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RESEARCH ARTICLE

Earthworm-controlled CO_2, N_2O and N_2 emissions from a silt loam soil amended with senescent litter of two energy crops (*Silphium perfoliatum* and *Zea mays*)

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Abstract

Aims Cup plant (*Silphium perfoliatum*) contributes to sustainable bioenergy production. However, little is known about the role of intensive litter fall and increased soil biodiversity on respiration and denitrification in its cultivation. This study aimed to assess CO_2, N_2O and N_2 emissions of annual and perennial energy cropping systems affected by earthworms when supplied with senescent cup plant or maize litter.

Methods In a 32-day laboratory incubation, the ^{15}N gas flux method was applied in an N_2-reduced atmosphere to a repacked silt loam soil, inoculated with *Lumbricus terrestris* L., and amended with senescent maize and cup plant litter. During incubation, CO_2, N_2O and N_2 emissions were measured. After incubation, NO_3^-, NH_4^+, total C, and total N was analysed in soil and casts. Litter removal from the soil surface and net nitrification rates were calculated.

Results Earthworms caused a fivefold increase in maize litter removal and a threefold increase regarding cup plant litter. Highest N_2O+N_2 fluxes were observed in treatments with earthworms and cup plant litter, although the high CN ratio of senescent cup plant litter implied higher N immobilisation than the CN ratio of senescent maize litter. Earthworms increased CO_2, N_2O, and especially N_2 emissions lowering the product ratio of denitrification (N_2Oi).

Conclusions Earthworm-controlled litter incorporation increased labile C from decomposition of recalcitrant litter as substrate for denitrification. Mineralisation patterns of senescent litter cannot be explained by CN ratio alone. The combination of both mechanisms affected denitrification, lead to elevated N_2O and N_2 emissions and N loss from the soil.

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Introduction

Sustainable energy production is by design aimed at climate change mitigation and adaptation. In light of the growing climate change awareness (Gärtner and Schoen 2021), perennial energy plants with a low resource demand (low external input) and a variety of ecological benefits as an alternative to resource-intensive (high external input) annuals became popular. They were established with the premise to lower N inputs and greenhouse gas emissions (GHG) and to promote biodiversity. One such promising perennial is the cup plant (*Silphium perfoliatum*), known for high yields and low requirements for pesticides (Gansberger et al. 2015). Early canopy closure and ample litter fall provide suitable habitat conditions and food sources for a variety of earthworms including anecic species (Schorpp and Schrader 2016). These earthworms are prominent for incorporating and feeding on surface-applied organic residues, thus substantially contributing to organic matter decomposition and nutrient turnover (Blouin et al. 2013; Lee 1985).

Besides all the beneficial ecosystem services earthworms provide, they simultaneously affect important parameters for denitrification and the emission of climate-threatening nitrous oxide (N_2O) (Blouin et al. 2013; Lubbers et al. 2013a). It has been shown that earthworms enhance decomposition and subsequently increase labile carbon compounds, which may serve as an energy source for denitrifying bacteria (Rummel et al. 2020; Senbayram et al. 2012; Senbayram et al. 2020; Weier et al. 1993). A second aspect of earthworm activity – especially by anecic earthworm species such as *Lumbricus terrestris* L.—and its effect on denitrification is the incorporation of litter as mentioned by Giannopoulos et al. (2010) and recently by Kemmann et al. (2021). The incorporation of litter accelerates decomposition leading to oxygen depletion and transient anaerobic microsites, which offer suitable conditions for denitrification (Marhan et al. 2015; Millar and Baggs 2005; Rummel et al. 2020; Surey et al. 2020).

Earthworms, especially anecic species, contribute further to N_2O emission because they build permanent, typically surface-connected burrows, thereby compacting the surrounding soil, which creates additional anoxic hotspots (Lubbers et al. 2011). Apart from that, earthworms are able to emit N_2O as well as N_2 due to denitrifying bacteria and suitable conditions in their gut (Drake and Horn 2006, Horn et al. 2006). Earthworm casts may also play a role as they are rich in organic matter and nutrients (Lee 1985). Even though mineral N in casts occur mainly as ammonium (NH_4^+), which is unfavourable for denitrification (Senbayram et al. 2012), earthworm casts may be potential hotspots of N_2O derived from nitrification by means of autotrophic ammonia oxidising archaea and bacteria (Hu et al. 2015; Liu et al. 2016) or from heterotrophic nitrification (Wrage-Mönnig et al. 2018). Yet, earthworm effects on GHG emissions remain elusive. Studies have reported both increased and decreased emission of N_2O due to earthworm activity. For example, Lubbers et al. (2013a), evaluated 237 observations from 57 studies and concluded that earthworms enhance soil N_2O emissions by 42% and soil carbon dioxide (CO_2) emissions by 33%. In an earlier study, Lubbers et al. (2011) reported increased plant-available nitrogen (N) and plant-N uptake as well as increased N_2O emissions depending on functional group association highlighting potential trade-offs between earthworm-controlled services and disservices. Other studies reported the opposite effect and demonstrated that earthworms may also suppress N_2O emissions depending on fertilizer regime, weather conditions and functional group association (Chen et al. 2014b; Kuiper et al. 2013; Lubbers et al. 2013b). In addition, earthworms – especially anecic species – may be mutualists of soil bacteria and fungi and increase their abundance and richness (Blouin et al. 2025). The earthworm-induced increase of soil microbial activity, however, may be temporally and locally limited and affected by a complex set of factors such as the presence of plants, addition of organic amendments and the pH of the soil (Blouin et al. 2025). Earthworms can locally elevate the soil pH due to their calciferous glands and epidermal mucus and thereby improve conditions for soil microorganisms enhancing their activity (Blouin et al. 2025; Schrader 1994). Hence, the occurrence of hotspots and hot moments must be kept in mind and earthworm effects must therefore always be treated in context of the biotic and abiotic characteristics of their environment.

To our knowledge most studies consider fresh crop residues, whereas senescent litter from arable crops has long been neglected, even though it may draw a more realistic image of litter fall and decomposition in agroecosystems. Due to maturity-related relocation of lipids, protein, nucleic acids and other available C and N from leaves (Bleecker and Patterson 1997, Lashermes et al. 2021), senescent litter from arable crops differs greatly from fresh organic residues. These differences in composition and CN ratio may strongly control the effect of senescent litter on production and emission of N_2O (Abalos et al. 2022) and moreover N_2. Fresh green plant material in general may be a source of carbon (C) and N and during its decomposition, oxygen (O_2) is consumed in microsites while nitrate (NO_3^-) is provided and labile C becomes available as an electron donor, supporting denitrification (Senbayram et al. 2012, 2018). This leads to emissions of N_2O but also N_2, which is often overlooked, although it may present significant N loss from soils. In contrast, the addition of senescent litter may trigger the immobilisation of N due to chemical characteristics of the organic material, especially the wide CN ratio (Chen et al. 2014a). In turn, the immobilisation of N may alter substrate availability in the aforementioned process and impact its outcome. Overall, the effect of litter, especially senescent litter, and its intrinsic properties on the $N_2O/(N_2+N_2O)$ product ratio of denitrification (N_2Oi) is not clear, as increasing labile C lowers N_2Oi due to enhancement of N_2O reduction to N_2, while increasing soil NO_3^- by adding organic amendments or from mineralisation of organic matter and nitrification has an opposite effect due to the inhibition of N_2O reduction (Grosz et al. 2022; Senbayram et al. 2018). Earthworm-mediated incorporation of senescent litter from arable crops with wide CN ratios and specific composition may create hotspots of interconnected nitrification and denitrification processes, that are yet to be understood. The interaction of earthworm activity and the quality of litter provided may affect N_2O, N_2 and CO_2 fluxes in a way that could even elevate N loss from soils and the subsequent need to increase N fertilisation counteracting the benefits of perennial energy crops. And since mitigating climate change became more urgent than ever, it is crucial to reiterate the potential effect of earthworm activity and senescent litter from arable crops in annual and perennial energy cropping systems on the production and emission of GHG. Hence our study was conducted to determine the combined impact of senescent cup plant and maize (*Zea mays*) litter as well as earthworm activity on CO_2, N_2O and N_2 emissions and mineral N content in relation to decomposition. Furthermore, our aim was to distinguish N_2O from denitrification and other sources. In a 32 days laboratory incubation experiment, we applied the ^{15}N gas flux method and a N_2-depleted atmosphere (Lewicka-Szczebak et al. 2017) to a silt loam soil. This method allows for an increased sensitivity while measuring N_2, since it is conducted in a N_2-depleted atmosphere compared to the natural N_2-rich atmosphere. The soil was homogenously labelled with potassium nitrate ($K^{15}NO_3$), repacked to columns, inoculated with the anecic earthworm species *Lumbricus terrestris* and finally amended with senescent cup plant and maize litter, respectively. CO_2 and N_2O emissions as well as N_2O and N_2 emissions from the labelled pool were regularly measured. Further, litter removal from the soil surface as a proxy for mineralisation, net nitrification and product ratio of denitrification (N_2Oi) were determined to test the following hypotheses:

I. Earthworm activity increases removal of senescent litter from the soil surface and thus CO_2 fluxes from soil.
II. N_2O and N_2 fluxes are more pronounced with maize litter and in the presence of earthworms.
III. Earthworm activity and earthworm-controlled decomposition increase the fraction of N_2O originating from denitrification.
IV. Earthworm activity and earthworm-controlled decomposition enhance N_2O reduction to N_2 and thus lower N_2Oi.

Material and methods

Soil

Soil was taken from the upper 27 cm (Ap horizon) of a four-year-old cup plant field near Oberthal (49.51°N; 7.07°E) in Western Germany in September of 2020. This field was selected due to low mineral nitrogen content as prerequisites for ^{15}N-labelling of the soil. The soil type was a Hypereutric Stagnic Cambisol (IUSS Working Group WRB 2015). The soil texture was a silt loam (SiL, 17.8 ± 0.4% sand, 57.9 ± 0.5% silt, 24.3 ± 0.2% clay). The soil was sieved (2 mm), moistened, thoroughly mixed and rested for a week at 4 °C to ensure even moisture distribution.

Two days prior to the start of the incubation, the soil was labelled using a spray can with KNO_3 solution consisting of 4.08 L distilled water, 6.00 g $CaCl_2$ to counteract dispersion of soil particles as well as 8.66 g $K^{14}NO_3$ and 14.56 g $K^{15}NO_3$ to match a ^{15}N enrichment of the NO_3^- pool of approximately 60 atom%. The following day, the soil was mixed a second time to allow for an even distribution of the label and soil moisture. After labelling, the soil was pre-incubated at 15 °C for one day until start of the incubation. The final gravimetric soil water content was 24.77 ± 0.05% equalling a water filled pore space (WFPS) of 50.3% at bulk density of 1.15 g cm^{-3}. At the start of the incubation, mineral N of the experimental soil averaged 26.62 ± 0.29 mg NO_3^- kg^{-1} and 1.46 ± 0.04 mg NH_4^+ kg^{-1}. Total C amounted to 16.16 ± 0.07 g kg^{-1} and total N to 1.92 ± 0.02 g kg^{-1}. Soil pH ($CaCl_2$) was 4.8.

Litter

Litter was sampled from the above-mentioned cup plant field and from a neighbouring maize field in September 2020. Visibly senescent maize and cup plant leaves were detached from the bottom parts of the plants, dried at 60 °C for 48 h and chopped into 1 cm^2 pieces. Total C and N in litter, earthworm casts and soil were analysed via dry combustion using a TruMac CN Analysator (LECO, St. Joseph, USA). Characterisation of total C and N of the applied leaf litter are presented in Table 1.

Earthworms

Adult individuals of the anecic earthworm species *Lumbricus terrestris* L. were purchased from a commercial supplier (Herrmann & Herrmann GbR, Braunschweig, Germany). The earthworms were gradually accustomed to the experimental conditions. In the first week, earthworms were kept in loose experimental soil and fed with excess experimental cup plant or maize litter. In the second week, earthworms were kept in soil of experimental soil density and moisture for adaption to experimental conditions. The adaptation process was done at 15 °C in the dark. Earthworms were washed in tap water to remove adhesive residues and mucus and then weighed before the experiment. Gut emptying was omitted to ensure earthworm vitality.

Table 1 Total C and N [g kg^{-1}] (± SE) content of the applied cup plant (*Silphium perfoliatum*) and maize (*Zea mays*) litter

	total C [g kg^{-1}]	total N [g kg^{-1}]	CN ratio
Cup plant	418.32 ± 0.58	6.55 ± 0.03	63.9
Maize	406.65 ± 5.18	13.50 ± 0.24	30.1

Experimental setup

Seven treatments with $n = 4$ replicates (28 soil columns in total) were set up. To mimic litter abundance in cup plant and maize fields, soil columns were amended with 3 g of dried maize litter and 3 g and 6 g of dried cup plant litter on the soil surface which corresponds to field conditions of 1.84 Mg ha^{-1} and 3.68 Mg ha^{-1}, respectively. To better compare earthworm effects and greenhouse gas emissions, we introduced another cup plant treatment amended with the same amount used in maize treatment. A second maize treatment with an elevated litter amount was not introduced, since it is unrealistic for such ample litter fall to occur in maize systems. All three litter treatments were studied with and without earthworms. A control ($n = 4$) with neither earthworms nor litter was added. A tabular overview of the experimental design is presented in the Supplementary Material, Table S1.

After pre-incubation, Plexiglas cylinders were fixed with a rubber seal onto Plexiglas plates. 4673.6 g labelled soil was filled into the Plexiglas cylinders (inner radius = 7.2 cm, height = 30 cm). Compaction was achieved by inserting a cylindrical spacer with a volume equal to the target headspace volume that was forced to fit the headspace by pressing the mesocosm cover plate using the threaded rods designed to hold base and cover plates of closed mesocosms. After this compaction procedure, the height of the soil monolith in the Plexiglas cylinders was 20 cm and the spacer was removed. This ensured a head space of 1.63 L and a bulk density of 1.15 g cm^{-3} in all soil columns. After packing the columns, remaining soil was stored at −18 °C immediately for mineral N analysis. The surface of all columns was roughened manually with a small fork. In earthworm treatments, a thin rod with a diameter of 0.5 cm was used to push two 6 cm deep holes in the surface centre, in which two adult individuals of *L. terrestris* were inserted which corresponds to 122.8 individuals per m^2. Combined mean earthworm biomass was 10.21 ± 0.07 g (3 g maize), 10.15 ± 0.36 g (6 g cup plant) and 9.78 ± 0.34 g (3 g cup plant). All columns were sprayed with 5 ml distilled water to prevent possible desiccation of the soils surface and the earthworms and stored with a lid on top at 15 °C overnight. After all earthworms had vanished into the soil, maize and cup plant litter was applied on the soil surface.

Afterwards, all columns were sprayed again with 5 ml distilled water to moisturize the dried litter, furnished with a rubber seal and closed airtight with a Plexiglas plate fitted with an inlet and an outlet for the gas flow system (Hantschel et al. 1994). Columns were attached to a flow through system as described by Hantschel et al. (1994) and flushed with gas mixture of 20.7% O_2, 2.8% N_2; 76.5% He, 0.2 ppm N_2O and 317.4 ppm CO_2 at a rate of 8 to 12.5 mL min^{-1} (Kemmann et al. 2021) Outflowing gas from each soil column was directed to a flowmeter and a gas chromatograph (GC) via polyamide tubing. Gas flow was measured with a digital flowmeter (model GFM17 0–50 mL min^{-1}, Analyt-MTC, Müllheim, Germany) every two minutes and measurements of N_2O and CO_2 concentrations via a gas chromatograph (GC) (model GC-2014ATF, Shimadzu, Kyoto, Japan) were conducted every six minutes, resulting in intervals of 4.6 and 1.5 h per column, respectively. For N_2O, instrumental precision of 0.2% was determined by repeatedly measuring standards of 0.19, 0.55, 2.01, 6.94, 40.40, and 131.00 ppm N_2O. For CO_2, instrumental precision of 0.1% was determined by repeatedly measuring standards of 100.1, 704.0, 2479.0, 10000.0, 20100.00 and 49600.0 ppm CO_2.

During incubation, correctness of gas flow and progress of litter decomposition were checked every day. Gas samples for ^{15}N gas analysis were taken on day 3, 5, 11, 15, 19, 23, 27 and 31 in 12 mL flat bottom exetainer vials with a rubber septum (Labco Limited, Lampeter, United Kingdom). The incubation temperature was 15 °C.

After incubation of 32 days, soil columns were detached from the gas flow system and deconstructed. Earthworm casts and remaining litter were collected from the soil surface. The soil was removed from Plexiglas cylinders. Since anecic species construct deep vertical burrows and change pore geometry and thereby potentially gas diffusivity (Lubbers et al. 2013a), the soil columns were cut into a top and a bottom half of the soil monolith of 10 cm length each for subsequent depth-specific analysis of earthworm effects due to change of pore geometry and subsequent gas diffusivity. Earthworms were removed, washed in tap water and weighed. Top and bottom halves of the soil monolith were thoroughly mixed each and sampled. Soil and cast samples were stored at −18 °C immediately. Litter samples were dried at 60 °C for 48 h and ground afterwards.

Chemical parameters

Frozen soil and cast samples were thawed at 4 °C overnight. Mineral N (NO_3^--N and NH_4^+-N) from soil and cast samples was extracted with 2 M KCl in a ratio of 1:4 according to ISO 14256–2 (2005). The soil solution was placed in an overhead shaker for 1 h and filtered. The filtrate was measured photometrically with a continuous flow analyser SA 5000 (Skalar Analytical B.V., Netherlands). For gravimetric determination of soil water content, the soil was dried for 48 h at 105 °C.

Air-dried soil was mixed with 0.01 M $CaCl_2$ in a ratio of 1:4 and shaken for pH measurement with a FE20 pH-meter (Mettler Toledo, Urdorf, Switzerland).

Isotope mass ratios

The soil was labelled with $K^{15}NO_3$, as described in 2.1. Since N naturally occurs with an isotope mass of 14 most of the time, the label allows the distinction and quantification of N_2 and N_2O from the soil. Hence, this method makes it possible to draw conclusions regarding the underlying N transformations, such as nitrification and denitrification by measuring and calculating stable isotope ratios and quantifying N_2 and N_2O fluxes from the ^{15}N-labelled N pool as described by Lewicka-Szczebak et al. (2013) and Kemmann et al. (2021). Gas samples from the exetainer vials were transferred to a GasBench II (Thermo Scientific, Bremen, Germany) via PAL system autosampler (CTC Analytics, Zwingen, Switzerland) with a two-hole needle. Isotope mass ratios of

^{29}R ($^{29}N_2/^{28}N_2$) and ^{30}R ($^{30}N_2/^{28}N_2$) were determined from N_2, N_2+N_2O and N_2O with a connected triple connector isotope ratio mass spectrometer (IRMS) (MAT 253, Thermo Scientific, Bremen, Germany) as described by Kemmann et al. (2021). The analytical precision of the IRMS equalled a standard deviation of $< 1e^{-6}$ for both, ^{30}R and ^{29}R.

Calculations

Removal of litter from the soil surface

To account for litter mineralisation and to allow for proper comparison of treatments, we used the equation for the decomposition rate k according to Olson (1963) for decay without production i.e., where litter fall was not continuous.

$$\textit{daily rate of litter removal from the soil surface} = \frac{\ln\left(\frac{X}{X_0}\right)}{(-t)} \quad (1)$$

The calculation was done via the natural logarithm of the quotient of the remaining litter biomass X on the soil surface [g], the initially offered litter biomass X_0 applied to the soil surface [g] and the duration of the incubation t [d]. X may include the mass of large chunks of litter sticking out of the burrows but exclude all fragmented litter ensiled in casts or too small to be manually removed. Therefore, the calculated rate is used in the following chapters not as decomposition rate but as a proxy for mineralisation and will be called daily rate of litter removal from the soil surface.

Net nitrification rate

Net nitrification rates in relation to dry soil weight were calculated according to Hart et al. (1994) using the final and the initial NO_3^- content under the consideration of the date of initial NO_3^- measurement prior to incubation and the date of experiment termination (difference of 41 days).

CO_2 and N_2O flux rates F

Equation 2 was used afterwards to calculate flux rates F for CO_2 [mg CO_2-C m^{-2} h^{-1}] and N_2O [μg N_2O-N m^{-2} h^{-1}] according to Säurich et al. (2019):

$$F = \frac{M}{V_m} * c * \frac{273.15}{273.15 + T} * v * 10^{-6} * 60 * \frac{1}{A} \quad (2)$$

Flux rates F were calculated using gas concentrations c [ppm], gas flow data v [ml min^{-1}], the molar masses M [g mol^{-1}] of N M_N and C M_C, respectively, the molar volume V_m of 22.4136 L mol^{-1} of an ideal gas under standard conditions and the surface area of the soil column A of 1.6286 $* 10^{-2}$ m^2 and temperature during incubation T [C°].

Some flow data were biased due to Exetainer sampling, indicated by flow measurements deviating from the adjusted flow of 8 to 12.5 ml min^{-1}. These samples amounted to < 1.5% of all flow data. Biased flow data und respective concentrations were excluded from the data.

N_2 and N_2O fluxes from the ^{15}N labelled pool

Regarding N_2 and N_2O fluxes from the ^{15}N labelled pool, non-random distribution of isotopocules was assumed according to Hauck and Bouldin (1961). Evaluation of isotopic data was done using the equations given in Kemmann et al. (2021, supplemental material). Briefly, the determination of the ^{15}N abundance in the soil pool undergoing denitrification (*apN_2O*) and of the fractions of gas species originating from the ^{15}N-labelled NO_3^- pool undergoing denitrification were quantified for N_2 (Fp_N_2), N_2+N_2O ($Fp\_N_2+N_2O$) and N_2O (Fp_N_2O) according to Spott et al. (2006), where the Fp notation refers to fraction (F) from the pool (p) of ^{15}N-labelled NO_3^-. Fp values were transformed to volume fractions of the respective gas species in the gas samples as fp = Fp*cNt, where cNt is the total N_2O or N_2+N_2O concentration of the gas sample. Fp values are obtained for N_2 (*fp_N_2,*), N_2+N_2O (*$fp\_N_2+N_2O$*) and N_2O (*fp_N_2O*). In cases of N_2 and N_2O below IRMS detection limit, half of the detection limit was adopted for *fp_N_2O* and *$fp\_N_2+N_2O$* for following calculations instead, as mentioned by Kemmann et al. (2021). Fractions of pool-derived N_2 (*fp_N_2*), the fraction of N_2O derived from the labelled pool of total emitted N_2O (*Fp_N_2O*) and the $N_2O/(N_2+N_2O)$ product ratio of denitrification (*N_2Oi*) were calculated by means of cumulated fluxes (Kemmann et al. 2021). Precision of N_2O analysis was better by GC than by IRMS. Therefore, to obtain consistency between the values of total N_2O

and fp_N_2O, fp_N_2O were derived by combing GC and IRMS data as

$$fp\_N_2O = \text{total } N_2\text{O} * Fp\_N_2O \tag{3}$$

fp_N_2 and $fp\_N_2 + N_2O$ are then obtained as

$$fp\_N_2 + N_2O = fp\_N_2O / N_2Oi \tag{4}$$

and

$$fp\_N_2 = fp\_N_2 + N2O - fp\_N_2O \tag{5}$$

N_2O fluxes from non-labelled N sources are obtained as

$$fn\_N_2O = \text{ total } N_2\text{O } - fp\_N_2O \tag{6}$$

Only in cases when Fp_N_2O or N_2Oi were below detection, *fp_* values were derived directly from stable isotope ratio using Eqs. 4 and 5 in Kemmann et al. (2021, supplemental material).

Mass fractions of fp_N_2, fp_N_2O, $fp\_N_2+N_2O$ and fn_N_2O [ppm] were translated into flux rates [μg N m^{-2} h^{-1}] according to (2). All fluxes, including those derived from GC, were linearly interpolated between measurement timepoints and integrated over the incubation period using the trapezoidal rule to calculate cumulative emissions. If initial flux or final values for individual incubation vessels were missing, the treatment mean was used to obtain a start or end flux value. The total experiment run-time was 32 days, but for flux aggregation, a total of 29 days was chosen for calculation to match IRMS sampling data, thus excluding the first two and last one day of the total incubation. All figures display the total experiment run-time of 32 days to provide an overview over the complete runtime.

Flux calculation and calculation of isotope ratios was conducted with Excel Office 2019 (Microsoft Corporation 2019).

Statistical evaluation

Statistical evaluation was conducted in RStudio with R version 4.0.3 (R Core Team 2020). The following parameters were fitted with linear models using the R function “lm” from the R package “car” (Fox and Weisberg 2019) and subjected to analysis of variance (ANOVA): removal of litter from the soil surface, aggregated GC-derived flux rates, aggregated flux rates fp_N_2, fp_N_2O and $fp\_N_2+N_2O$, net nitrification rates, total C and N in litter and cast, cast NO_3^- and NH_4^+ and mean Fp_N_2O and N_2Oi. Data were log10-transformed or sqrt-transformed, if necessary to obtain normal distribution of model residuals. For compact letter display in figures, simplified linear models with treatment as sole independent variable were used for CO_2 and N_2O fluxes. Independent variables included: earthworms (yes/no), litter type (none/maize/cup plant), litter amount (none/3 g/6 g) and additional interactions of earthworms and litter (earthworms:litter type, earthworms:litter amount). NH_4^+ in the top half (0—10 cm) and bottom half (10–20 cm) of the soil column were examined by taking initial soil mineral N into account. For that matter and to ensure normal distribution, the log-transformed quotient of final and initial substance content (+1) was used as dependant variable. For post-hoc testing, the Tukey´s HSD test was conducted by means of the R packages “emmeans” (Lenth 2020) and “multcomp” (Hothorn et al 2008). Correlations were analysed using the R package “stats” and Spearman´s method of correlation (R Core Team 2020). Initial and final earthworm biomass as well as initial and final soil mineral N were evaluated by applying a paired student´s t-test (“t.test”). A linear mixed-effects model (“lme”) from the R package “nlme” was used to analyse trends of pool enrichment apN_2O (Pinheiro et al. 2020). Autocorrelation was accounted for using the R function “corCAR1” (Pinheiro et al. 2020). Subsequent post-hoc testing of treatments regarding apN_2O was conducted using the “emtrends” function from the “emmeans” package. Figures were created with the packages “dplyr” (Wickham et al. 2021) and “ggplot2” (Wickham 2016).

Results

Earthworm survival

The survival rate of earthworms was 96% since only one individual perished. The respective replicate (3 g cup plant) was consequentially excluded from further evaluations. Earthworms lost up to 22% (3 g cup plant) of their initial biomass during 32 days of incubation. Earthworm biomass decrease was significant for both litter types ($P<0.01$).

Litter removal from the soil surface and CO_2 gas fluxes

After incubation, maize and cup plant litter (3 g) were mostly vanished from the soil surface, while a larger amount of residues was found in 6 g cup plant treatments. Litter removal from the soil surface was significantly enhanced by earthworms ($P < 0.0001$) and were three to four times as high as in the respective treatment without earthworms (Fig. 1). Most litter was removed from the soil surface of the 3 g maize litter treatment with earthworms. Differences due to litter type had no significant effect, but the litter amount significantly affected litter removal from the soil surface ($P < 0.0001$). More litter removal was observed when earthworms were fed with low amounts of litter and vice versa ($P < 0.01$). With earthworms, in the 6 g cup plant treatment, litter removal from the soil surface was 44% lower compared to the 3 g cup plant treatment. In litter-only treatments, differences in litter removal from the soil surface were negligible.

CO_2 fluxes, in general showed a steady decline until reaching a constant flux around day 14 (Supplementary Material, Fig. S1). Temperature varied slightly during the course of the experiment (Supplementary Material, Fig. S2). The initial temperature was found to be too high with values around 18 °C and, hence, readjusted to 15 °C several times. Slight fluctuations in temperature appeared to coincide with slight variations in CO_2 fluxes, e.g., approximately around day 10 and 24 (Supplementary Material, Fig. S1 and S2). Cumulated CO_2 fluxes (Fig. 2) were lowest for the control (22.98 ± 0.11 g m^{-2}), while litter addition significantly increased cumulated CO_2, depending on litter type and amount ($P < 0.0001$; Fig. 2) in the following order: 3 g cup plant < 3 g maize < 6 g cup plant. Addition of 6 g cup plant nearly doubled CO_2 emissions compared to the control. Earthworms further enhanced CO_2 emission ($P < 0.0001$). In the earthworm treatment with 3 g maize, CO_2 fluxes were lower than in the 3 g cup plant treatment as interactions of earthworms and litter type were significant ($P < 0.01$, Fig. 2, Table 3). Highest emissions were linked to the earthworm treatment with 6 g cup plant (56.84 ± 0.61 g m^{-2}).

Soil physical and chemical parameters

The pH level remained unchanged during the experiment. WFPS decreased by 1.6% absolute (Supplementary Material, Table S2). Total N in the litter remaining on the soil surface after incubation increased in treatments without earthworms and decreased in treatments with earthworms ($P_{earthworms} < 0.0001$, Table S3) compared to the initial litter N (maize: 13.50 ± 0.24 g N kg^{-1}; cup plant: 6.55 ± 0.03 g N

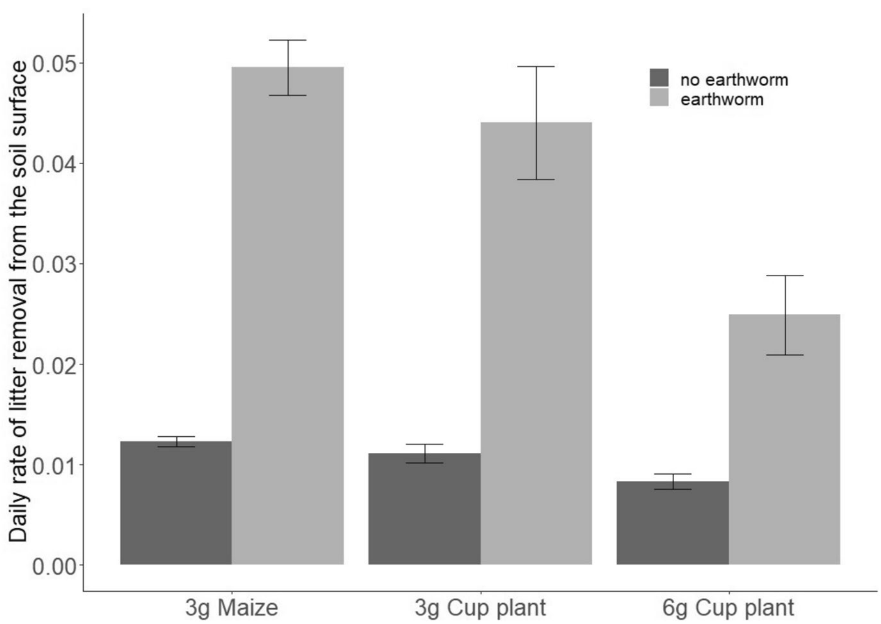

Fig. 1 Daily rate of litter removal from the soil surface k (mean ± SE) in treatments ($n = 4$) amended with 3 g maize litter as well as 3 g and 6 g cup plant litter with and without earthworm species *Lumbricus terrestris*

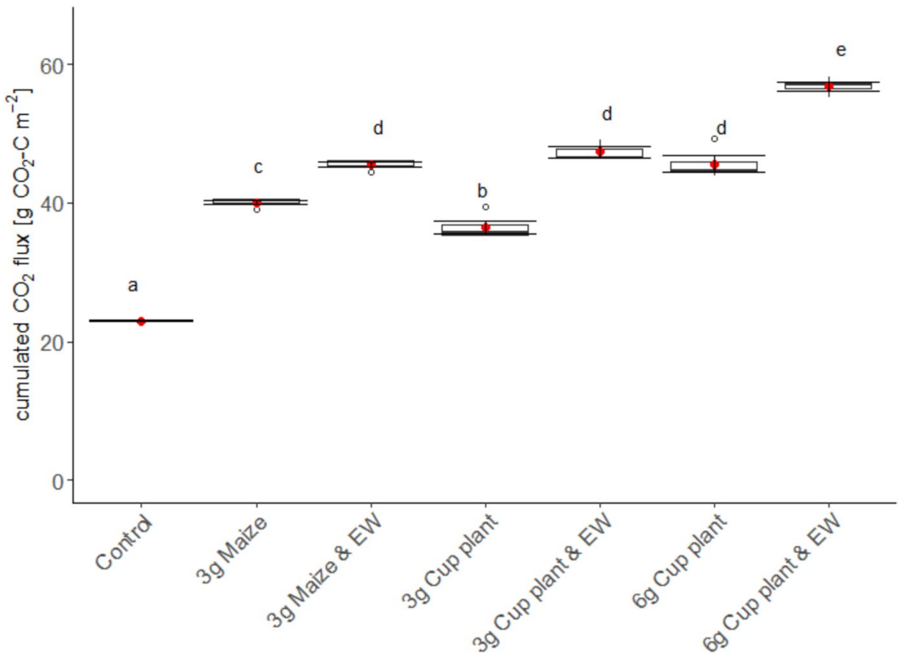

Fig. 2 Cumulated CO_2 [g CO_2-C m.$^{-2}$] emissions of 29 days (mean ± SE (model-derived), $n = 4$) with compact letter display derived from a simplified model for the control, as well as for treatments with 3 g Maize, 3 g and 6 g Cup plant and the respective earthworm (EW) treatments. Means are indicated by a red dot. Different letters indicate statistical differences ($P < 0.05$)

kg^{-1}; Table 2). Although earthworm presence and litter type as well as their interaction were found to be significant for total litter C contents as well (Table 2), total litter C declined across all treatments compared to initial litter C, with cup plant treatments being more affected (maize: 406.65 ± 5.18 g kg^{-1}; cup plant: 418.32 ± 0.58 g kg^{-1}; Table 2; Supplementary Material, Table S3).

The total C content in casts collected after incubation was 37% to 52% higher than in the surrounding soil (cf. mean of total soil C 16.07 ± 0.08 g C kg^{-1}), but neither litter type nor amount were significant factors. Only a trend was found regarding litter amount ($P < 0.1$). Cast N was increased by 8% to 13% compared to the surrounding soil (cf. mean of total soil N 1.86 ± 0.01 g C kg^{-1}) with litter type being the only significant factor ($P < 0.01$) as highest cast N contents were associated with the maize treatment.

The NO_3^--N content in earthworm casts was less than in surrounding soil, while cast NH_4^+-N levels were six to twenty times higher than in surrounding soil (Fig. 3). Most NO_3^--N and NH_4^+-N was found in casts of the 3 g maize treatment as they were significantly affected by litter type ($P_{NO3-} < 0.001$; $P_{NH4+} < 0.01$). Cast NO_3^--N correlated with decomposition rates (ρ 0.67; $P < 0.05$).

After incubation, soil NO_3^--N and NH_4^+-N contents in 0–10 cm and

Table 2 Total C and N of earthworm casts and litter in treatments with 3 g maize as well as 3 g and 6 g cup plant (mean ± SE, $n = 4$) and in respective earthworm (EW) treatments after incubation

Treatment	Total cast C [g kg^{-1}]	Total cast N [g kg^{-1}]	Total residual litter C [g kg^{-1}]	Total residual litter N [g kg^{-1}]
3 g Maize	–	–	405.43 ± 3.52	15.78 ± 0.43
3 g Maize & EW	24.22 ± 0.88	2.27 ± 0.05	245.19 ± 14.78	4.83 ± 0.22
3 g Cup plant	–	–	398.83 ± 5.50	9.27 ± 0.44
3 g Cup plant & EW	21.77 ± 0.91	2.04 ± 0.06	326.23 ± 3.33	6.31 ± 0.29
6 g Cup plant	–	–	388.28 ± 4.68	7.59 ± 0.30
6 g Cup plant & EW	24.70 ± 0.82	2.06 ± 0.02	329.51 ± 6.27	5.81 ± 0.34

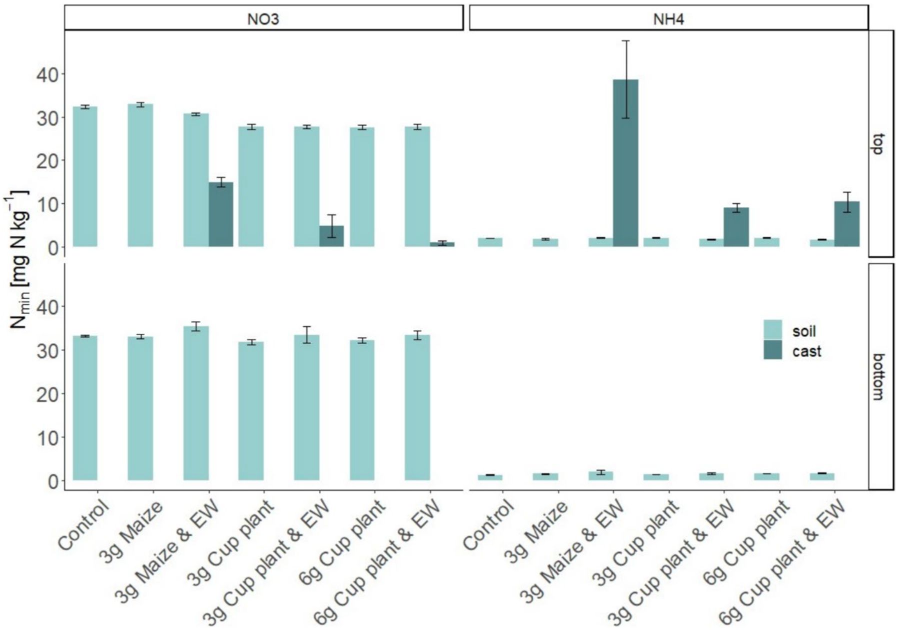

Fig. 3 NO_3^- and NH_4^+ [mg N kg^{-1}] in soil and casts of the top 0–10 cm and bottom 10–20 cm of the soil columns (mean ± SE, $n=4$) of treatments with 3 g maize, 3 g cup plant and 6 g cup plant as well as with respective earthworms (EW)

NO_3^- in 10–20 cm soil depth were significantly increased ($P_{top,\ bottom\ NO3-;\ top\ NH4+} < 0.0001$) compared to pre-incubation levels of 26.62 ± 0.29 mg NO_3^--N kg^{-1} and 1.46 ± 0.04 mg NH_4^+-N kg^{-1} (Fig. 3). Differences were caused primarily by earthworm interactions with litter type ($P < 0.01$). In the bottom part (10–20 cm), highest NH_4^+-N levels were associated with earthworm treatments ($P < 0.001$).

Initial and final NO_3^--N resulted in net nitrification rates shown in Fig. 4. Net nitrification in the top half of the soil columns was significantly affected by litter type ($P < 0.0001$) with highest rates associated with maize treatments. Net nitrification rates in cup plant treatments were less than a third compared to the control or respective maize treatments. In comparison to the control and litter-only treatments, net nitrification rates in the bottom 10–20 cm increased in the presence of earthworm activity ($P < 0.001$), most noticeably in maize treatments. For further statistical information on soil NH_4^+-N and net nitrification rates, see also Supplementary Material, Table S4.

N_2O fluxes

Daily N_2O fluxes in treatments without earthworms declined until day 14 and became constant afterwards, while earthworm treatments peaked between day 1 and day 10 (Supplementary Material, Fig. S3). Cumulated N_2O emissions were lowest in the control (2.00 ± 0.04 mg N_2O-N m^{-2}). Neither litter type nor amount were significant factors, but earthworms caused a two- to five-fold increase in cumulated N_2O emissions compared to their litter-only counterparts ($P < 0.0001$, Fig. 5). The earthworm treatment with 3 g cup plant emitted roughly twice as much N_2O compared to the earthworm treatment with 3 g maize (6.95 ± 0.82 mg N_2O-N m^{-2}) which illustrates the highly significant interaction between earthworms and litter type ($P < 0.001$, Table 3).

N_2O emissions significantly correlated with litter removal from the soil surface (ρ 0.75; $P < 0.0001$) as well as with cumulated CO_2 (ρ 0.8; $P < 0.0001$) and cast NH_4^+-N (ρ −0.62; $P < 0.05$). Regarding soil mineral N in maize treatments, cumulated N_2O negatively correlated with NO_3^--N in the top half of the soil columns (ρ −0.76; $P < 0.05$) and positively in the bottom half of the soil columns (ρ 0.86; $P < 0.05$). In treatments with cup plant litter, N_2O emissions correlated negatively with NH_4^+-N in the top 0–10 cm of the soil column ($\rho_{cup\ plant}$ −0.79; $P_{cup\ plant} < 0.001$).

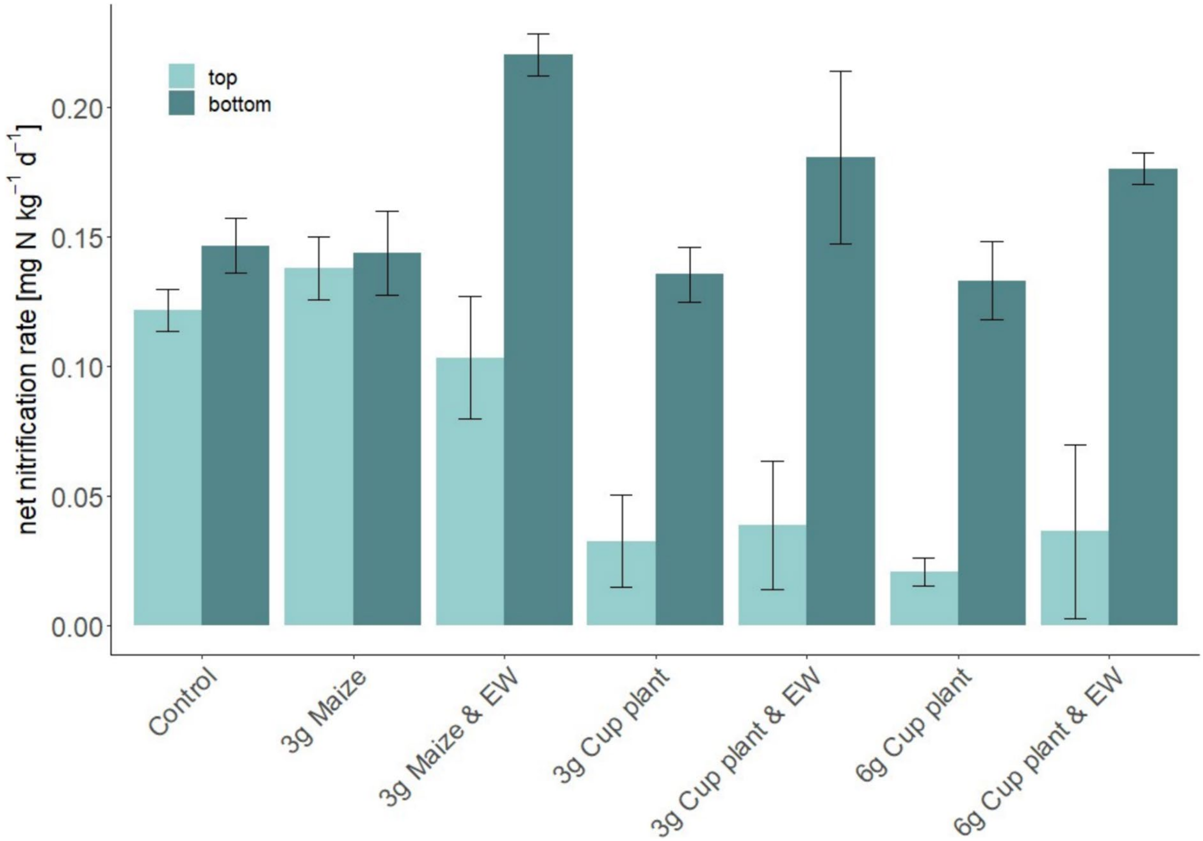

Fig. 4 Net nitrification rates [mg NO_3^--N kg^{-1} d^{-1}] (means ± SE, $n=4$) in the top 0–10 cm and bottom 10–20 cm of the soil columns for control (no earthworms/no litter), and the following treatments: 3 g maize, 3 g cup plant and 6 g cup plant with and without earthworms (EW)

Stable isotope data

N fluxes from the labelled and non-labelled N pools

An overview of cumulated fluxes is presented in Table 4. Fluxes across the incubation period ranged from 1.55 ± 0.08 mg N m^{-2} in treatments with 3 g Maize to 1.76 ± 0.23 mg N m^{-2} in treatments with 6 g cup plant. Data for fp_N_2, Fp_N_2O and N_2Oi were determined from cumulated fluxes of fp_N_2O, $fp\_N_2+N_2O$ and are presented in Table 4 as well. For details of daily development of fluxes from the labelled and non-labelled pool see Supplementary Material, Fig. S4.

Pool-derived N_2O (fp_N_2O) aligned with cumulated total N_2O (see also Fig. 5). As presented in Tables 4 and 5 as well as in Fig. S5, earthworm presence significantly affected all pool-derived fluxes and, also N_2Oi ($P<0.0001$). Earthworms facilitated a three- to almost sevenfold increase of fp_N_2O compared to their litter-only counterparts (Table 4). The respective increase of $fp\_N_2+N_2O$ was 12- to 33-fold ($P<0.0001$). The substantial difference between fp_N_2O and $fp\_N_2+N_2O$ in earthworm treatments is due to a high share of emitted N_2 from the labelled pool. Consequentially, due to earthworm presence, N_2Oi derived from cumulated fluxes was significantly decreased by 73% to 81% to a range of 0.08 to 0.11 (Table 4), showing that presence of earthworms enhanced N_2O reduction to N_2 ($P<0.0001$).

Cumulated Fp_N_2O were not significantly affected by earthworms. However, litter type and amount were found to be of importance for Fp_N_2O ($P_{litter\ type,\ amount}<0.05$). Regarding the pool-derived fluxes of fp_N_2O and $fp\_N_2+N_2O$, litter amount and type caused significant differences in both cases

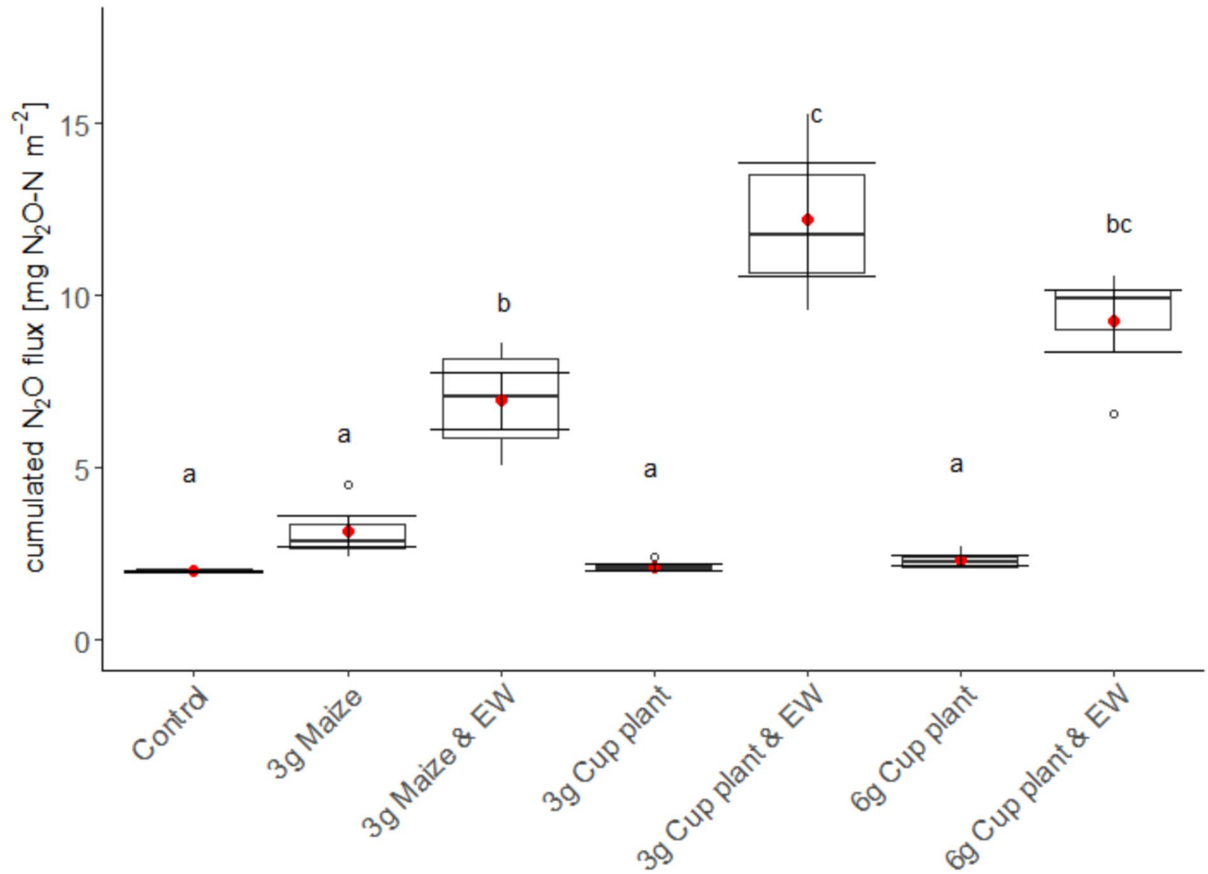

Fig. 5 Cumulated N_2O [mg N_2O-N m^{-2}] emissions (mean ± SE (model-derived), $n=4$) of 29 days with compact letter display derived from a simplified model for the control as well as for treatments with 3 g maize, 3 g and 6 g cup plant and the respective earthworm (EW) treatments. Means are indicated by a red dot. Different letters indicate statistical differences ($P<0.05$)

Table 3 Ratio of variance among groups and variance within groups (F) and the corresponding p-value (Pr > F) of the complex linear models with cumulated CO_2 and N_2O as dependant log-transformed variables and earthworm presence, litter type, litter amount and interaction of earthworms with litter type and amount as independent variables

	log(CO_2)		log(N_2O)	
	F	Pr(> F)	F	Pr(> F)
Earthworms	714.73	< 2.2e-16***	317.28	9.738e-14***
Litter type	446.34	< 2.2e-16***	1.91	0.1743808
Litter amount	144.18	1.345e-10***	0.44	0.5130559
Earthworms:litter type	14.33	0.001163**	19.86	0.0002419***
Earthworms:litter amount	1.40	0.251395	3.21	0.0882763$^{(*)}$

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '' 1

($P_{litter\ type,\ amount}<0.05$), while N_2Oi was not affected. Earthworm presence affected fluxes, as also indicated by a significant interaction of earthworms and litter type ($P_{earthworm:litter\ type}<0.05$). Regarding fp_N_2, litter effects and their interactions with earthworms were less pronounced, as only trends were found for litter

Table 4 Cumulated fluxes originating from the labelled (*fp_N$_2$O, fp_N$_2$+N$_2$O, fp_N$_2$*) and non-labelled (*fn_N$_2$O*) N pools (means ± SE, $n=4$); Fp_N$_2$O and *N$_2$Oi* derived from cumulated aggregated fluxes

	Total N_2O [mg N m^{-2}]	*fp_N$_2$O* [mg N m^{-2}]	*fp_N$_2$+N$_2$O* [mg N m^{-2}]	*fp_N$_2$* [mg N m-2]	*fn_N$_2$O* [mg N m^{-2}]	Fp_N$_2$O	N$_2$Oi
Control	2.00 ± 0.04	1.41 ± 0.09	3.00 ± 0.25	1.59 ± 0.18	0.59	0.70 ± 0.04	0.47 ± 0.02
3 g Maize	3.16 ± 0.46	1.54 ± 0.24	4.21 ± 1.18	2.67 ± 1.02	0.49	0.50 ± 0.08	0.41 ± 0.08
3 g Maize & EW	6.95 ± 0.82	4.69 ± 0.61	51.59 ± 6.09	46.89 ± 5.50	2.3	0.68 ± 0.04	0.09 ± 0.00
3 g Cup plant	2.13 ± 0.10	1.78 ± 0.26	4.22 ± 0.44	2.44 ± 0.43	0.45	0.85 ± 0.15	0.43 ± 0.07
3 g Cup plant & EW	12.21 ± 1.66	11.73 ± 1.86	140.39 ± 18.37	128.66 ± 16.74	0.5	0.95 ± 0.03	0.08 ± 0.01
6 g Cup plant	2.32 ± 0.14	1.52 ± 0.31	3.70 ± 0.31	2.18 ± 0.17	0.8	0.64 ± 0.09	0.40 ± 0.05
6 g Cup plant & EW	9.26 ± 0.90	6.88 ± 0.63	67.86 ± 10.03	60.98 ± 10.00	2.38	0.75 ± 0.04	0.11 ± 0.02

Data are given as means (± SE, $n=4$) for the control and the following treatments: 3 g maize, 3 g and 6 g cup plant with and without earthworms (EW). For statistical evaluation, see Table 5

amount as well as for the interaction of earthworms and litter amount ($P_{litter\ amount,\ earthworm:litter\ amount} < 0.1$). As for *Fp_N$_2$O*, major differences were attributed to the significant effect of litter type and amount ($P_{litter\ type,\ litter\ amount} < 0.05$). In particular, the *Fp_N$_2$O* of maize treatments were up to 70% lower than cup plant counterparts without earthworms and up to 40% lower than cup plant counterparts with earthworms (Table 4), indicating a lower share of denitrification in the total N_2O flux. No significant differences were found regarding earthworm presence. Treatments with 3 g cup plant, however, were considerably higher, indicating the fraction of N_2O derived from the labelled pool to be 0.85 ± 0.15 (3 g cup plant) and 0.95 ± 0.03 (3 g Cup plant & EW). This implied that almost all of the emitted N_2O was derived from the labelled pool.

An illustration of the time course of *Fp_N$_2$O* and *N$_2$Oi* for earthworm treatments can be found in the Supplementary Material (Fig. S5), showing that *N$_2$Oi* declined in some treatments during first half. *Fp_N$_2$O* remained relatively constant, although a slightly increasing trend in cup plant treatments with earthworms were found around day 8.

^{15}N enrichment of the labelled N pool

At the first date of isotopic analysis of gas samples (day 3) the enrichments of the nitrate pool undergoing denitrification (*apN$_2$O*) of all treatments were lower (40 to 50 at % ^{15}N) compared to the ^{15}N enrichment of the added NO_3^- (60 at% ^{15}N). Subsequently, ap_N$_2$O showed a steady decline throughout incubation ($P < 0.0001$) (Fig. 6). Cup plant treatments with earthworms were found to be significantly different ($P < 0.05$) from all other treatments, although the slopes of earthworm treatments with 3 g maize and 3 g cup plant shared similarities ($P < 0.05$). The two cup plant treatments with earthworms showed lower *apN$_2$O* in the first 12 to 14 days of incubation and remained constant afterwards, while the other treatments including the control similarly started out with a higher enrichment and steadily declined in the following days.

Discussion

Turnover of organic matter affected by earthworm activity and litter quality and quantity

Organic matter decomposition proceeds – with and without earthworms – in close relation to its composition. During senescence, protein, nucleic acids and lipids are relocated from the leaves, leaving them with less available C and N (Bleecker and Patterson 1997, Lashermes et al. 2021) and, thus, being less attractive for mineralisation. Earthworms, however, were found to increase the decomposition of low-quality litter (Jiang et al. 2018). Accordingly, in our study, earthworm activity significantly increased the decomposition of senescent litter from arable crops and, thus,

Table 5 Ratio of variance among groups and variance within groups (F) and the corresponding p-value (Pr > F) of the linear models with Fp_N_2O, N_2Oi, cumulated N_2O and N_2+N_2O fluxes from the labelled pool (*fp_N_2O* and *fp_N_2+N_2O*) and N_2 fluxes from the labelled pool derived from cumulated fluxes (*fp_N2*) as dependent log-transformed variables as well as earthworms, litter type, litter amount and interaction of earthworms with litter type and amount as independent variables

	Fp_$N_2O_{cumulated}$		log($N_2Oi_{cumulated}$)		log(*fp_N_2O*)		log(*fp_N_2+N_2O*)		log(*fp_$N_{2\ cumulated}$*)	
	F	Pr(> F)	F	Pr(> F)	F	Pr(> F)	F	Pr(> F)	F	Pr(> F)
Earthworms	2.77	0.11144	208.83	4.776e-12 ***	198.56	7.575e-12***	758.33	< 2.2e-16***	504.86	1.157e-15***
Litter type	4.38	0.02636*	0.66	0.5258	4.04	0.03359*	4.26	0.02878*	2.56	0.10209
Litter amount	6.48	0.01923*	0.29	0.5971	5.24	0.03313*	7.56	0.01237*	3.32	0.08360(*)
Earthworms:litter type	0.26	0.61848	0.00	0.9940	6.01	0.02354*	5.78	0.02599*	2.70	0.11586
Earthworms:litter amount	0.0000	0.97923	1.04	0.3208	1.44	0.24458	4.59	0.04471*	3.03	0.09693(*)

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

caused 14 to 30% higher CO_2 emissions compared to litter-only treatments. These results are comparable with the 22% increase in CO_2 emission caused by *L. terrestris*, that was observed by Engell et al. (2021) and is in line with a meta-analysis by Lubbers et al. (2013a), who found a 33% increase of CO_2 emissions caused by earthworms.

Earthworms increase respiration and CO_2 emissions due to their burrowing and feeding activities but also by secreting mucus and excreting urine (Lee 1985; Lv et al. 2020). Anecic earthworms in particular drag fresh organic matter into their burrows and incorporate it into their casts by feeding on it (Lee 1985). Thereby, they promote litter fragmentation and enhance the surface of the organic residues as well as the contact between soil and organic matter, thus facilitating microbial attack and respiration (Domínguez et al. 2010; Engell et al. 2021; Lee 1985). These activities increase the availability of easily degradable carbon (Lee 1985; Lv et al. 2020), i.e. water-extractable C like non-cellulosic polysaccharides, proteins or carboxylic groups (Majumder and Kuzyakov 2010; Surey et al. 2020), as a substrate for respiration (Rummel et al. 2020; Senbayram et al. 2012). The availability of these substrates was significantly controlled by litter type and amount. As evident, the treatment with 6 g of cup plant litter emitted more CO_2 than its counterpart with only 3 g of cup plant litter. Litter type matters as the maize treatments show in comparison to cup plant. Without earthworms, the addition of 3 g maize lead to higher cumulated fluxes of CO_2 than the addition of 3 g cup plant (Fig. 4). In the presence of earthworms, cumulated CO_2 fluxes increased for both treatments and differences subsided. This result may be linked to the respective litter decomposition. In our study, the CN ratio of the maize litter was 30.1 while the CN ratio of cup plant litter amounted to 63.9. This indicates, that although organic matter with narrow CN ratios may be preferably mineralised (Curry and Schmidt 2007), earthworms improve decomposition of organic matter with wide CN ratios or recalcitrant compounds, i.a., by creating hot spots and triggering priming effects (Bityutskii et al. 2012; Jiang et al. 2018; Kuzyakov et al. 2000; Kuzyakov and Blagodatskaya 2015). This dynamic, in turn, may affect CO_2 emission patterns leading to higher earthworm-mediated CO_2

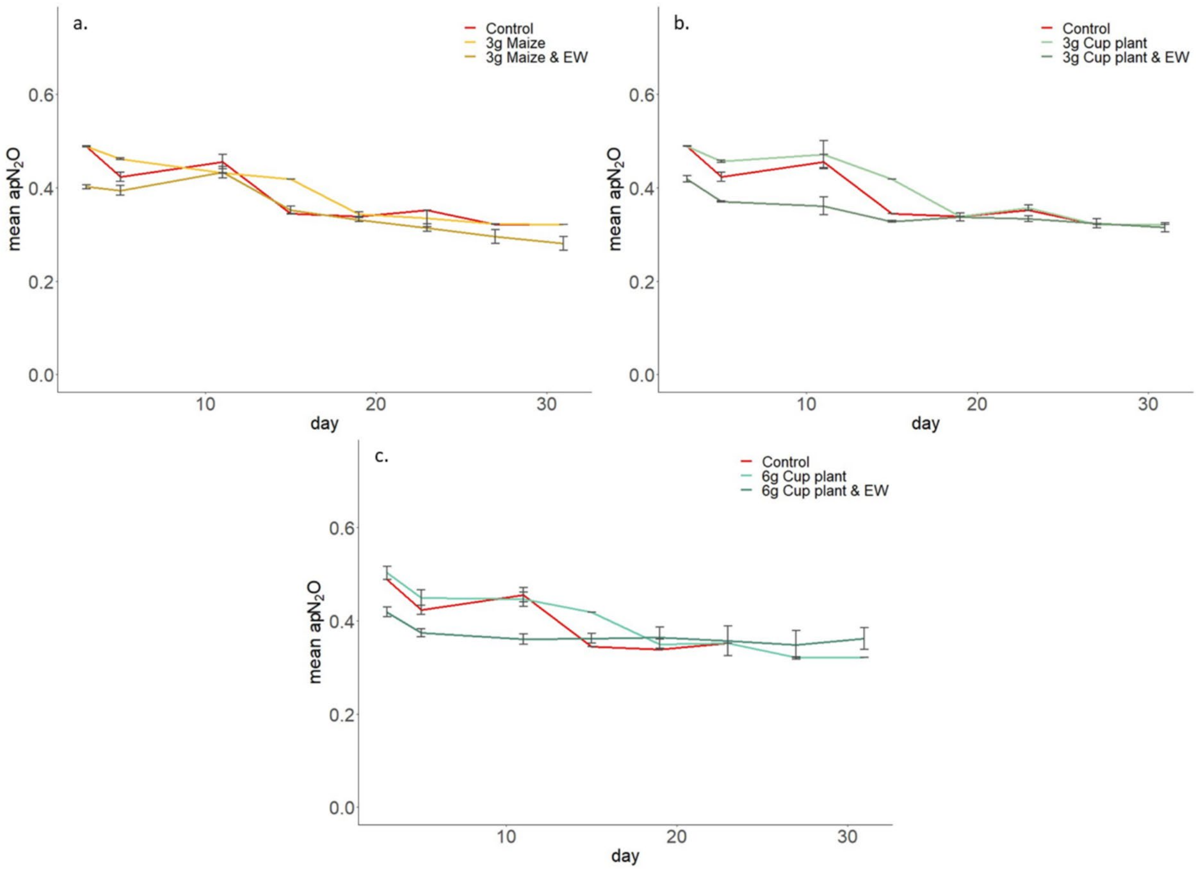

Fig. 6 Temporal development of the [15]N enrichment of the pool undergoing denitrification (*apN_2O*, mean SE, $n=4$) of (**a.**) the control and the 3 g maize treatment with and without earthworm species *L. terrestris* (EW), (**b.**) the control and the 3 g cup plant treatment with and without earthworm species *L. terrestris* (EW) and (**c.**) the control and the 6 g cup plant treatment with and without earthworm species *L. terrestris* (EW)

emissions from recalcitrant organic matter (here: cup plant), in comparison to litter of higher quality such as maize. This was emphasized by the remaining total C in the litter after incubation, which was generally lower in earthworm treatments. Additionally, underlying processes of respiration may be further affected by litter palatability and accessibility as well as the possible occurrence of varying phenolic compounds in cup plant and maize litter, which may alter earthworm feeding behaviour and, thus, decomposition (Wöhl et al. 2023). Hence, our first hypothesis, that earthworm activity increases removal of senescent litter from the soil surface and thus CO_2 fluxes from soil as an expression of soil respiration depending on litter type and amount, was confirmed.

Effects of litter quality and quantity as well as earthworm activity on N_2O and N_2 emissions

While most previous studies on earthworms affecting N_2O dynamics focused on total N_2O fluxes only, our study also distinguished N_2O sources and quantified N_2O reduction to N_2. This allows us to evaluate combined effects of earthworms and senescent litter on these processes which has not been done previously to the best of our knowledge.

In our study, we used senescent cup plant litter (CN ratio of 63.9) and senescent maize litter (CN ratio of 30.1). Even though both CN ratios suggest N immobilization (Trinsoutrot et al. 2000), it is also implied, that maize litter, much like its fresh form, supplies more available N. Nevertheless, litter type and amount as standalone factors did not significantly affect overall cumulated N_2+N_2O emissions. Moreover, these factors did also not affect N_2O reduction to N_2, since N_2Oi was also not different between litter only treatments. The absence of notable effects was likely due to the surface application of the litter, where creation of anoxic conditions around decomposing litter was hindered by the direct contact with the atmosphere, whereas complete O_2 depletion in pore space is typically limited to conditions when litter is not in contact with air filled pores (Lucas et al. 2024). Moreover, the low impact of the senescent litter in absence of earthworms might to some extent also be due to the wide CN ratios causing N immobilisation, recalcitrant compounds as well as low microbial attack of the litter at the surface due to a limited soil-litter interface (Engell et al. 2021; Trinsoutrot et al. 2000).

While cumulated fluxes of total N_2O and also of pool-derived N_2 and N_2O from litter-only treatments did not differ much from the control, earthworms drastically increased those fluxes in both cup plant and maize litter treatments. Cumulated N_2+N_2O fluxes correlated with the litter removal from the soil surface and cumulated CO_2 fluxes, with highest cumulated N_2+N_2O fluxes always associated with earthworm presence, showing that earthworm-mediated litter removal from the soil surface and subsequent decomposition and respiration was a main driver of denitrification rates in our study. With respect to total N_2O fluxes, our results are in line with studies of Marhan et al. (2015) and Schorpp et al. (2016). The first observed a 62% to 122% increase in N_2O emissions using endogeic *Aporrectodea caliginosa* and elevated soil temperatures, while for the second, the addition of *L. terrestris* resulted in doubled N_2O emissions from sandy soil and a tenfold increase of emissions from loamy soils (Marhan et al. 2015; Schorpp et al. 2016). Earthworm-mediated decomposition increases labile carbon compounds as an energy source for denitrifying bacteria and increase NO_3^- in deeper parts of the soil as substrate for denitrification (Rummel et al. 2020; Senbayram et al. 2012; Senbayram et al. 2020; Weier et al. 1993). Furthermore, *L. terrestris* was observed to be very efficient in incorporating the surface applied litter into the soil. Due to the limited O_2 diffusion between the atmosphere and the deeper soil layers this leads to hotspots of oxygen depletion and transient anaerobic microsites (Schlüter et al. 2024), which offer suitable conditions for denitrification (Marhan et al. 2015; Millar and Baggs 2005; Rummel et al. 2020; Surey et al. 2020).

The increase in N_2O and N_2 fluxes due to earthworms was affected by litter type since emissions from treatments with 3 g cup plant litter were almost 3 times higher compared to 3 g maize litter. As mentioned before, this circumstance may be traced back to differences in litter composition and CN ratio and aforementioned higher respiration of cup plant.

As a result of litter incorporation, O_2 diffusion between litter-induced hot spots and the soil surface takes longer, enhancing O_2 depletion around incorporated litter and thus increasing the anaerobic volume and N_2+N_2O production by denitrification (Schlüter et al. 2024). In summary, the first part of our second hypothesis has to be rejected since there were no significant differences regarding N_2O and N_2 fluxes when applying senescent cup plant and maize litter. Earthworm activity, on the other hand, did increase N_2O and N_2 fluxes from the soil, hence the second part of the hypothesis was validated.

Overall N_2O emissions in our study were small, as they only amount to up to approximately 122 g N per ha in 30 days. This would be equivalent to 1.48 kg of N per ha and year, which is comparatively low for cropland (Jungkunst et al. 2006). The emission of N_2 combined with N_2O in our study correspond to a loss of up to 16 kg N from the soil per ha and year implying comparatively moderate denitrification (Pan et al. 2022). But it must be noted that upscaling of our laboratory results to field conditions is limited in view of the absence of plant, management and climate effect.

N_2O production pathways in relation to substrate availability and earthworm activity

The main source of N_2O emissions was denitrification, as the fraction of N_2O originating from the labelled N pool (Fp_N_2O) ranged between 0.5 and 0.93, meaning 50 to 95% of the total emitted N_2O came from the ^{15}N-labelled nitrate pool. The remainder originated from unlabelled N pools like organic N, NH_4^+-N and the N-oxides produced from those pools (Müller and Clough 2014) and could thus be produced by nitrification or nitrifier denitrification (Wrage-Mönnig et al. 2018). Nitrification directly contributing to N_2O emissions may occur by autotrophic ammonia oxidising archaea and bacteria

(Hu et al. 2015; Liu et al. 2016) or as heterotrophic nitrification given the low soil pH (Butterbach-Bahl et al. 2013; Wrage-Mönnig et al. 2018). For all litter treatments, although not significant, Fp_N_2O was found to be higher in earthworm treatments. Hence, our third hypothesis that earthworm activity and earthworm-controlled decomposition increase the fraction of N_2O originating from denitrification was validated. This can be explained by the enhancement of denitrification by litter incorporation while N_2O production from unlabelled sources (fn_N_2O) was unchanged or even declined in the 6 g cup plant & earthworm treatment (Table 4). The type of litter used in the respective treatment also affected the Fp_N_2O with higher values for cup plant compared to maize. Litter incorporation has previously been shown to increase Fp_N_2O (Pfülb et al 2024) and was explained by the intensification of N_2+N_2O production by denitrification. Hence, the higher Fp_N_2O of cup plant treatments might be due to the higher denitrification rates of cup plant treatments compared to maize treatments when the litter was incorporated by earthworms (Table 4). The fact that, in contrast to the response of N_2+N_2O fluxes from denitrification, fn_N_2O fluxes were quite low and exhibited little variation (Fig. S5, Supplementary Material) suggests that N_2O from nitrification and nitrifier denitrification was not strongly affected by earthworm activity and associated litter dynamics. One explanation could be the fact that the N_2O yield of nitrification is quite low (Hink et al. 2017). Moreover, while decomposition of incorporated litter lead to anoxic denitrifying microsites and, thus, strong earthworm and litter effects on denitrification, nitrification proceeds in the well aerated soil matrix. This larger part of the soil volume changed only to a minor extent, whereas the anaerobic volume where denitrification occurs is typically only a small fraction of the total soil volume (Schlüter et al. 2024), especially when induced by litter in relatively dry soil. Autotrophic and heterotrophic nitrification could have also led to a potential release of NO_3^- from SOM, as shown by net nitrification rates. Even though net nitrification rates were low, the released unlabelled NO_3^- may have acted as a precursor for N_2O via denitrification (Prosser et al. 2020). Nitrate from nitrification could either directly produce unlabelled N_2O (thus being attributed to fn_N_2O flux) or dilute the ^{15}N-labelled pool and its N_2O production would be attributed to the fp_N_2O flux. We obtained some evidence on differences in the dilution of the labelled N pool from the non-random distribution of emitted N_2O isotopologues which was used to calculate the ^{15}N enrichment of the labelled NO_3^- pool producing N_2O (ap_N_2O). In all treatments, initial ap_N_2O values were lower than the enrichment of the added NO_3^-, showing that the added NO_3^- mixed with unlabelled NO_3^- already present in the soil or produced by nitrification during the first days of incubation. However, this difference was largest in the earthworm treatments with cup plant litter since initial ap-values were lower compared to the other treatments (Fig. 6). Because pre-treatment and ^{15}N-labelling was identical for all treatments, these differences point towards initially enhanced mineralisation-nitrification processes in the earthworm treatments with cup plant litter, which we cannot explain so far based on our data. Further studies would be needed to reveal whether the quality of senescent cup plant leaves, their microbial biome or specific interactions with earthworms could be an explanation. Moreover, subsequent ap-values of the earthworm treatments with cup plant litter were constant while N_2+N_2O fluxes gradually increased (Fig. S4, Supplementary Material). This suggests that in the denitrifying microsites initiated by incorporated cup plant litter, unlabelled NO_3^- was produced or incorporated and the ^{15}N enrichment in the denitrifying microsite was thus diluted. We assume that the subsequent consistency in ap_N_2O was due to inhibition of nitrification in the microsite due to absence of oxygen. In all other treatments, however, we found a steadier decline in ap_N_2O which has often been observed in previous soil incubation studies using the ^{15}N gas flux method (Kemmann et al. 2023; Pfülb et al. 2024; Rummel et al. 2021) and which had been explained by continuous dilution of the ^{15}N pools by unlabelled NO_3^- from by nitrification in aerated parts of the soil matrix. Earthworm casts may also play a role, as a relationship was found between cumulated N_2O fluxes and NH_4^+ in earthworm casts. Even though mineral N in casts occurred mainly as NH_4^+, which is unfavourable for denitrification (Senbayram et al. 2012), earthworm casts may be potential hotspots of N_2O derived from nitrification by autotrophic ammonia oxidising archaea and bacteria (Hu et al. 2015; Liu et al. 2016) or from heterotrophic nitrification (Wrage-Mönnig et al. 2018).

N_2O reduction to N_2 in relation to substrate availability and earthworm activity

Since earthworms promote the production of N_2O but even more so the production of N_2 by incorporating and breaking down low quality litter, their presence strongly lowered the $N_2O/(N_2+N_2O)$ product ratio of denitrification (N_2Oi). In presence of earthworms, N_2O fluxes were only 8 to 11% of N_2+N_2O fluxes compared to 40 to 47% in absence of earthworms. Our fourth hypothesis, that earthworm activity and earthworm-controlled decomposition enhance N_2O reduction to N_2 and thus lower N_2Oi, was thus validated. The production of N_2O and its reduction to N_2 are both partial processes of denitrification and thus their main drivers are identical. However, low O_2 is known to increase N_2O reductase activity (Morley et al. 2008) and N_2Oi also depends on the actual denitrification rate and its dependence on the balance between the contents of available NO_3^- and labile organic C, inhibiting and enhancing N_2O reduction, respectively (Senbayram et al. 2019, 2022). In line with Grosz et al. (2022), Pfülb et al. 2024 and Giannopoulos et al. (2010), the incorporation of litter or organic manure into the soil is a key factor in driving N_2O and N_2 production. While in those studies the organic substrates were mixed in or injected by simulated agricultural management practice, in our case, earthworms incorporated the litter which was accompanied by a steady earthworm-controlled provision of labile C. These combined earthworm effects favored N_2O reduction to N_2 and thus lowered N_2Oi. Apparently, these factors outweighed the counteracting effects of high NO_3^- content (Senbayram et al. 2019) and low pH (Nadeem et al. 2020). On this note, it may further be assumed that earthworms also counteracted inhibitory effects of low soil pH levels on N_2O reductase to a certain extent. Low pH levels are known to promote N_2O rather than N_2, because acidic conditions suppress the synthesis of N_2O reductase and thereby the reduction of N_2O to N_2 (Kemmann et al. 2021; Liu et al. 2014; Nadeem et al. 2020). Earthworms, however, modify the pH in the drilosphere of their burrows towards neutral by secreting epidermal mucus (Schrader 1994).

While the application of senescent litter in conjunction with earthworm activity caused a clear and drastic lowering of N_2Oi and increasing of N_2+N_2O fluxes, the exact regulation of this effect could not be elucidated by our study as it is not clear to which extent the interplay of earthworm-modified pore structure, spatial distribution of substrates, pH, microbial communities, water and oxygen affected process dynamics (Schlüter et al 2024). Moreover, future studies should include both fresh and matured litter of the same crop (Süß et al. 2024; Surey et al. 2020) and may employ ^{13}C-labelled leaf litter (Rummel et al. 2021) to better understand the fate of labile C in natural mineralization processes.

Implications of experimental setup

The measured earthworm effects in our study may be short-term, as described in a field study by Ejack et al. (2021), who found a short-term increase of N_2O after earthworm addition, which faded after a year. Although the authors assumed earthworm mortality as one reason, they also stressed the importance of initial burrowing activity after earthworm addition. In experiments with repacked soils, the effects of this initial burrowing activity may be of even greater importance (Lubbers et al. 2013a). To test this hypothesis, a control with earthworms only but without litter should be included. This would allow investigation of the hypothesised effect of initial burrowing and enable more robust conclusions regarding the results and their interpretation under controlled conditions. Burrowing activity is furthermore an intrinsic feature of the specific functional earthworm group. Earthworm effects in the present study were caused by the anecic behaviour of adult *L. terrestris*, thus effects of earthworm species of other ecological groups are expected to differ. This instance may also be valid for other factors affected by initial burrowing activity, such as CO_2 emissions and effects on the soil decomposer community. Future studies may assess the factors with a larger variety of earthworm species; especially since earthworm communities in agricultural soils often include larger populations of *Aporrectodea spp*. (Emmerling et al. 2021; Schorpp and Schrader 2016).

Furthermore, the contribution of formation pathways of N_2O, such as nitrifier denitrification or fungal denitrification were not assessed. However, these may not be ruled out. Nitrifier denitrification is linked to nitrite (NO_2^-) availability as well as to low soil C contents and pH levels (Hu et al. 2015, Wrage-Mönnig et al. 2018), which probably occurred at some points in time during incubation, as this is often the case at soil-litter interfaces (Rummel et al. 2020; Wrage-Mönnig et al. 2018). Fungal denitrification can be linked to the application of crop residues, as shown by Senbayram et al. (2018). Therefore, it may have occurred in our case as well. However, the assessment of fungal denitrification requires the examination of ^{15}N site preference (Rohe et al. 2021), which was not conducted in this study. To better explain litter and earthworm effects on N_2 and N_2O fluxes, future studies should thus additionally aim to distinguish those processes. Furthermore, microbial activity in casts may be short-lived (Blouin et al. 2025); hence, N_2O formation in casts may be limited to hot moments, whose contribution to the total emissions may be examined in future studies.

Finally, due to experimental constraints, living plants could not be included. However, root uptake and root exudates, for example, affect nitrification and denitrification processes. Future studies examining these processes may take into account the effect of both, living plants and the intricate characteristics of senescent plant residues, for a more holistic approach.

Conclusion

Earthworms provided a valuable ecosystem service in decomposing recalcitrant senescent litter and potentially adding to nutrient turnover. By increasing litter decomposition earthworms increased denitrification and N_2O reduction to N_2 as well when provided with recalcitrant senescent maize and cup plant litter. Higher emissions, however, were not linked to the higher litter N content of maize. The interaction of various nitrification and denitrification processes in the backdrop of earthworm-mediated decomposition of senescent litter is extraordinary complex. In contrast to fresh organic matter, senescent litter and the physiological and behavioural response of earthworms to its subpar palatability seems to have a greatly different effect on priming effects, mineralisation patterns and substrate availability regarding the aforementioned nitrification and denitrification processes. In our study, this led to a point, where the N content and CN ratio of the litter could not explain N emissions anymore. Furthermore, the N_2 emissions we found were substantially higher in earthworm treatments and even overtook N_2O emissions indicating a loss of N from the soil. Earthworms indeed help gain nutrients from recalcitrant field residue. But for these nutrients not to be lost with detrimental effects on the climate, an integrated fertilization management with respect to boundary conditions such as soil moisture, pH and plant demand is required. In view of the limitations of our soil incubation approach it is important to include organic fertilization and living crops in addition to earthworms and senescent litter in future research to assess fertilization regimes, that protect the climate while sufficiently providing nutrients for the cultivated crop.

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Author contribution Lena Wöhl: study conception, experimental design, conduction of experiment, data collection and analysis, statistical evaluation of data, writing of first draft and editing. Björn Kemmann: study conception, contribution to first draft. Thorsten Ruf: advice to experiment, contribution to first draft. Christoph Emmerling: project coordinator, advice to experiment, contribution to first draft. Reinhard Well: supervision, advice to study conception and experimental design, evaluation and interpretation of isotopic data, contribution to first draft. Stefan Schrader: supervision, advice to study conception and experimental design, contribution to first draft. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interest The authors have no relevant financial or non-financial interests to disclose.

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