Crop residue displacement by soil inversion: Annelid responses and their impact on carbon and nitrogen dynamics in a lab-based mesocosm study

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ABSTRACT

In the context of sustainable agriculture, a deeper knowledge of the effects of soil management on soil annelids is needed, as they play an important role in many soil processes. In a laboratory mesocosm experiment, we compared the simulated effect of ploughing by inverting the top soil (crop residues at 15 cm depth) to a non-inversion treatment (crop residues on the soil surface) using the soil type Haplic Luvisol. We investigated the response of earthworms and enchytraeids and the consequences for microbial and chemical soil parameters. Four treatments with soil fauna were established by adding: (i) endogeic earthworms (*Octolasion cyaneum*), (ii) anecic earthworms (*Lumbricus terrestris*), (iii) a combination of two enchytraeid species (*Enchytraeus crypticus* and *Enchytraeus christenseni*) and (iv) having control columns (without annelids). Feeding behaviour of annelids was investigated using isotopic analysis (δ^{13}C, δ^{15}N), and chemical and microbial soil properties were measured. Carbon and nitrogen losses in the form of gas emissions (CO_2, N_2O) and leachate were recorded during the time of incubation.

We found no interactions of soil inversion and annelid addition on chemical and microbial soil properties; these properties were closely related to crop residue placement, indicated by the effect of soil inversion between 0 and 20 cm. Below 20 cm, this effect disappeared. Here, the presence of enchytraeids enhanced soil microbial properties, regardless of soil inversion. Stimulating microbial activity and increasing soil aeration seem to be the most important factors that increase CO_2-C emissions in the presence of anecic earthworms. N_2O-N emissions were consistently higher (+188%) in the inverted columns. Our results show that regardless of the placement of crop residues, anecic earthworms and enchytraeids fed more on crop residue derived carbon than endogeic earthworms, while endogeic earthworms appeared to avoid feeding at the soil surface. Moreover, it was found that the inversion and the annelid effects did not interact in an experimental setting where soil inversion is carried out in a form without detracting or impairing the annelids directly.

1. Introduction

Major knowledge gaps regarding earthworm behaviour, their biology, and their impact on soil function and ecosystem services persist (Blouin et al., 2013). Soil annelids (earthworms, enchytraeids) react sensitively to human impacts, e.g. agricultural management practices. Many studies have reported that conventional ploughing decreases abundance and biomass of anecic earthworms in the field (e.g. van Capelle et al., 2012; Briones and Schmidt, 2017). Anecic earthworms

preferably feed at the soil surface (Bouché, 1977), and soil inversion due to ploughing relocates their food source belowground. In addition, ploughing destroys the vertical burrows of anecic earthworms and takes away their day time shelter (Briones and Schmidt, 2017). In contrast, biomass and abundance of endogeic earthworms increases in ploughed fields (Chan, 2001). In order to recommend soil management practices that support annelids and their functions, it is imperative to understand how these management practices affect annelids activity (e.g. feeding behaviour as a response to ploughing) and how microbial and chemical

soil properties are altered.

Earthworms act like 'engineers' in the soil system (Lavelle et al., 1997; Jouquet et al., 2006; Blouin et al., 2013). They translocate soil particles and organic matter during feeding and egesting (Taylor et al., 2018), contribute to the transfer of carbon in the soil (Jégou et al., 1998), and boost the turnover of soil organic material by stimulating microbial activity (Hoang et al., 2017). Briefly, anecic earthworms are primary decomposers, which feed directly on partially decayed organic material, whereas endogeic earthworms are secondary decomposers and feed on soil and its organic matter (Curry and Schmidt, 2007). Seeber et al. (2006) showed that earthworms belonging to primary decomposers (*L. rubellus*) strongly increased litter breakdown, whereas secondary macro-decomposers (*O. lacteum*) had no or even negative effects on litter breakdown. To investigate feeding ecology and trophic relationships of annelids, analysing stable isotope ratios (C, N) offers a powerful instrument (Scheu and Falca, 2000; Schmidt et al., 2004; Curry and Schmidt, 2007).

Greenhouse gas emissions in agriculture are known to be influenced by soil fauna and soil disturbances. For instance, studies have shown an increase in CO_2 (Lubbers et al., 2013) and a decrease in N_2O (Kuiper et al., 2013) due to the presence of earthworms. Gorbunova et al. (2020) even report on suppressive effects of earthworms on the emission of CO_2. The impact of soil invertebrate fauna on soil gas emissions seems variable as three different experiments of Kuiper et al. (2013) demonstrate; soil fauna can suppress, increase or on a temporal scale delay and accelerate N_2O emissions.

Enchytraeids are an often neglected part of the soil mesofauna. Compared with earthworms, much less is known about enchytraeids even though they are widely distributed in agricultural systems (Vavoulidou et al., 2009; Severon et al., 2012) and react sensitively to changes in management practices (Pelosi and Römbke, 2016). Enchytraeids contribute to the comminution and mixing of litter and soil. Thus, they enhance the surface area for microbial colonization along with passively transporting microorganisms through the various soil depths (Scheu et al., 2005). Enchytraeids are mainly secondary decomposers (Gajda et al., 2017) and feed on partially degraded plant fragments. Additionally, they regulate microbial communities by grazing on bacteria, fungi and microalgae (Gajda et al., 2017). This strong interaction of enchytraeids with microorganisms in soil is able to induce both increasing microbial activity by stimulation and also reducing activity due to stronger reductions of the microbial biomass by feeding (van Vliet, 2000). Concerning the effects of enchytraeids on CO_2 and N_2O emissions from soil van Vliet et al. (2004) could show strong dependencies to soil type and litter placement. Furthermore, the results of John et al. (2019) indicate that enchytraeids can promote the immobilization of carbon.

The influence of annelids on microorganisms is not yet fully understood. Many studies have reported that annelids decrease microbial biomass (Butenschoen et al., 2007; Sandor and Schrader, 2012; Wachendorf et al., 2014) due to feeding and may as a result alter microbial community composition (Eck et al., 2015). Microorganisms are considerably involved in many processes of the carbon and nitrogen cycle. For instance, they contribute to the storage and loss of nutrients. Earthworm burrows are known to be microbial hotspots (Kuzyakov and Blagodatskaya, 2015) and can improve the efficiency of organic matter decomposition by microorganisms (Hoang et al., 2017). Microbial parameters like basal respiration, microbial biomass and fungal abundance are indicators that reflect the ability of the soil system to store and cycle organic carbon over time (Creamer et al., 2016).

The aim of this study was to investigate how soil inversion including different crop residue placements in the soil profile (buried vs. unburied) affects ecological and functional impacts of certain annelids known to be dominating the annelid fauna in agricultural soils. We chose three different organisms or organism groups to represent (1) anecic earthworms, (2) endogeic earthworms and (3) endogeic enchytraeids. We further aimed to assess if this has consequences for soil microbial and chemical properties. To investigate impacts on nutrient fluxes, we quantified gas emissions (CO_2 and N_2O) and leachate losses (C and N). Corn (a C4 plant) leaves were selected as crop residues to follow the feeding behaviour of annelids, which was tracked using isotopic shifts in $\delta^{13}C$. We expected ecological groups to differ based on the proportion of crop residue derived C in the annelids tissue. We hypothesised that incorporating crop residues into the soil will be beneficial for endogeic earthworms, whereas litter on the surface will be advantageous for anecic species. We assumed that endogeic earthworms and enchytraeids (secondary decomposers, feeding on organic material that is already decomposed and colonised by microorganisms), absorb less crop residue derived C than anecic earthworms (primary decomposer, feeding on fresh organic material). Overall, we expected combined effects of both factors (annelid addition and soil inversion) on the chemical and microbial properties of the soil.

2. Material and Methods

2.1. Sampling for soil mesocosms

After harvest of winter wheat in August 2017 we sampled undisturbed soil columns for the mesocosm experiment at the study site "Garte Süd" (5°29′ N, 9°56′ E), which is located in the south of Göttingen, Lower Saxony, Germany (Jacobs et al., 2009). Referring to the complete randomized block design of "Garte Süd" we took 32 undisturbed soil columns from the minimum tilled plots (8 columns per block).

Plexiglass cylinders (15 cm diameter, 30 cm length, 0.018 m^2 surface area) were inserted into the soil and then carefully extracted using a spade. For annelid-defaunation, the columns were frozen for one week at $-20\,°C$. Defaunation of the soil columns was successful. This was verified at the Institute of Applied Soil Biology, Hamburg, Germany by extracting enchytraeids from 12 randomized sub-samples of four columns from the three depths (0–10 cm, 10–20 cm, 20–30 cm) using a wet-funnel technique without heating (ISO 23611-3, 2007).

The soil type in our columns was a Haplic Luvisol (Ehlers et al., 2000; Reiter et al., 2002) derived from loess with a pH of 7.2. It consists of 15.1% clay, 72.7% silt and 12.2% sand (Ehlers et al., 2000). Average annual precipitation and temperature at the sample location was 649 mm and 9.5 °C, respectively (Climate Data Center (CDC), 2018). Prior to column sampling, the soil was fertilized at a rate of 20.7 kg ha^{-1} mineral N in spring 2017 and winter wheat (2017 and 2016) and a mixture of peas and oat (2015) was grown.

2.2. Experimental design

The two factors soil inversion and annelid treatment were investigated under laboratory conditions in a randomized block ($n = 4$) experiment. The factor 'soil inversion' consisted of two levels: (i) Soil inversion with crop residues buried at 15 cm depth (SI) and (ii) non-inversion with crop residues at the soil surface (NI). For SI, the upper soil layer (0–15 cm) was inverted manually without destroying its natural structure by turning the layer over and replacing it back in the soil column. For NI, the undisturbed soil columns were left in their natural state and crop residues were placed on the soil surface. However, one has to keep in mind that mechanical or manual tillage in an arable field always leads to stochastic destruction and inversion.

In both, the SI and the NI treatment, 5 g of corn (*Zea mays* L.) leaves with a C/N ratio (mean $\pm$ standard deviation) of 42 $\pm$ 5 (43 $\pm$ 0.2% C, 1 $\pm$ 0.1% N) were used as crop residues. Leaves were collected in September 2017 from a field-site in the South of Germany, dried for 24 h at 60 °C and cut into pieces of 2–4 cm. The amount of corn leaves added to each soil column corresponded to ~2.8 Mg ha^{-1} on a field level. Compared to other earthworm mesocosm studies, such as Frazão et al. (2019) (2.2 Mg ha^{-1} winter wheat stubble + straw, radish residues) and Giannopoulos et al. (2010) (~2.6 Mg ha^{-1} radish residues) the amount

of crop residues was in a similar range.

The factor 'annelid treatment' consisted of four levels: Addition of (i) anecic earthworms (EW$_{anecic}$), (ii) addition of endogeic earthworms (EW$_{endo.}$), (iii) addition of a combination of two enchytraeid species (ENCH), and (iv) a control (CON) without any addition of worms to the SI and NI mesocosms. Adult individuals of earthworms from two different species (*Lumbricus terrestris*, *Octolasion cyaneum*) were collected five days prior to the start of the experiment. *L. terrestris* is one of the most abundant earthworm species in temperate Europe and belongs to the ecological group of anecic earthworms; it is widely spread in grassland, pastures and arable land (Sims and Gerard, 1999; Krück, 2018). As an endogeic earthworm *O. cyaneum* is widely spread over most soil habitats dwelling in forest soils, grassland and arable soils and has no preference for certain soil types (Sims and Gerard, 1999, Krück, 2018).

Earthworms were collected by hand-sorting from the same field-site, where the soil columns were sampled and were kept fresh at 4 °C. The indicators established by Fründ et al. (2010) were used to check if the earthworms were in good condition. Earthworms were cleaned with cold water and placed on a wet tissue for 24 h for defecation. For the EW$_{anecic}$ treatment, two individuals of L. *terrestris* were added per column and for EW$_{endo.}$ four individuals of *O. cyaneum*, which corresponded to 113 and 226 individuals m^{-2}, respectively. The chosen number of individuals m^{-2} was in the same range as abundances in the field. Ulrich et al. (2010) reported 221 worms m^{-2} in reduced tilled (chisel plough) plots at a long-term experimental field-site in Germany. For the ENCH treatment, a combination of 150 individuals of *Enchytraeus crypticus* and 200 individuals of *Enchytraeus christenseni* was used, which corresponded to ~20,000 ind. m^{-2} and was similar to values reported under conventional tillage (22,567 ind. m^{-2}) and reduced tillage (12,318 ind. m^{-2}) at a field-site in Germany (Severon et al., 2012). The biology and ecology of both species is similar. Both enchytraeid species originated from the same lab culture of the Institute of Biodiversity, Thünen-Institute, Braunschweig, Germany.

2.3. Incubation

The soil columns were placed on ceramic plates (pore diameter of 1 μm), to which a constant suction of 50 bar was applied. The leachates were collected weekly in glass bottles and frozen in polyethylene bottles until measurements began. All mesocosms were hermetically sealed and were randomly placed in a dark climate chamber at 10 °C. They were continuously supplied with fresh air and automatically sprinkled (3 times day^{-1} 10 mL; refers to 1.67 mm precipitation per day, which corresponds to annual precipitation at the field-site, where soil columns were sampled) with a 0.01 M CaCl$_2$ solution – to simulate the ionic strength of the soil solution. This irrigation procedure resulted in a soil moisture of 46% of the maximal water holding capacity of our soil. However, one has to keep in mind that the results of these mesocosm experiments are not directly transferable to field conditions with variations in moisture and temperature.

The lids were connected to a gas chromatograph by plug valves. Gas fluxes (CO$_2$ and N$_2$O) were measured every 4.5 h utilizing a gas chromatograph (Shimadzu Gas Chromatograph GC-14A, Duisburg, Germany), with an electron-capture detector (ECD) for determination of CO$_2$ and a flame ionization detector (FID) for N$_2$O (Loftfield et al., 1997). After a pre-incubation period of 20 days, the experiment run for 114 days.

2.4. Extraction of annelids

At the end of the incubation experiment, mesocosms were sampled destructively, keeping three soil depths (0–10 cm, 10–20 cm, 20–30 cm) separately. Earthworms were hand-sorted and weighed (fresh weight). Enchytraeids were extracted from soil samples by using a wet-funnel technique without heating by placing soil subsamples under water for

48 h (ISO 23611-3, 2007). In modification to ISO 23611-3 (2007), the water-solution containing the enchytraeids was sieved (20 μm) for collecting the enchytraeids. Thereafter, they were counted using a grid and a dissecting microscope with magnification 10 to 40 times.

2.5. Analyses of soil samples and leachate

Soil samples were taken at the three different depths from the mesocosms at the end of the experiment: 0–10 cm, 10–20 cm, 20–30 cm. Soil dry weight was measured by oven-drying (105 °C, 24 h). Ergosterol was measured as marker of saprotrophic fungi applying the method of Djajakirana et al. (1996). The determination of ergosterol was done by high-performance liquid chromatography (HPLC), followed by UV detection at 282 nm (Dionex UVD 170 L). Microbial biomass carbon (C$_{mic}$) and nitrogen (N$_{mic}$) were determined by Chloroform-Fumigation-Extraction (CFE) (Brookes et al., 1985; Vance et al., 1987; Wu et al., 1990; Joergensen, 1996). Half of the samples (10 g of homogenized sediment) were fumigated (24 h, 25 °C) with chloroform (CHCl$_3$). Afterwards, samples (non-fumigated and fumigated) were extracted with 40 mL 0.05 M K$_2$SO$_4$ on a horizontal shaker (200 rpm) and filtered. Estimations for the microbial activity (mg CO$_2$-C g C$_{mic}^{-1}$ d^{-1}) were calculated based on the metabolic quotient from Anderson and Domsch (1990); not using basal respiration rates, but total CO$_2$-C fluxes from the soil columns. The soil pH was measured in deionized water with a soil to solution ratio of 1:2.5. Percolates were analysed for DOC (dissolved organic carbon), TDN (total dissolved nitrogen) and DIN (dissolved inorganic nitrogen: NH$_4^+$, NO$_3^-$). DOC and TDN extracts were analysed by dry combustion with a multi N/C 2100 S (Analytik Jena, Jena, Germany). Dissolved inorganic nitrogen (DIN) was measured by ion chromatography (930 Compact IC Flex, Metrohm, Filderstadt, Germany). Dissolved organic nitrogen (DON) was calculated as TDN minus DIN.

2.6. Isotopic analysis

Isotopic ratios (δ^{13}C, δ^{15}N) as well as total masses for soil organic carbon (C$_{org}$) and total nitrogen (N$_{total}$) were measured for soil samples, for corn leaves and for annelid body tissues after defecation as described above (see 2.2). For soil, total C corresponded to C$_{org}$ as no carbonates were detectable. Measurements were done by isotope-ratio mass spectrometry Delta V Advantage (Thermo Fisher Scientific, Bremen, Germany). Soil samples were dried, ball milled and weighed in 5 × 9 mm sized tin capsules. Four samples of non-incubated corn leaves (dried at 60 °C, 24 h) were ball milled and analysed in the same way. Annelid tissues were dried (60 °C) before analysis; prepared as single subsamples of each worm, using the first body segments of every individual earthworm and a number of whole bodies of the enchytraeids (without separating the two species) until reaching a sample weight suitable for analysis (mean: 151 μg). Control samples (four replicates) for earthworms consisted of non-incubated earthworms from the same field-site; both earthworm species were tested separately and for enchytraeids the control were animals from the lab-culture (non-incubated). The analyses took place at the Centre for Stable Isotope Research and Analysis, University of Göttingen, Germany. Dual stable isotopes (δ^{13}C and δ^{15}N) were measured on the same sample. The results are expressed as the delta notation in parts per thousand: δ ^{13}C/^{15}N [‰]. V-PDB was used as the standard for ^{13}C and atmospheric dinitrogen for ^{15}N, respectively. The proportion of crop residue derived C in annelid tissue C (ATC$_{crd}$ %) was calculated with the following equation based on Balesdent and Mariotti (1996):

$$ATC_{crd}\% = \left(\delta^{13}C_{AT} - \delta^{13}C_{CAT} \right) / \left(\delta^{13}C_{CR} - \delta^{13}C_{CAT} \right)$$

where δ ^{13}C$_{AT}$ is the δ ^{13}C value from each individual annelid tissue at the final sampling day, δ ^{13}C$_{CAT}$ is the initial δ ^{13}C value of the control annelid tissues at day 0, δ ^{13}C$_{CR}$ is the δ ^{13}C value of the crop residues (corn leaves) at day 0.

2.7. Statistics

Data were analysed using the statistical software R (Version 3.6.1, R Core Team, 2019) with packages *car* (V. 3.0-3, Fox and Weisberg, 2019) and *multcomp* (V. 1.4-13, Hothorn et al., 2008). Analysis of variance (ANOVA) was carried out to determine if there were significant differences between treatments. ANOVAs were carried out separately for each depth with $n = 32$. The effects of block (4 factor levels), soil inversion (2 factor levels), annelid treatment (4 factor levels) and the interaction of soil inversion and annelid treatment were tested. Stepwise model reductions were carried out. We first eliminated a non-significant interaction, then non-significant main effects (Crawley, 2012), independent of the factor 'block'. Residuals of the final model for each variable were checked for homoscedasticity graphically and using Levene's test and for normal distribution by the Shapiro–Wilk test and graphically by inspecting QQ-plots. In the case of a significant treatment effect in the ANOVA, Tukey's HSD tests were applied to conduct pairwise comparisons between treatment levels.

For soil analysis, the three different soil depths were evaluated separately. In order to meet parametric assumptions, the following variables were transformed using logarithmic transformation (ergosterol (0–10 and 10–20 cm), ergosterol:C_{mic} (10–20 cm), DOC, TDN, NO_3^--N) or Box-Cox transformation (C_{org} (10–20 cm)). Data were considered significant for $p \leq 0.05$. For $C_{mic}:C_{org}$ ratio, Pearson product-moment correlation (C_{org}) and Spearman rank correlation analyses (N_{total}) were carried out.

3. Results

3.1. Survival and growth of annelids

After the incubation period, the total number of enchytraeids was increased approximately tenfold; from 350 to 3481 ± 976 (mean $\pm$ standard deviation) individuals (total number of juvenile and adult individuals per column (0.018 m^2)) in the NI treatment and up to 3432 ± 649 (mean $\pm$ standard deviation) individuals in the SI treatment. In the SI treatment, the enchytraeid distribution was decreasing with soil depth: 44% of the total community at 0–10 cm, 38% at 10–20 cm and 18% at 20–30 cm soil depth. For the NI treatment, distribution was quite balanced throughout the soil column, containing 35%, 30% and 35% of the total community from the top to the bottom for the three soil depths, respectively.

Earthworm mortality was low during the experiment; on average, only 6% of the anecic earthworms (corresponding to one individual earthworm) died while all endogeic earthworms survived the incubation. Biomass (mean $\pm$ standard deviation) of anecic earthworms for SI was 5.17 ± 0.18 g fresh weight (FW) before incubation and 5.66 ± 0.96 g FW after the experiment and for NI it was 4.97 ± 0.19 g FW and $5.21 \pm$

0.67 g FW, respectively. In contrast, biomass of endogeic earthworms increased in SI columns during the experiment from 3.47 ± 0.07 g FW to 4.82 ± 0.20 g FW. For NI, it was from 3.76 ± 0.16 g FW to 4.63 ± 0.62 g FW. The activity of the anecic earthworms in the respective columns could be observed with the naked eye. Already after one month, the soil surface area had changed visibly due to the presence of anecic earthworms in the non-inverted columns; at the end of the experiment, nearly all crop residues had disappeared from the soil surface (Fig. 1).

3.2. Isotopic ratios of annelids

Averaged over all treatments, isotopic values of soil samples ranged between -27 to -25 δ^{13}C (‰) and 4 to 6 δ^{15}N (‰). The corn leaves had an isotopic signature of -13.65 δ^{13}C (‰) and 7.12 δ^{15}N (‰). A comparison of isotopic ratios of body tissues of earthworms from the field-site where the soil originated (control) and annelids in the experiment showed a shift in δ^{13}C (Fig. 2). However, anecic earthworms differed significantly ($F(2, 6) = 49.38; p < 0.001$) in δ^{13}C values; Tukey post-hoc analysis indicated that only control earthworms ($p < 0.001$) differed from anecic earthworms in the lab (SI and NI) without differences between soil inversion treatments (Fig. 2). On average, anecic earthworms had assimilated crop residue derived C corresponding to 29% of their body tissue C in the NI and 30% in the SI treatment.

Endogeic earthworms differed in δ^{13}C values ($F(2, 6) = 144.47; p < 0.001$). Tukey post-hoc analysis revealed not only a differences between control earthworms and earthworms from the lab experiment for SI ($p < 0.001$) and NI ($p < 0.001$), but also differences between earthworms from SI and NI columns: endogeic earthworms had a significantly ($p = 0.02$) greater proportion of crop residue derived C in their tissue C in the SI columns (18%) compared with NI (14%) (Fig. 2).

Similar to the earthworms, isotopic ratio (δ^{13}C) of enchytraeid tissues differed significantly ($F(2, 6) = 21.91, p < 0.01$). The results of the Tukey post hoc analysis showed that enchytraeids from SI ($p < 0.01$) and NI columns ($p < 0.01$) had higher δ^{13}C values compared with control enchytraeids from laboratory culture. Assimilated crop residue derived C of enchytraeids corresponding to approximately 25% (SI) and 30% (NI) of their body tissue C (Fig. 2).

3.3. Chemical and microbial soil parameters

We found no significant interactions of soil inversion and annelid addition at any of the soil depths. At the soil depths 0–10 cm and 10–20 cm, only soil inversion affected microbial and chemical soil parameters (Tables 1, 3), regardless of annelid addition. At 20–30 cm, only differences in annelid treatments occurred, but no soil inversion effect (Tables 2, 3).

Concentrations of C_{mic} and N_{mic}, ergosterol and the ergosterol:C_{mic} ratio were, like C_{org} and N_{total} concentrations higher in NI columns (vs.
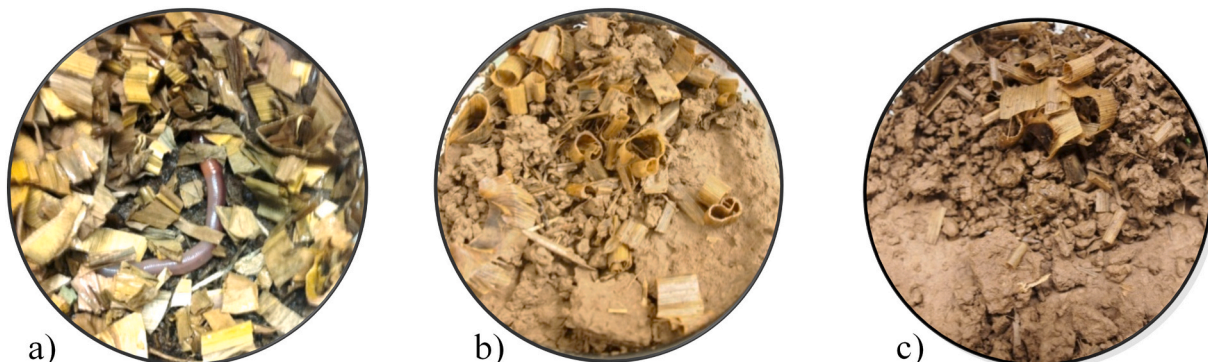

Fig. 1. Change of the surface area (diameter: 15 cm) over time (a) start of the experiment, (b) after 23 days, (c) after 114 days, from a soil mesocosm of the non-inversion treatment with chopped corn residues on the soil surface (NI) and addition of anecic earthworms (*Lumbricus terrestris*).
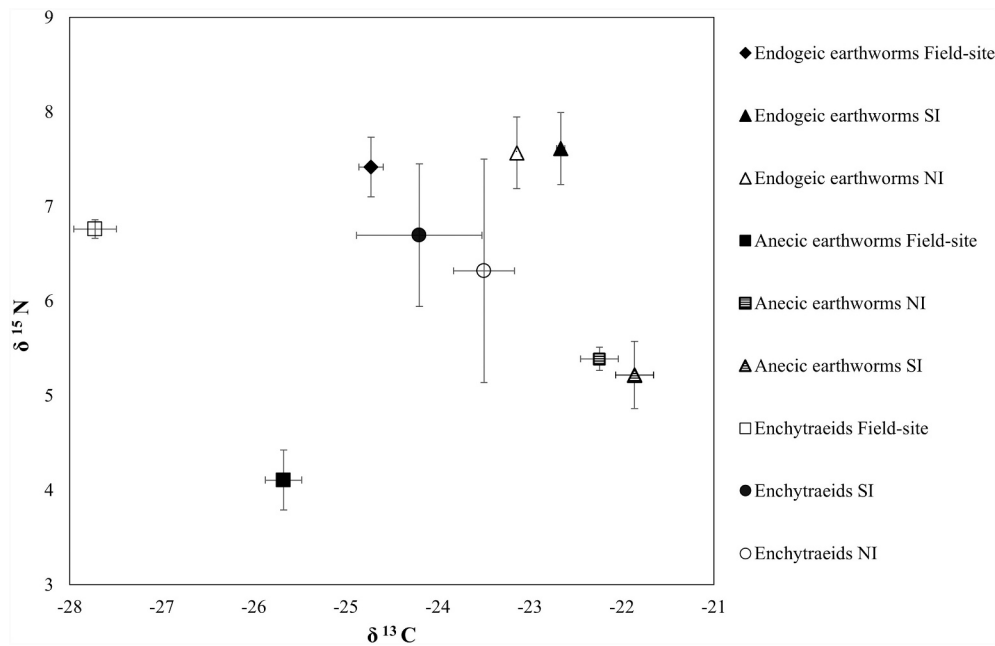
Fig. 2. Means ± standard errors of dual stable isotopes ($n = 4$) of annelid tissues before (start) and after incubation in soil columns treated either with soil inversion with residues buried (SI) or non-inversion with residues on the soil surface (NI). EW_{anecic} = Anecic earthworms (*Lumbricus terrestris*), $EW_{endo.}$ = Endogeic earthworms (*Octolasion cyaneum*), ENCH = Enchytraeids (*Enchytraeus crypticus* and *Enchytraeus christenseni*). Shift of isotopic values reflect an uptake of corn leaves as residues during the time of incubation (114 d). Values of corn leaves (non-incubated) were 7.12 δ^{15}N, −13.65 δ^{13}C, for soil samples before incubation 5.2 δ^{15}N, −26.7 δ^{13}C and soil samples after incubation 5.3 δ^{15}N, −26.4 δ^{13}C. Means of δ^{13}C followed by different letters are significantly ($p < 0.05$) different; lower case letters indicate differences for endogeic earthworms, upper case letters for enchytraeids and framed letters for anecic earthworms.

Table 1
Effect of the factor soil inversion on concentrations of soil organic carbon (C_{org}), total nitrogen (N_{total}), microbial biomass carbon (C_{mic}) and nitrogen (N_{mic}), the ratio of C_{mic} to C_{org}, ergosterol and the ratio of ergosterol to C_{mic} at the soil depths 0–10 cm and 10–20 cm. Means ($n = 32$) for the levels non-inversion (NI) and soil inversion (SI) and standard errors for differences of means are shown. Means followed by different letters are significantly ($p < 0.05$) different from each other at each soil depth.

Soil property	Soil depth	NI	SI	Standard errors for differences of means
C_{org} [%]	0–10 cm	1.57 a	1.37 b	0.02
	10–20 cm[†]	0.19 b	0.48 a	0.04
N_{total} [%]	0–10 cm	0.17 a	0.15 b	0.00
	10–20 cm	0.13 b	0.14 a	0.00
C_{mic} [µg g^{-1}]	0–10 cm	358 a	278 b	26
	10–20 cm	174 b	255 a	20
N_{mic} [µg g^{-1}]	0–10 cm	49.3 a	40.9 b	4.5
	10–20 cm	25.7 b	40.7 a	3.5
C_{mic}:C_{org} [%]	0–10 cm	2.27	2.04	0.17
	10–20 cm	1.49 b	1.88 a	0.14
ergosterol [µg g^{-1}]	0–10 cm[‡]	0.38 a	−0.33 b	0.16
	10–20 cm[‡]	−0.67 b	0.20 a	0.11
ergosterol:C_{mic} [%]	0–10 cm	0.44 a	0.30 b	0.05
	10–20 cm[‡]	−5.78 b	−5.27 a	0.10

In these depths, neither the interaction between the factors soil inversion and annelid treatment nor the annelid treatment were significant. There were no significant effects of the factor soil inversion at 20–30 cm.

[†] Data were Box-Cox transformed.
[‡] Data were log-transformed.

Table 2
Effect of the factor annelid treatment on concentrations of microbial biomass carbon (C_{mic}) and nitrogen (N_{mic}) and the ratio of C_{mic} to soil organic carbon (C_{org}) at 20–30 cm. Means (n = 32) for the levels anecic earthworms (EW_{anecic}), endogeic earthworms ($EW_{endo.}$), enchytraeids (ENCH) and control columns (CON) and standard errors for differences of means are shown. Means followed by different letters are significantly ($p < 0.05$) different from each other.

Soil property	EW_{anecic}	$EW_{endo.}$	ENCH	CON	Standard errors for differences of means
C_{mic} [µg g^{-1}]	80.94 ab	91.15 ab	101.36 a	68.00 b	10.82
N_{mic} [µg g^{-1}]	11.71 ab	11.21 ab	14.83 a	8.55 b	1.99
C_{mic}:C_{org} [%]	0.89 ab	1.04 ab	1.11 a	0.75 b	0.13

In this depth, neither the interaction between the factors soil inversion and annelid treatment nor the factor soil inversion were significant. There were no significant effects of the factor annelid treatment at 0–10 and 10–20 cm.

Table 3
ANOVA for the effect of soil inversion on concentrations of soil organic carbon (C_{org}), total nitrogen (N_{total}), microbial biomass carbon (C_{mic}) and nitrogen (N_{mic}), the ratio of C_{mic} to C_{org}, ergosterol and the ratio of ergosterol to C_{mic} at the soil depths 0–10 cm and 10–20 cm plus ANOVA for the effect of annelids on microbial biomass carbon (C_{mic}) and nitrogen (N_{mic}) and the ratio of C_{mic} to C_{org} at the soil depth 20–30 cm. Non-significant (n.s.) results are not shown.

Soil depth	Source	DF	C_{org} [%]		N_{total} [%]		C_{mic} [µg g^{-1}]		N_{mic} [µg g^{-1}]		ergosterol [µg g^{-1}]		ergosterol:C_{mic} [%]		C_{mic}:C_{org} [%]	
			F ratio	P	F ratio	P	F ratio	P	F ratio	P	F ratio	P	F ratio	P	F ratio	P
0–10 cm	Soil inversion	1	95.11	<0.001	68.33	<0.001	9.61	<0.01	3.37	0.08	41.09	<0.001	8.91	<0.01	1.94	0.43
	Block	3	3.84	0.02	8.10	<0.001	0.55	0.65	1.94	0.15	6.54	<0.01	7.01	<0.01	0.94	0.18
	Residual	27														
10–20 cm	Soil inversion	1	35.16	<0.001	30.22	<0.001	17.47	<0.001	18.77	<0.001	58.18	<0.001	26.00	<0.001	8.10	<0.01
	Block	3	8.32	<0.001	13.04	<0.001	8.32	<0.001	10.03	<0.001	1.61	0.21	3.07	<0.05	6.65	<0.01
	Residual	27														
20–30 cm	Annelids	3	n.s.		n.s.		3.48	<0.05	3.35	<0.05	n.s.		n.s.		3.33	<0.05
	Block	3					4.72	<0.01	2.05	0.13					6.79	<0.01
	Residual	25														

SI) at 0–10 cm and lower at 10–20 cm. C_{mic}:C_{org} was significantly higher in SI columns compared with NI at 10–20 cm. The C_{mic}:C_{org} ratio was closely correlated with C_{org} ($r = 0.74$) and N_{total} ($r = 0.76$) among soil depths. At 20–30 cm, C_{mic} and N_{mic} contents as well as the C_{mic}:C_{org} ratio was lowest in the control columns, compared with the ENCH treatment.

3.4. Carbon and nitrogen losses

On average, among all treatments, the cumulative emissions of CO_2-C was 128.5 g m^{-2} after 114 days of incubation; without differences in soil inversion treatments. The amount of cumulative CO_2-C emitted in the soil columns was 22% higher in the EW$_{anecic}$ columns compared with control columns ($F_{(3, 25)} = 3.51$; $p = 0.03$). In contrast, cumulative N_2O-N emissions increased due to SI (vs. NI) by 188%, independent from annelids ($F_{(1, 27)} = 13.81$; $p < 0.001$). The ratio of CO_2 flux to C_{mic} was significantly ($F_{(3, 25)} = 4.28$; $p = 0.014$) increased by 37% in the presence of anecic earthworms, compared with enchytraeids (Table 4). After adding the substrate at the start of the experiment, the daily CO_2-C emissions led to a steadily increasing curve of the cumulative CO_2-C emissions, which flattens out slightly over time. In the case of the N_2O-N emissions, differences over time can be seen between the two inversion levels: with soil inversion, the increases of cumulative values are on average constantly higher than for the values of non-inversion columns, which is also reflected in the cumulative sums (Table 4).

The amount of soil solution that had leached after 114 days of incubation was 3263 g in NI and 3178 g in SI columns. For DOC, TDN and DIN no effect of soil inversion or annelid treatment was found. Leaching of DON was small; averaged over all treatments, 71% of the N had leached as DIN in form of NO_3^-. Cumulative TDN and NO_3^--N losses averaged 10.39 g m^{-2} and 7.33 g m^{-2}. Ammonium-N concentrations were below detection rates. Cumulative DOC values were in a range from 0.82 to 2.49 g m^{-2}.

4. Discussion

4.1. Conditions for annelids

Compared with other laboratory experiments, earthworm mortality in this mesocosm experiment was very low (0–6%). This underlines that earthworms had suitable conditions during the experiment, although one single earthworm died. Giannopoulos et al. (2010) reported a mortality of 15% and 14% for L. *rubellus* and *A. caliginosa,* respectively, in a 90-days mesocosm experiment. In contrast to our hypothesis, we could not find a body weight loss in any soil inversion treatment for anecic earthworms. This result contrasts the mesocosm study of Frazão et al. (2019). In their study, running for 61 days, incorporation (burying) of crop residues led to a 30% reduction in body weight for L. *terrestris*; the body weight loss of the endogeic earthworm species (*A. caliginosa*) did not differ between the treatments (incorporated crop residues vs. surface applied). Thus, in addition to crop residue placement, other factors might play a role for changes in earthworm biomass during laboratory experiments, such as the number of individuals per column, the study duration and possibly also the choice of crop residues.

Experimental conditions seemed also suitable for enchytraeids. The strong (tenfold) increase of enchytraeid individuals during the experiment can be explained by sufficient food supply and the lack of predators. Under favourable conditions, enchytraeids are known for their rapid reproduction rates, as reported by van Vliet et al. (2004) for *E. minutus* and by Sandor and Schrader (2012) for the two species, that were also used in this experiment. In our case, the enchytraeids were evenly distributed over the entire length of the soil columns in the NI treatment, whereas they were concentrated in the upper 20 cm in the SI treatment. Under field-conditions, Severon et al. (2012) reported an even distribution of enchytraeids in the entire soil profile examined (0–20 cm) in conventionally tilled plots (inverted soil), whereas for reduced tilled plots (non-inversion), enchytraeids were mainly found

Table 4

Effect of the factors soil inversion (SI: soil inversion with residues buried, NI: non-inversion with residues on the surface) and annelid treatment (EW$_{anecic}$: anecic earthworms, EW$_{endo.}$: endogeic earthworms, ENCH: enchytraeids, CON: control columns) on cumulative N$_2$O-N and CO$_2$-C emissions after 114 days and on the ratio of CO$_2$ and microbial biomass carbon (C$_{mic}$). Values followed by different letters are significantly ($p < 0.05$) different (n = 32 for each response variable in the ANOVA and subsequent Tukey's HSD test).

	N$_2$O-N [mg m^{-2}]	CO$_2$-C [g m^{-2}]	CO$_2$-C:C$_{mic}$ [mg CO$_2$-C g^{-1} C$_{mic}$ d^{-1}]
Factor soil inversion: NI	45 b	132	16.0
SI	129 a	125	15.2
Standard errors for differences of means	23	7	1.3
Factor annelid treatment: EW$_{anecic}$	77	141 a	18.0 a
EW$_{endo.}$	114	135 ab	17.1 ab
ENCH	109	122 ab	13.2 b
CON	49	115 b	14.1 ab
Standard errors for differences of means	38	9	1.6

n.s.: not significant.

There were no significant interactions between the soil inversion and annelid treatment factors.

treatment factors.

between 0 and 10 cm. In our experiment, the uneven distribution of enchytraeids in the SI columns could be due to the manual soil inversion, which caused an interruption at a soil depth of 15 cm, so that the deepest layer (20–30 cm) could no longer be reached by the enchytraeids and their dispersion was limited.

4.2. Annelid feeding behaviour

The isotopic signature (δ^{13}C, δ^{15}N) showed a clear separation between ecological earthworm groups (anecic vs. endogeic). This difference between the anecic species (*L. terrestris*) that is predominantly a litter feeder and therefore a primary decomposer and the endogeic species (*O. cyaneum*) that as secondary decomposer predominantly feeds on soil affirms the findings of Scheu and Falca (2000) in beech forests and of Schmidt et al. (1997) in arable soils. Similar to the results of Schmidt et al. (2004), litter-feeding earthworms were notably lighter in both, δ^{13}C and δ^{15}N, compared with soil feeders. In accordance with Schmidt et al. (2016), δ^{15}N values of enchytraeids were similar to those of endogeic earthworms, but not to anecic earthworms.

The shift in isotopic δ^{13}C values for all investigated groups of annelids reflect that earthworms and enchytraeids had a preference for fresh crop residue material over older native soil organic matter (Briones et al., 1999), but with differences between the ecological groups. Anecic earthworms showed a greater uptake of crop residue derived C, compared with endogeic earthworms. Our results indicate that even though the secondary decomposer *O. cyaneum* fed on crop residues, it preferred to feed on well mineralised organic matter (cf. Eck et al., 2015), whereas anecic earthworms and enchytraeids favoured the fresh organic material or grazed on microorganisms dwelling on the litter. These findings are also in line with other studies (Briones et al., 1999; Bossuyt et al., 2006). In contrast to our hypothesis, *L. terrestris* (known as a species feeding at the soil surface) did not show a higher uptake of crop residue derived C in the NI columns.

Our results showed that deep burrowing earthworms access and feed on buried crop residues to an equal extend as if crop residues are placed at the soil surface. This result indicates that under field conditions it is more likely the destruction of natural bio-pores and the killing and injuring of worms by machinery that has a negative effect on anecic earthworms than the belowground placement of food sources due to ploughing. Nevertheless, in this mesocosm study our conclusions with regard to crop residue placement are only valid for adult individuals. In order to predict the community development under such conditions, other factors such as reproductive activity and hatchling survival should also be taken into account (cf. Briones and Schmidt, 2017).

4.3. Microbial biomass and soil organic matter

An important result is the absence of any interaction among the soil inversion treatment and annelids on the response variables, indicating that soil inversion, as carried out here, and annelid treatment have simple additive effects on the response variables. Most literature reflects the combined detractive effects of tillage on earthworm abundance and their activity (Briones and Schmidt, 2017). Our experiment shows that the two effects (annelids and pure soil inversion) are independent, when the inversion is not detracting or impairing the annelids directly as a side effect of the used machinery. For this reason, the findings of this model study cannot be directly translated to field conditions, where soil inversion is hardly realisable without soil structure destruction.

Concentrations of C$_{org}$ and N$_{total}$ were only affected by soil inversion and enhanced where crop residues were buried, which indicate that both parameters were closely related to the organic material. Similar results were observed for ergosterol contents and the ratio of ergosterol to C$_{mic}$. Incorporation of crop residues in the soil is known to promote fungi (Allison and Killham, 1988) as it provides a habitat for decomposing microorganisms (Potthoff et al., 2005). A decrease of ergosterol has been reported in the presence of the endogeic earthworm species *O. tyrtaeum* by Butenschoen et al. (2007) and *A. caliginosa* by Wachendorf et al. (2014). Such an effect of annelids on fungal biomass was not detected in our experiment. As shown for total microbial biomass reducing and supporting effects of annelids are balanced. Hence, the annelid feeding on fungi might be compensated or less pronounced due to a variety of other food sources in our setting using quite large soil columns (~5.8–7 kg of dry soil) containing undisturbed soil.

In the present experiment, higher C$_{mic}$ and N$_{mic}$ concentrations and a greater C$_{mic}$:C$_{org}$ ratio were found in columns with enchytraeids, compared with control columns, at 20–30 cm for both, NI and SI. Sandor and Schrader (2012) reported for a mesocosm experiment that the presence of enchytraeids reduced microbial biomass. Van Vliet et al. (2004) reported quite small impacts of enchytraeids on microbial biomass in a lab incubation experiment. As known for earthworms two effects directed towards microbial communities might be balanced also for enchytraeids. On the one hand, there is a reduction of microbial biomass by direct feeding (Potthoff et al., 2001) and, on the other hand, earthworm bioturbation might enhance availability of resources and support microbial growth.

The ratio C$_{mic}$:C$_{org}$ was in the upper 10 cm on average 2.1%, similar to values reported by Heinze et al. (2010) and Murugan et al. (2014) for Luvisols. From the top to the bottom, C availability for microorganisms decreased by approximately 55%, indicating a decreasing C availability for microorganisms (Anderson and Domsch, 1989; Anderson and Domsch, 2010). As the C$_{mic}$:C$_{org}$ ratio was closely correlated to C$_{org}$ and N$_{tot}$ concentrations, it reflects the reduced amount of organic material at 20–30 cm soil depth.

4.4. Gas emissions and leaching

For all treatments, C losses were mostly due to gas emissions in the form of CO_2-C. Generally, DOC levels were in the same range like in the mesocosm study of Sänger et al. (2011). We found no effect of annelids on NO_3^--N unlike reported in other studies (Araujo et al., 2004; Sandor and Schrader, 2012). Furthermore, we found no effect of annelids on N_2O emissions. In contrast, Nieminen et al. (2015) showed an increase in N_2O (27%) and CO_2 (13%) in the presence of L. terrestris in a mesocosm experiment running for 15 weeks. Lubbers et al. (2013) reported that earthworms increase CO_2 and N_2O emissions in soils by an average of 33% and 42%, respectively. Our results showed a strong and pronounced soil inversion effect (+188%) for N_2O-N emissions. This might have masked the annelid effect, as this effect could be expected as much smaller. Comparable results were reported by Giannopoulos et al. (2010), who investigated the role of earthworms (A. caliginosa and L. rubellus) in a treatment with incorporated crop residues vs. a treatment with crop residues placed on the soil surface. Here, the incorporation of crop residues led to much higher N_2O emissions than when crop residues were placed on the soil surface.

We observed a 22% increase in cumulative CO_2-C emissions in the presence of L. terrestris, compared with control columns. Lubbers et al. (2015) noticed an increase of 25% in no-till mesocosms (A. caliginosa, L. rubellus) after 750 days. In one of our columns one L. terrestris died throughout incubation, which might induce a temporary flush of CO_2-C emissions. We did not observe such an effect. The increase in CO_2-C might be explained by stimulation of the microbial turnover. Concerning the microbial respiration in this study, in the presence of anecic earthworms 37% more CO_2-C was respired per unit C_{mic} over time, compared to when only enchytraeids were present. This was less pronounced for endogeic earthworms, since anecic earthworms as primary decomposers are closer related to plant residues where microbial mineralization runs faster and in higher rates than for soil organic matter. Therefore, the anecic impact takes place at a hot spot of decomposition with high rates. In addition, Binet et al. (1998) name higher soil aeration due to bioturbation due to L. terrestris in their experiment as an explanation for an increase in respiration rates. A reduced availability of O_2 reduces CO_2, but enhances N_2O fluxes (Vor et al., 2003).

Earthworms boost soil aeration due to their burrowing activity that creates soil pores. We assume soil aeration to be increased in the presence of both earthworm species (L. terrestris and O. cyaneum), though bioturbation rates of anecic species are known to be generally lower than for endogeic species (Taylor et al., 2018). As both earthworm species differ in their feeding and burrowing behaviour (Bouché, 1977), their burrow constructions are not the same. While 40–50% of the burrows of endogeic earthworms are refilled with earthworm casts, this is only the case for 20% of the burrows by anecic earthworms that preferably cast on the soil surface (Capowiez et al., 2014). Therefore, soil aeration might be greater in soil columns with anecic compared with endogeic earthworms. For enchytraeids, we found no significant effect on gas fluxes. Van Vliet et al. (2004) also report no effect on CO_2 emissions. However, N_2O emissions were increased by enchytraeids in a loamy sand soil and decreased in a sandy clay loam. In contrast, John et al. (2019) observed a reduction of the average CO_2 emissions by 35% by E. buchholzi in a paddy soil. In summary, a strong interaction of enchytraeid effects with soil type is indicated. Based on the results from their 64-day mesocosm experiment, Wu et al. (2015) highlight, that only earthworms enhanced CO_2 emissions, whereas the smaller mesofauna had less impact, which indicate that effects may also be size-dependent.

5. Conclusion

The experiment showed that O. cyaneum benefit when crop residues are buried in the soil profile (due to soil inversion). Contrasting our hypothesis, L. terrestris took no advantage from the non-inversion treatment. However, the amount of crop residue derived C in the earthworm tissue and its isotopic signature clearly differed between L. terrestris on the one hand and O. cyaneum and enchytraeids on the other hand, which may indicate, that there is a difference between primary and secondary decomposers. Surprisingly, there was no interaction between both factors (soil inversion x annelid treatment) for soil microbial and chemical parameters. In the upper 20 cm, the concentrations were only dependent on soil inversion, indicating a strong relationship to the organic material. We observed an effect of annelids regarding nutrient losses only for carbon, but not for nitrogen. Here, stimulation of microbial activity by annelids seems to be an important factor increasing CO_2-C emissions. In contrast, N_2O-N emissions were dependent on soil inversion. Results indicate that the effect of annelids on soil processes clearly depend on life form of earthworms and might be also partially size-dependent, being lower for mesofauna than for macrofauna.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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