in agricultural soils has been previously found in some other experiments [70,76]. At the same time, we do not yet have a clear understanding of the differences in ecology (e.g., oxygen tolerance threshold or growth kinetic parameters) of denitrifying microorganisms with the *nirK* and *nirS* genes [77]. However, we found that neither the application of increasing doses of mineral nitrogen fertilizer, nor the presence of earthworms in the soil significantly changes the ratio of microorganisms with these genes.

At a high application rate of mineral N fertilizer, we found a decrease in nitrous oxide emission from the soil in the presence of earthworms (Figure 2). On the one hand, this could be caused by a decrease in the activity of earthworms due to the toxic effects of high doses of nitrates since a statistically significant decrease in the earthworm wet biomass was observed only in this variant of the experiment. However, we did not find a statistically significant difference in the content of, for example, DOC in the presence of earthworms and the application of nitrogen fertilizers at low and high rates. One of the possible explanations for the observed phenomenon may be the formation of a microbial community capable of consuming nitrous oxide and thus reducing its emission. An increase in the abundance of the N_2O reducers when high doses of nitrates are applied to the soil has previously been shown in some studies [36,78]. At the same time, the presence of earthworms could create the necessary conditions for the formation of not only a community of denitrifiers, but also microorganisms that consume nitrous oxide.

5. Conclusions

Our study showed a significant role of the earthworm *L. terrestris* activity in changing the intensity of nitrous oxide emission after the application of mineral N fertilizers. The main factor leading to an increase N_2O emission in the presence of earthworms is the stimulation of free-living soil denitrifiers by the organic matter of earthworms' excretions, as well as the thorough mixing of plant residues and soil. Contrary to our expectations, earthworms did not increase the representation of nitrite reductase genes, although the earthworm's gut can be considered a refugium for denitrifiers. Although great care should be taken in extrapolating the results of a controlled study to processes occurring in nature,

we believe that the main established effects also occur in the field. Therefore, our results indicate a possible risk of increased N_2O emission from arable temperate soils with an increase in earthworm populations as the climate warms, even if application rates of mineral nitrogen fertilizers are reduced. Overall, our data highlight the importance of studying the role of earthworms in the greenhouse gas balance in agroecosystems.

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