No evidence that earthworms increase soil greenhouse gas emissions (CO 2 and N 2 O) in the presence of plants and soil moisture fluctuations

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Article

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Abstract

Earthworms can stimulate plant productivity, but their impact on soil greenhouse gases (GHG) is still debated. Methodological challenges of measuring GHG in experiments with plants are presumably contributing to the status quo, with the majority of studies being conducted without plants. Here we report the effect of earthworms (without, anecic, endogeic and their combination) and plants (with and without) on GHG (CO_2 and N_2O) emissions in an experiment. N_2O emissions were also 34.6 and 44.8% lower when both earthworm species and only endogeic species were present, respectively, while plants reduced the cumulative N_2O emissions by 19.8%. No effects on CO_2 were found. Estimates of soil macroporosity measured by X-ray tomography show that the GHG emissions were mediated by their burrowing activity affecting the soil aeration and water status. Both GHG emissions decreased with the macropore volume in the top soil, presumably due to reduced moisture and microbial activity. N_2O emissions also decreased with macropore volume in the deepest layer, likely caused by a reduction in anaerobic microsites. Our results indicate that, under experimental conditions allowing for plant and earthworm engineering effects on soil moisture, earthworms do not increase GHG emissions and that endogeic earthworms may even reduce N_2O emissions.

Introduction

Soil biota controls the decomposition of organic matter entering the soil system [1,2], with consequences for carbon-cycle feedbacks on climate change [3,4]. Ever since Darwin published “The formation of vegetable mould through the action of worms” [5], earthworms have been considered as indicators of soil fertility and plant productivity [6,7], with 25% increase in crop yield [8] in the presence of earthworms. However, this comes with the drawback of increasing CO_2 emissions by 33% and N_2O emissions by 42% [9], which is notable since the N_2O emissions have 265 times the global warming potential of CO_2 [10]. Such consideration may affect the choice of soil biodiversity-friendly agricultural policies that increase earthworm biomass such as no-tillage, superficial tillage, reduced pesticide use and organic fertilizer applications. However, although additional studies have been published that support the findings of Lubbers et al. (2013) meta-analysis [11–15], a substantial number of laboratory [16,17] and field studies included in the Lubbers et al. (2013) meta-analysis as well more recent ones [18–22] are not in line with the overall conclusion that earthworms increase GHG emissions. Collectively, these observations suggest that we do not fully understand the factors thought which earthworms affect CO_2 and N_2O emissions, calling for additional studies addressing some of the limitations of the previous studies in order to gain a mechanistic understanding of the factors leading to contrasting results.

The most notable limitation of the majority of the previous studies is the absence of plants: we quote “*Almost every experiment in our dataset measured earthworm effects on GHG emissions in the absence of growing plants*” [19]. As primary producers, plants are at the heart of biogeochemical cycles [23] since they control the quantity and quality of the carbon that enter the soil system. Furthermore, inputs of root-

derived C substrates [24] leads to high transient O_2-demand and can cause sub-oxic microsites, thus favoring denitrification [25]. Conversely, they compete for nitrogen acquisition [26], reduce soil water content by transpiration, modify soil porosity by root growth [27] and thus can change the preponderance of the controlling N_2O emission processes (nitrification, denitrification and nitrate ammonification) [25,28]. Earthworms interact with these processes by altering plant growth via increasing the mineralization rate of soil organic matter [29,30] and by consuming roots [6,30]. Therefore, these three biotic components, microorganisms, plants, and earthworms form a complex system in constant interaction [30].

Soil moisture is a long recognized primordial factor affecting soil greenhouse gases production in soil [31,32] and can explain up to 95% of emissions [33]. It determines the diffusion of gases and nutrients and hence the availability of labile oxygen, nitrates, ammonium and carbon to microorganisms, thereby modulating their activity. Under anoxic conditions, at high soil water content, N_2O emissions are stimulated, preferentially by denitrification, while nitrification will be more predominant under aerobic conditions when the soil water content is unsaturated [34]. Soil moisture fluctuations such as the frequency and intensity of drying-rewetting cycles can also affect the proportion of denitrified nitrogen being converted in N_2O or N_2, thus modulating the N_2O/N_2 ratio that will be emitted into the atmosphere [35,36]. To the best of our knowledge, the only two studies that looked at the impacts of soil moisture fluctuations on soil greenhouse gas emissions in the presence of earthworms, although without plants, showed that cumulative N_2O and CO_2 emissions were reduced in the presence of earthworms [18,21]. Given that preliminary evidence suggest that maintaining constant soil moisture conditions through frequent watering will likely minimize the earthworm-mediated effects on the soil water status with consequence for greenhouse gas emissions [18,21], more studies that incorporate naturally occurring soil moisture fluctuations are needed.

Lumbricid earthworms are broadly classified in three main ecological categories based on their feeding and burrowing characteristics: (1) anecic species feed on surface litter, have permanent vertical burrows and often pull the litter into their burrows and create surface middens (mixing casts with litter), (2) epigeic species are surface-dwellers that also feed on litter and make very few non-permanent burrows and (3) endogeic species live and feed on mineral soil, rhizosphere and the associated belowground organic matter and make numerous non-permanent burrows [37]. Generally, the burrowing and casting activity of earthworms as well as the associated changes in microbial communities can lead to both increased carbon and nitrogen mineralization as well as stabilization of soil organic matter [38,39]. However, no consensus has been reached on what is the net outcome of the two processes and how this is affected by the different ecological categories. There is evidence that the CO_2 and N_2O emissions depend on the earthworm ecological category, with significantly higher emissions for the anecic group (Lubbers et al., 2013), but whether this finding holds in presence of plants and soil moisture fluctuations remains to be tested.

In this study, we assessed the impact of two earthworm species belonging to two ecological categories (endogeic, anecic) and their combination on CO_2 and N_2O fluxes in a greenhouse mesocosm experiment designed to investigate the combined effect of earthworms engineering and litter burial activities, plants, and soil moisture fluctuations on N_2O and CO_2 fluxes. Additionally, we measured the aboveground plant biomass and multiple soil parameters representing potentially relevant predictors including soil nitrogen and water status, microbial biomass and respiration, denitrification potential and multiple metrics of soil macroporosity using X-ray tomography. We hypothesized that: 1) the CO_2 and N_2O emissions will be lower in the presence of earthworms relative to controls as increased carbon and nitrogen mineralization will be offset by the earthworm soil engineering effect (burrowing) on soil water status due to increased water infiltration and drainage, 2) plant presence will reduce N_2O emissions due to nitrogen and water uptake but will increase CO_2 emissions due to increased carbon substrates entering the soil via rhizodeposition, and 3) differences in N_2O and CO_2 fluxes among the two earthworm ecological categories are mediated by the burrowing patterns affecting soil (macro) porosity and water status.

Results

Soil water content

The soil water content (SWC, expressed as percentage of field capacity) varied not only with the experimentally imposed soil water fluctuations (reaching a minimum of 52.0% of soil field capacity), but also with the plant and earthworm treatments as indicated by the Plant×Week and Ew×Week interactions (Table 1, Fig. 1). The SWC was significantly lower in presence of plants during weeks 6 and 11, with overall 5% lower values (averaged over the whole experiment) in the presence of plants. The presence of anecic earthworms in both treatments where they were present (anecic and both) led to significantly lower fitted estimates for SWC relative to control during weeks three to six and eight to 12. Averaged over the whole experiment, the SWC was 81.5% of field capacity in the presence of anecic earthworms, whereas in the presence of endogeic earthworms and control the average SWC was higher at 85.9 and 87% of field capacity, respectively. Overall, the differences between the control and the treatment combinations with anecic earthworms increased with the time form the last watering and peaked at weeks six and 11 (Fig. 1).

Table 1
Minimal adequate models for weekly time series (SWC, Litter cover, N_2O, and CO_2) and cumulative emissions (cN_2O and cCO_2) as affected by the earthworm (Ew), plant (Plant), sampling week (Week), soil water content (SWC) and their interactions. “NA” stands for non-applicable, “ns” stands for variables that were not significant (P > 0.1) and were not retained in the minimal adequate models whereas ${}_mr^2$ represents the marginal coefficient of determination. ***P < 0.001; **P < 0.01; *P < 0.05; $^+$P < 0.1.

Source	SWC	Litter	N_2O	cN_2O	CO_2	cCO_2
Ew	46.68***	182.89***	82.97***	7.07***	2.53$^+$	1.25
Plant	173.83***	2.91$^+$	34.58***	3.82$^+$	5.98*	ns
Week	2758.70***	68.85***	112.9***	NA	230.28***	NA
SWC	NA	NA	0.04	6.35*	128.85***	7.54**
Ew:Plant	2.63$^+$	3.65*	9.52***	2.05	2.01	ns
Ew:Week	9.40***	198.63***	5.61***	NA	1.97**	NA
Plant:Week	24.01***	ns	9.32***	NA	4.93***	NA
Ew:SWC	NA	NA	14.84***	4.45**	5.16**	3.16*
Plant:SWC	NA	NA	1.43	ns	ns	ns
Week:SWC	NA	NA	8.34***	NA	ns	NA
Ew:Plant:Week	ns	ns	1.57*	NA	ns	NA
Ew:Plant:SWC	NA	NA	9.51***	ns	ns	ns
Ew:Week:SWC	NA	NA	3.81***	NA	ns	NA
Plant:Week:SWC	NA	NA	5.52***	NA	ns	NA
Ew:Plant:Week:SWC	NA	NA	ns	NA	ns	NA
${}_mr^2$	0.81	0.91	0.67	0.49	0.43	0.27

N_2O and CO_2 fluxes

The weekly measured N_2O emissions ranged from no detectable emissions in the weeks with low SWC to a maximum recorded value of 0.10 g N_2O m^{-2} day^{-2} (Fig. S1A) and were significantly affected by all possible three-way interactions between our two experimental treatments (Ew, Plant and SWC; Table 2) and time (i.e. the sampling week). Overall, the N_2O emissions increased with SWC with higher emissions detected after each irrigation (Fig. S1A), and peaked in the second week of the experiment (0.04 g N_2O m^{-2} day^{-1}), followed by a gradual decline with time, reaching almost no emissions in the eleventh week

before the final irrigation (Fig. S1). The cumulative N_2O emissions (Figs. 1 and 2) over the 12 weeks of the experiment were the highest in the control without earthworms (0.135 g m^{-2}). Relative to control, the cumulative N_2O emissions were 17.04, 34.59 and 44.81% lower in the anecic, both and endogeic treatments, respectively (Fig. 1E). The presence of plants reduced the cumulative N_2O emissions in average by 19.82% (Fig. 1F). The Ew×SWC interaction (Table 1) indicates that cumulative N_2O emissions increased with average SWC for control, anecic and both treatment levels, but decreased with SWC in the presence of endogeic earthworms (Fig. 2A).

Table 2
Covariables considered as predictors for the N_2O and CO_2 emissions from the last week of the experiment in addition to the experimental treatments. See Fig. S3 for a correlation matrix of all tested predictors and Fig. S2 and S5 for the effects of experimental treatments on these predictors. DW: dry weight.

Abbreviation	Description	Unit
Ew_bm	Earthworm biomass	g FW mesocosm^{-1}
Ew_no	Number of added earthworms per mesocosm	number
SWC	Soil water content relative to field capacity	% of field capacity
Plant_bm	Aboveground plant biomass	g DW mesocosm^{-1}
Litter	Percentage of soil surface covered by litter	%
DEA	Potential denitrification enzymatic activity	µg N g^{-1} soil DW h^{-1}
C_{mic}	Microbial biomass C	µg C_{mic} g soil $^{-1}$ DW
BR	Microbial basal respiration	µg C-CO_2 g^{-1}soil DW^{-} h^{-1}
Met_Q	Microbial metabolic quotient	µg C–CO_2 µg C_{mic} $^{-1}$ h^{-1}
NH_4^+	Ammonium content in soil at the end of the experiment	mg kg^{-1}
NO_3^-	Nitrate content in soil at the end of the experiment	mg kg^{-1}
Vpores_L1-4 & tot	Macropore (burrow + cracks) volume estimated from CT scan in the 0-8.5 cm layer (L1), 8.5–17 cm layer (L2), 17-25.5 cm layer (L3), 25.5–34 cm layer (L4), and in the whole mesocosm (tot).	cm^3
Vburrows_ L1-4 & tot	Burrow volume estimated from CT scan in the 0-8.5 cm layer (L1), 8.5–17 cm layer (L2), 17-25.5 cm layer (L3), 25.5–34 cm layer (L4), and in the whole mesocosm (tot).	cm^3
Vcracks_L L1-4 & tot	Cracks volume estimated from CT scan in the 0-8.5 cm layer (L1), 8.5–17 cm layer (L2), 17-25.5 cm layer (L3), 25.5–34 cm layer (L4), and in the whole mesocosm (tot).	cm^3

Table 4

Minimal adequate models presenting the results explaining the CO_2 and N_2O fluxes from the last sampling (week 12) where the soil porosity-related variables were included in the model as potential predictors (compared with the models without the soil-porosity variables). “NA” stands for non-applicable, “ns” stands for variables that were not significant and were not retained in the minimal adequate models and $_mr^2$ represents the marginal coefficient of determination. F-values are shown with significance levels: ***P < 0.001; **P < 0.01; *P < 0.05; $^+$P < 0.1.

	N_2O model			**CO_2 model**	
Source	without porosity	With pore volume L1	with pore volume L4	without pore volume	with pore volume L1
Ew	15.46***	24.51***	16.15***	ns	2.1
Plant	ns	1.77	ns	6.95*	15.9***
SWC	11.93**	28.25***	13.15***	4.12*	14.33**
Vpore	NA	0.63	ns	NA	36.98***
Ew×Plant	ns	1.22	ns	ns	1.27
Ew×SWC	12.19***	18.73***	12.81***	ns	2.86^+
Plant×SWC	ns	2.00	ns	ns	0.32
Ew×Vpore	NA	5.60**	ns	NA	0.14
Plant×Vpore	NA	1.04	ns	NA	0.15
SWC×Vpore	NA	0.66	3.83^+	NA	0.49
Ew×Plant×SWC	ns	1.19	ns	ns	1.81
Ew×Plant×Vpore	NA	2.04	ns	NA	1.14
Ew×SWC×Vpore	NA	11.79***	ns	NA	1.39
Plant×SWC× Vpore	NA	1.95	ns	NA	1.94
Ew×Plant×SWC×Vpore	NA	ns	ns	NA	3.76*
Marginal r^2	0.69	0.86	0.72	0.18	0.83

CO_2 weekly emissions were significantly affected by the Ew×Week, Plant×Week and Ew×SWC interactions (Table 1), and were generally higher after re-watering at higher SWC (Fig. S1B). The cumulative CO_2 emissions showed no significant overall main effects of earthworms or plant presence (Fig. 1G-H) however, a significant interactive effect of earthworms with SWC was found (Table 1). The Ew×SWC interaction (Table 1; Fig. 2B) indicates that when SWC was relatively high (> 85.9% SWC in average), as in the endogeic and earthworm-control treatment, cumulative CO_2 emissions generally decreased with increasing SWC. In contrast, in the treatment with anecic or both earthworm species, where SWC was

generally lower (81.1% and 81.7% of field capacity for anecic and both species, respectively), CO_2 emissions increased with SWC when anecic earthworm were present, or showed no clear trend when both earthworm species were present (Fig. 2B).

Plant, soil and litter response variables

The effects of the earthworm and plant treatments on the measured response variables at the end of the experiment are available in Table 3 and Fig. S2. The presence of earthworms led to generally higher aboveground plant biomass compared to control (+ 57, + 25 and + 41% for anecic, endogeic and both species, respectively), but due to the high intra-treatment variability these effects were not statistically significant (Table 3). We found a tendency of a higher denitrification potential in the mesocosms with anecic earthworms (+ 22%) relative to control, but no plant effects. Microbial biomass (C_{mic}), microbial basal respiration (BR) and microbial metabolic quotient (Met_Q) were not significantly affected by any experimental treatment. Soil ammonium content (NH_4^+) was marginally significantly lower (-58%) in the mesocosms with endogeic earthworms. Irrespective of the earthworm treatment, soil nitrate content (NO_3^-) was found to be significantly lower (-80%) in the presence of plants. Furthermore, the soil nitrate content also increased in the presence of earthworms (+ 184%) relative to the control, irrespective of the plant treatment. The percentage of litter remaining at the soil surface also depended on the presence of earthworms and varied with time as indicated by the Ew×Week interaction (Table 3, Fig. S2). As expected, the consumption and burying of litter by anecic earthworms significantly reduced the proportion of soil that was covered by litter by the end of the experiment to 5.7% of the total surface and to 25.7% when both endogeic and anecic earthworms were present. In the mesocosms containing endogeic earthworms and the no-earthworm control, the percentage of soil that remained covered by litter remained at 100%. The SWC measured at the end of the experiment was positively correlated with the proportion of soil that remained covered by litter ($r = 0.55$, $t = 4.86$, $P < 0.001$, $n = 56$; Fig. S3), suggesting that, at least in part, the earthworm effect on the SWC also acted via the effect of the bare ground on water evaporation.

Table 3
Effects earthworms (Ew) and plant treatments on the response variables tested as predictors of N_2O and CO_2 fluxes (See Table 2 for detailed variable description). The "ns" abbreviation stands for variables that were not significant and were not retained in the minimal adequate models whereas $_mr^2$ represents the marginal coefficient of determination. ***P < 0.001; **P < 0.01; *P < 0.05; $^+$P < 0.1.

Source	Plant_bm	NH_4^+	NO_3^-	Litter	
Ew	ns	2.22^+	15.95***	74.39***	
Plant	NA	ns	46.41***	0.93	
Ew×Plant	NA	ns	ns	3.08*	
$_mr^2$	0.0	0.11	0.73	0.91	
Source	DEA	C_{mic}	BR	Met_Q	
Ew	2.75^+	ns	ns	ns	
Plant	ns	ns	ns	ns	
Ew×Plant	ns	ns	ns	ns	
$_mr^2$	0.14	0.00	0.00	0.00	
Source	Vpores_L1	Vpores_L2	Vpores_L3	Vpores_L4	Vpores_tot
Ew	ns	9.04***	32.04***	191.71***	11.91***
Plant	ns	26.63***	21.36***	13.83***	15.78***
Ew×Plant	ns	ns	ns	ns	ns
$_mr^2$	0.00	0.48	0.68	0.94	0.59

Examples of X-ray based 3D reconstruction of soil macroporosity are displayed in Fig. 3. The volume of macropores in the top soil layer (L1) was not significantly affected by any experimental treatment (Table 3), however, we found lower macropore volumes in the presence of plants in the other three layers (-26.0% in L2, -23.8% in L3, -18,1% in L4). The macropore volumes measured in L2-L4 were also affected by the earthworm treatment, with the highest volume in the mesocosms with endogeic earthworms, followed by both, anecic and control (Fig. S4). Similar trends were observed when macropores were differentiated in burrows and cracks with no effect (or small) of the treatment in the L1 (Table S1, Fig. S4). Burrow volume was largely driven by the earthworm treatment with high coefficients of determination in L2-4 and in total, while the plant treatment did not affected burrow volume in L3 and L4. The volume of cracks was influenced by the treatments in the same manner that total porosity.

Exploration of multiple predictors for N_2O and CO_2 fluxes

Alongside SWC, we further explored the importance of all measured potential predictors on the N_2O and CO_2 fluxes from the last week of sampling (n = 56). Out of the 16 tested potential predictors (Table 2), the results of the MCP-penalized multiple regression for N_2O emissions indicate that, in addition to a retained positive coefficient for SWC ($4.663e^{-04}$), the volume occupied by macropores in the first and fourth soil layer (Vpores_L1 and Vpores_L4) were also retained with negative coefficient ($-1.01e^{-05}$ and $-4.18e^{-05}$ respectively) at minimum cross validation error with lambda = 0.001). For the CO_2 fluxes the MCP-penalized multiple linear regression selected the total volume of macropores in the top soil (Vpores_L1) as the best predictor with a negative coefficient (− 0.034) at minimum cross validation error with lambda = 0.321). These selected predictors also had among the highest correlation coefficients with CO_2 and N_2O fluxes (Fig. S3). See also Fig. 2C to G for univariate regressions depicting the relationship between SWC, Vpores_L1, Vpores_L4 and N_2O, and SWC, Vpores_L1 and CO_2 fluxes, respectively. In a final step we compared how the inclusion of porosity metrics affected the performance of the models explaining the emissions from the last experimental week by comparing mixed effects models with and without the best subset variables alongside the earthworm, plant and soil water treatments (Table 4). In the case of N_2O emissions, Vpores_L1 and Vpores_L4 could not be included together in the model in interaction with the other predictors without leading to overfitting and convergence issues, leading us to ran one model for each porosity variable.

The minimal adequate models for N_2O emissions without any porosity variable retained the Ew×SWC interaction, with overall lower SWC and lower emissions in the presence of endogeic earthworms (r^2m = 0.69, Table 4). The competing model including Vpores_L1 explained more variation (r^2m = 0.86) and retained two other interactions. The Ew×SWC×Vpores_L1 three-way interaction was kept, with positive fitted coefficients meaning that in presence of earthworm and higher macropore volume (which are the highest in L1 in control due to cracks) and highest value of soil water content, emissions were higher. The second model with porosity including Vpores_L4 variable was intermediate in the amount of explained variation (r^2m = 0.72). In addition to the Ew×SWC interaction, only the SWC×Vpores_L4 two-way interaction was marginally significant (pval = 0.054, Table 4), with negative fitted coefficient indicating that the N_2O emissions decreased with increasing Vpores_L4, and more strongly so when the SWC was higher. Regarding CO_2 emissions, the minimal adequate model without Vpores_L1 showed that the CO_2 emissions increased in the presence of plants and with increasing SWC (mr^2 = 0.18, Table 4). In contrast, the model including Vpores_L1 largely increased in the amount of variance explained (mr^2 = 0.83), and the four-way interaction Ew×Plant×SWC×Vpores_L1 was significant with positive fitted coefficients, with higher CO_2 emissions in the presence of earthworms (each of the three modalities) and plants when porosity was higher at high SWC levels.

Discussion

To further advance our understanding of the effects of earthworms on GHG emissions, our study was designed to simultaneously investigate the effect of earthworms, plants, and soil moisture fluctuations, notably with an experimental set-up allowing earthworm and plants to affect the soil water status. In line with our first hypothesis, we found not only that the presence of earthworms did not increase the CO_2 and N_2O cumulative emissions over 12 weeks, but also that the endogeic species *A. icterica* actually reduced the cumulative N_2O emissions by 44.8% on its own and by 34.6% when the anecic *L. terrestris* was also present. Whilst our results are in contrast to the conclusion of Lubbers et al. (2013) meta-analysis and several other studies that emerged after 2013 [11–15], they are however in line with several other studies indicating that earthworms can have either no significant effect [16,20,40] or even offset some of the CO_2 or N_2O emissions, with two of these studies including soil moisture fluctuations [18,19,21,22]. These highly variable findings stress the very complex relationships between environmental conditions, earthworms and GHG emissions, which can be heavily influenced by how close the experimental conditions are to natural conditions [20,41].

Soil water availability modulate N_2O and CO_2 emissions in complex ways. Regarding N_2O, it is generally assumed that denitrification under sub-oxic conditions (< 0.2 mg O_2. L^{-1}) [42] and nitrification under aerobic conditions are the main pathways leading to N_2O emissions. The proportion of nitrogen that is denitrified as N_2O (an obligate intermediate in the denitrification pathway) is also under the control of various environmental conditions (soil moisture, aeration, nitrate, organic carbon, pH) [43]. Conversely, nitrification is a strictly aerobic process since the NH_4^+ oxidation enzyme of nitrifying organisms requires O_2 for activation. Although the relationship between soil water status and N_2O flux is rather complex [44,45] a combination of limited substrate diffusion at very low water content and limited gas diffusion at high water content, the combined output of N_2O emissions via nitrification and denitrification is at a maximum at intermediate soil water content, ~ 70–80% water-filled pore space [46]. Similarly, a substantial body of evidence showed that carbon substrate limitation occurs in drier conditions and oxygen limitation under wetter conditions, with optimal conditions for respiration at intermediate water contents [47,48]. Our study confirmed the major importance of soil moisture as a primordial driver of greenhouse gases production in soil [31,32], as well as the existence of an optimal SWC (that depends on soil textures and its field capacity) value for greenhouse gases emission [46], and even more interestingly, highlighted how plant and earthworms modulated this relationship through complex interactions (Tables 1,3 and 4, Fig. 1 and S1).

In our experiment SWC values varied largely, being the lowest in the presence of plants and anecic earthworms, alone or mixed with endogeic earthworms (Fig. 1). The Ew×SWC interaction (Fig. 2A,B) observed for cumulative and weekly CO_2 emissions illustrate this SWC optimal value phenomena (around 85% of field capacity). The anecic treatment with earthworms that create large vertical burrows that are known to affect water infiltration, drainage, and evaporation [49] led to lower than optimum SWC value for microbial activity, hence the positive relationship between SWC and respiration for this species. Conversely, the presence of endogeic earthworm, similar to the control with no earthworm, maintained on

average higher than optimal SWC for soil respiration, thus explaining the negative slopes with increasing SWC. Finally, the presence of both earthworm species led to SWC values spanned across the optimum and no clear relationship between SWC and CO_2 could be detected. Simultaneously, the presence of *B. dystachion* lowered the average SWC (mean ± se = 82.0 ± 0.6% of WHC) and in this soil moisture range CO_2 emissions increased with SWC (Fig. S6). In the absence of *B. dystachion*, the average SWC was higher (86.2 ± 0.59% of WHC), but increasing SWC lowered the emission as the range of SWC was beyond optimum, and presumably soil respiration was limited by O_2 diffusivity under these conditions (Fig. S6). Concerning N_2O emissions, very similar patterns are found except for the earthworm-free treatment where emissions increase above the SWC optimal value. This only partially confirm the optimum SWC mechanism found for CO_2 and our first hypothesis, and suggests that although GHG emissions are strongly controlled by the interactions between earthworms, plants and SWC, other mechanisms are at play.

The inclusion of the soil porosity data showed that an even better predictor than the total SWC of the mesocosm is the total volume occupied by soil pores in the upper soil layer (with a negative coefficient, Fig. 2E,F,G?). This is in line with our third hypotheses suggesting that the increased porosity/aeration in in the top soil layer (0-8.5 cm) decreased the N_2O and CO_2 emissions by presumably reducing the SWC in the upper and most microbially-active soil layer. Interestingly, porosity in the bottom layer (25.5–34 cm) also was a good predictor of N_2O emissions (Fig. 2G) and is the only variable that is influenced by earthworm species in the same way as cumulative N_2O emissions. Indeed, the amount of burrow in the deepest layer was higher in presence of the endogeic *A. icterica* (alone or with anecic earthworm), a species with high affinity for the deepest layer [50], that prevents the development of denitrification-stimulating anaerobic sites. The reduction of N_2O emissions via an increased aeration of the soil was also previously suggested by several studies [19,22], but was not explicitly shown to our knowledge. Our results indicate that this effect is more prevalent in the presence of the endogeic species and seems to be related to the higher number of burrows that are produced by this species in contrast to the larger but less numerous semi-permanent burrows produced by the (epi) anecic species (Fig. S4) [49].

Our experiment also allows to discuss the importance of nutrient availability for GHG emissions. Indeed, the lowered N_2O emissions by 19.8% in presence of plants occurred likely also in part due to plant N uptake as we found that the amount of soil NO_3^- and NH_4^+ was 43% and 20% lower respectively in the presence of plants (in line with our second hypothesis) independently of earthworm presence. This support our hypothesis stating that plant compete with microorganisms for nutrient and therefore limit microbial activity [26], given the importance of nitrogen availability for nitrification and denitrification rate [51]. The absence of positive effect of plant on CO_2 fluxes (either weekly fluxes, or microbial potential activity at final harvest) is surprising, notably since our experimental design only allowed the combined measurements of CO_2 originating from heterotrophic and root respiration. This could be explained by the nutrient (nitrate and ammonium) limitations, or by an overall low plant effect due to the relatively low plant biomass production of *B. dystachion* in our experiment. Soil NO_3^- increased in the presence of

anecic and endogeic earthworms, and even more when both types were present with, in presence of the two earthworm species, a 2.1 and 3 fold increase compared to the control with and without plant, respectively. This can be explained by the combined effect of local vertical litter burial by *L. terrestris* and the horizontal redistribution in the large burrow system of *A. icterica* and nitrogen stabilization in earthworms cast [52,53]. The accelerated burying of surface litter by the anecic species therefore likely contributed to the higher N_2O emissions via increased N availability after the first watering, but this effect faded with time. Simultaneously, this higher nutrient availability partly contributed to higher plant growth in the presence of earthworm [8]. Despite this increase in nutrient availability, the decreased soil moisture due to earthworm burrowing and the formation of cracks in the top soil still reduced microbial activity and greenhouse gases production.

By measuring GHG emissions as affected by earthworms in a model system with plants, this study shows that alongside plants, soil moisture fluctuations, and the earthworm engineering effect on porosity are interacting to modulate GHG emissions. However, our experiment also has several limitations that need to be noted. First, since it is well known that soil properties can strongly influence GHG emissions [45] and greenhouse specific experimental conditions can strongly affect the direction of magnitude of the experimental effects in controlled environments [54], it is not inconceivable that our results are only valid under the specific experimental conditions and only for the soil used in this experiment. Furthermore, as only one earthworm species per ecological category was used, it is unknown whether our findings are transferable to other species from the same ecological categories, or whether these findings are also valid for epigeic earthworm species which have been reported to also increase N_2O emissions [9]. Earthworm ecological categories never aimed to describe functional role but rather ecological and morphological groups which can explain the high variability on earthworm effect among species of a same ecological category [37,55,56]. We also acknowledge that the size of our mesocosms, although larger than many other studies, could have still potentially interfered with the earthworm burrowing behavior, especially for the deep burrowing anecic earthworms [57]. Our study is the first to our knowledge to investigate the link between porosity and greenhouse gases fluxes, but the frequent moving of the mesocosm filled with a 4 mm-sieved soil led to the formation of cracks that unfortunately made more difficult this analysis. Cracks being even more unstable that biological burrows under drying-rewetting cycles [58], they should be avoided if possible or taken into account in analyses in future experiments. Last but not least, our experiment is relatively short and covers only one part of the growing season (the plant growing phase of late spring to early summer) and not the time when plant litter is decomposing during autumn and winter time.

In conclusion, notwithstanding the above-mentioned uncertainties, we argue that our study opens up potential avenues of research that may explain the inconsistent and often contrasting effects of earthworms on N_2O and CO_2 fluxes. First, in agreement with other studies, we observe that the weekly emissions were at the highest at the start of the experiment presumably due to higher carbon and nitrogen availability from the added plant residues, and decreased with time as these resources were becoming more limiting. In this incipient phase, earthworm treatments (especially the anecic *L. terrestris*)

displayed higher N_2O emissions, and particularly so in the treatment without plants (Fig. S3AB.) and following watering events. This suggests not only that shorter experiments [11,12,14] are likely to emphasize this transient stimulation of emissions due earthworm bioturbation effects on organic matter mineralization if there are no plants available, but also that experiments only sampling after watering events (which is a common practice) will likely lead to biased estimates. Our study provides evidence that under experimental conditions with plants and soil water fluctuations, the presence of earthworms does not increase greenhouse (CO_2 and N_2O) gas emissions and revealed that the endogeic earthworm ecological category has even the potential to reduce N_2O emissions. We show that these effects likely occur via the earthworm impact on soil water status and aeration due to their burrowing patterns. Although the generality and transferability of these findings needs to be further tested, this study also suggests that future earthworm experiments in controlled environment setups should consider experimental conditions with plants in setups where the earthworm soil engineering effect on soil water status and aeration is allowed to occur.

Materials And Methods

Soil and biological material

The soil, classified as a gleyic luvisol, was excavated from a field margin adjacent to a wheat-corn-alfalfa rotation at the EFELE experimental site (North West of France, 8°05'35.9”N, 1°48'53.1”W) belonging to the long-term observatories SOERE-PRO-network. Only soil from the upper 0–30 cm layer was used in this experiment. According to the analyses performed by the Soil Analysis Laboratory, INRA Arras, France, the soil is composed of 14.6% clay, 72.1% silt and 13.3% sand, with a pH of 6.14, and contains 1.5% total organic matter, 0.84% carbon, 0.1% nitrogen, with a C:N ratio of 8.4.

The individuals of adult *L. terrestris* were supplied by the German company Wurmwelten (Dassel, Germany) and weighed on average 4.8 ± 1.3 g fresh weight. Adult individuals of *A. icterica* were harvested from a pesticide-free orchard in Avignon by manual digging and weighed on average 0.4 ± 0.2 g fresh weight. The earthworms were kept in their original soil for 3 days at a temperature of 14 ± 2°C and then placed in a mixture of the original soil and the experimental soil for one week at 8°C in the dark before the onset of the experiment.

Brachypodium distachyon L. was the plant species selected for this study due to its short life cycle (less than 3 months) [59] and because it is frequently used in controlled environment experiments as a model grass species in functional genomics experiments [60]. Experimental research and field studies on plants (either cultivated or wild), including the collection of plant material, must comply with relevant institutional, national, and international guidelines and legislation. "The seeds of the wild type variety (Bd 21 WT), were supplied by Observatoire du Végétal, INRAE Versailles (Paris, France). After germination in seedling trays, four seedlings were introduced in each mesocosm, outside the central cylinder that was introduced as a base for flux measurements of greenhouse gases (see Table 1 with response variables

and Fig. S1). During the first 3 weeks, any dead seedlings were replaced. The experiment finished with a final destructive harvest.

Mesocosm design and experimental treatments

The mesocosms consisted of PVC tubes of 16 cm dia. and 37 cm height (Fig. S1). Each mesocosm was filled with 9.2 kg air-dried soil containing 10% gravimetric water content (up to 3 cm from the brim) sieved to 2 mm and compacted to a bulk density of 1.21 g cm^{-3}. The mesocosms were sealed at their base with a 1 mm mesh followed by a PVC led pierced with 5 holes (1 cm in dia.) that allowed the drainage of surplus water out of the mesocosm. A transparent plastic film of 10 cm height was attached around the top perimeter of the mesocosms in order to act as a barrier preventing the earthworms to leave the mesocosms. As a previous meta-analysis indicated that earthworm effects on plant growth are more prevalent in the presence of crop residues [8] which also serve as a food resource of earthworms, the soil surface of all mesocosms was covered with 4 g litter mixture (2.2% N, C/N = 24) consisting of 1.3 g dry weight of *Medicago truncatula* Gaertn. shoots and 2.7 g dry weight of *Zea mays* L. leaves, the equivalent of organic residue inputs of 1060 Kg C ha^{-1} and 44 Kg N ha^{-1}.

The mesocosm experiment presented in this study included an earthworm treatment (henceforth Ew) with four levels: a control without earthworms, an anecic earthworm species (*Lumbricus terrestris* L.) with two individuals and a total of 9.6 ± 1g fresh weight (FW) earthworm biomass per replicate, an endogeic earthworm species (*Aporrectodea icterica* Savigny) with 7 ± 1.1 individuals and a total of 2.9 ± 0.1g FW earthworm biomass per replicate, and a mixture of both species with one *L. terrestris* individual (4.9 ± 0.9 g FW biomass) and 5 ± 1.6 *A. icterica* individuals (1.7 ± 0.5 g FW biomass) per replicate. Expressed per m^2, the earthworm FW biomass is the equivalent of 480, 145 and 330 g m^{-2} for the anecic, endogeic and both earthworm treatments levels, respectively; note that *L. terrestris* was more recently classified as species displaying traits belonging to both anecic and epigeic species[36], but in the large majority of literature it is considered an anecic species. The earthworm treatment was factorially crossed with a plant (*Brachypodium distachyon* L.) treatment with two levels, with and without plants, with 7 replicates per treatment combination.

The experiment was carried out in a greenhouse kept at temperatures ranging between 20 and 23°C during day-time and 18–20°C during night-time with an air relative humidity of 80%. The natural light was supplemented during day-time by artificial lighting for 12 hours per day with the help of high-pressure sodium lamps. The mesocosms were divided into two blocks corresponding to their position on the north or south bench of the greenhouse, and their position within the block was randomly changed twice a week, limiting the bias of the position within the block. The experiment run for 12 weeks between March and May 2017.

The watering protocol

The watering protocol was specifically designed to include soil moisture fluctuations (analogous to what happens in natural conditions) and to allow the earthworm burrowing and casting activities to affect the

soil water content (SWC). To this end, at the beginning of the experiment, the mesocosms were abundantly watered with 1.7 L of reverse-osmosis water using a laboratory dispenser (two sessions of 850 ml each), a volume decided based on whether the amount was sufficient to observe water draining out of the mesocosms. Measurements of weight changes after 24 h were used to calculate the weight of the mesocosms at field capacity (knowing that the soil contained 10% gravimetric water, i.e. ~0.9 L), which also allows the estimation of the water holding capacity during the experiment. After a drying phase of 6 weeks (until the driest mesocosms reached 60% of field capacity), the volume of water lost by the driest mesocosm was determined and then added to all mesocosms in order to ensure that all mesocosms received sufficient water to return to 100% of their initial field capacity. Following this method, all mesocosms were subjected to two drying-rewetting cycles (Fig. 1A,B).

Response variables

The measurements of CO_2 and N_2O emissions were carried out weekly using a static sampling chamber approach allowing the quantification of the changes in gas concentration by accumulation following the recommendations of Rochette (2011). The sampling chamber (9 cm dia., 6 cm height, 370 ± 1 ml) was equipped with a bung of silicone rubber for gas sampling at the top. During sampling, the chamber was placed on the circular collar (Fig. S1) that was inserted in the center of the mesocosms during the setup of the mesocosms, and which allowed measuring soil N_2O and CO_2 fluxes without disturbing the plants. The collar was inserted into the soil down to 3 cm and consisted of frame that provided support but allowed the access of earthworms and roots into the inner soil core, whereas the part above the ground part contained a gutter-like double walled section/groove allowing the insertion of the sampling chamber into the groove to provide a air-tight sealed chamber. CO_2 and N_2O fluxes were measured at the Platform for Chemical Analysis in Ecology (LabEx CeMEB, Montpellier, France). CO_2 concentrations were measured with a gas chromatograph (MicroGC S-Series, SRA Intruments, Marcy l'Etoile, France) using a catharometric detector, quantifying the gases on the basis of their thermal conductivity. N_2O concentrations were measured by gas chromatography equipped with an electron capture detector (Varian CP-3800, Varian Inc., Palo Alto, USA). In the day of gas sampling, the mesocosms from each block were transported to the gas sampling laboratory with a trolley. Prior to sampling, 20 ml of distilled water was added in the groove to provide an air-tight sealing system when placing the sampling chamber on the collar. Air samples for determining the CO_2 and N_2O concentrations were taken at T0 (immediately after placing the chamber on the collar) and after 2 h to assess the changes in concentrations. Previous tests sampling after 1, 2, 3 and 4 hours revealed that the accumulation response was linear during this short accumulation time. A volume of 0.2 ml was sampled sequentially for CO_2 and N_2O measurements via the silicone bung using a plastic syringe equipped with a 25G needle and injected immediately in the gas chromatographs via a 1/32“ PFA line. A volume of 0.2 ml was sampled sequentially for CO_2 and N_2O measurements via the silicone bung using a plastic syringe equipped with a 25G“ needle and injected immediately in the gas chromatographs via a 1/32“ PFA line. Concentration changes in the sampling chamber between T0 and T0 + 2h were used to estimate the greenhouse gas emission rates as g CO_2 (or N_2O) m^{-2} day^{-1}.

At the end of the experiment, the mesocosms were transported to the INRAE center of Nancy to analyze soil macroporosity by X-ray tomography using a medical scanner (BrightSpeed Exel 4, General Electric). The settings were 120 kV and 50 mA for the current and 0.625 mm width for each image. The images were transformed into 16-bit image and binarized (i.e. converted in black and white) using a fixed threshold value (70) since the different peaks (for the soil matrix and for the porosity) were well separated [61]. The roots and pores created by the roots could not be included in the analysis due to their smaller average size compared to the resolution of the scanner (0.4 mm per pixel). The burrow system was then characterized by computing the volume and number of burrows in four soils layers (L1 for 0-8.5 cm, L2 for 8.5–17 cm, L3 for 17-25.5 cm and L4 for 25.5–34 cm depth). Drying-rewetting cycles contributed to the formation of cracks, i.e. macropores resulting from physical processes (shrinkage, swelling [58]) notably in the top soil layer (Fig. 3 and S4). We attempted to differentiate cracks from burrows according to the macropore circularity and range of area in 2D images using ImageJ [58], burrows made by earthworms being more circular compared to cracks. However, the partitioning method employed still identified burrows in the mesocosms without earthworm, with 25.42% of pores misidentified as burrow in the whole column, the error being higher in the first layer (32.15%) than in the bottom layer L4 (20.06%) (Fig. S2, Table S1), which limits our ability to investigate the earthworm burrowing effect only. Therefore, considering that gas fluxes will be influenced by the total porosity, regardless of its biological or physical origin, and the high correlation between the 15 different porosity variables (Fig. S3), although we investigated how the treatment affected the different porosity type (pores, burrows, and cracks), we decided to only use the total porosity data as a predictor in the models.

After the X-ray scan in Nancy the mesocosms were transported back to Montpellier for the final destructive harvest. The proportion of earthworms found at the final harvest was 62 and 90% for *L. terrestris* and *A. caliginosa*, respectively. The proportion of recovered earthworms was likely affected by the mortality occurring during the days of transport and storage for the X-ray scans (during which the temperatures and vibrations were not controlled) as several *L. terrestris* individuals were found freshly dead at the harvest. However, the X-ray scans together with the litter mass loss dynamic (Fig. 1C,D) provide strong evidence that the earthworms were active during the whole duration of the experiment.

With the exception of the remaining percentage of litter, which was assessed weekly by the same person using a visual estimation method with 5% intervals, other additional soil and plant-related response variables were measured at the end of the experiment. The soil analyses were performed on the homogenized soil (sieved at 2 mm) sampled from the upper 10 cm of the mesocosms inside the collar. Potential soil microbial denitrification enzymatic activity (DEA) was measured using the acetylene inhibition method described by a method that measures total potential denitrification (as N_2O and N_2) [62]. This is a complementary method to the fluxes measured during the experiment which are only measuring the N_2O emissions. The MicroResp™ method[43] was used to determine the microbial metabolic quotient. Approximately 0.39 g dry weight of soil was incubated in six replication wells with a solution of D-glucose (1.5 mg C g^{-1} soil), and six replication wells with deionized water (for basal respiration), so as to reach 80% of the field capacity in 96-DeepWell Microplates (Fisher Scientific E39199). Cresol red gel detection

plates were prepared as recommended by the manufacturer. After an initial two-hour pre-incubation at 25°C in the dark, each deepwell microplate was covered with a CO_2-trap microplate detection plate using a silicone gasket (MicroResp™, Aberdeen, UK). The assembly was secured with a clamp, and incubated for four additional hours. Optical density at 590 nm (OD590) was measured for each detection well before and after incubation using a Victor 1420 Multilabel Counter (Perkin Elmer, Massachusetts, USA). Calibration relying absorbance (OD590 readings) and CO_2 concentrations was performed using the gas chromatograph previously described. Final OD590 were normalized using pre-incubation OD590, and converted as respiration rates expressed in µg C-CO_2 respired per g^{-1} of soil h^{-1}. The glucose-induced respiration rate was used to estimate the soil microbial C (C_{mic}, µg $C_{microbial}$ g^{-1} dry soil) biomass [63]. Finally, the metabolic quotient (Met_Q) was determined as the ratio between basal respiration rates measured in the wells with water only and no C substrate (as a proxy of the microbial basal respiration) and C_{mic}. Soil mineral nitrogen was extracted from 10 g of freshly sampled soil with 40 mL of 1 M KCl solution. Nitrate and ammonium concentrations were measured by continuous flow spectrophotometry (SKALAR 3000 auto analyzer, Breda, The Netherlands). The plant shoot biomass was weighed after drying at 60^{o}C for three days. As the roots of *B. dystachyon* are extremely thin and fragile, it was not feasible to sample root biomass.

Statistical analyses

Statistical analyses were done using R version 4.0.2 (R Development Core Team, 2015) in Rstudio version 1.3.959 (RStudio Team, 2015). Weekly time series of CO_2 and N_2O emissions and SWC were analyzed with the “nmle” package version 3.1–145 [64] to perform repeated measurers analyses using a generalized mixed-effects model to test the effect of earthworms, plants, SWC (for GHG emissions only) and sampling week and their interactions on gas fluxes. The identity (ID) of the mesocosm and its position in the blocks were used as random factors to account for temporal pseudoreplication and the effect of the position in the north or south bench in the greenhouse (“random = ~ 1 | Block / ID”). To reach models which respect the assumption of homosedasticity of the residuals, we tested the model fit with varIdent (for plant and earthworm experimental treatments), varPower (for SWC) and varExp (for SWC) weighting functions [65] and selected the most appropriate models based on maxim likelihood (ML) model comparison tests. A similar approach was used for the cumulative CO_2 and N_2O data at the end of the experiment (estimated assuming constant emission rates between the weekly measurements), but without the sampling week among the fixed effects and the mesocosm ID in the random effects. For the later analysis, we used the mean of weekly SWC values as arguably this variable is more relevant to the cumulative fluxes. The “r.squaredGLMM” function from the MuMIn [66] was used to derive the proportion of the variation that was explained by the fixed factors (i.e. marginal r^2, mr^2) in mixed-effects models. The “multcom” package was used to perform Tukey's HSD (honestly significant difference) multicomparison posthoc test, but note that this test does not include the random effects and occasionally the results are not entirely in line with the fitted coefficients from the mixed effects models.

Additional analyses were effectuated aiming to link the large number of potential predictors (Table 1, Fig. S3) measured at the end of the experiment and the CO_2 and N_2O fluxes from week 12 (just before the experiment was stopped). As large number of response variables were measured in order to explore potential predictors, a method of best subset selection that is penalizing model complexity (i.e. regularization) during estimation was required. Regularization, aims to significantly reduce the variance of the model as well as model overfitting by varying the lambda (λ) parameter which is tuning the level of penalization for the complexity of the model. This approach has been proved to be a viable option for estimating parameters in scenarios with small sample size and many collinear/correlated predictors. Here we used a penalized regression method based on the minimax concave penalty (MCP) in order to select the best subsets [67] using the *ncvreg* package 3.11–1[68]. This approach was combined with a 10-fold cross-validation procedure to derive the lambda parameter (also called the regularization rate) which minimizes the cross-validation error. We report the fitted coefficients and the coefficient of determination (r^2) at lambda values that minimize the cross-validation error. The subset variables with retained non-zero coefficients where then tested in the generalized mixed-effects models which have the advantage of including random effects alongside the treatment factors.

Declarations

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Data Availability

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

Author contributions : AM and JN provided the funding, AM, JN and PG designed the experiment, PG carried out the experiment and measurements, YC, IB, BB, AS, NF provided methods and carried out specific measurements. PG and AM analyzed the data, PG and AM wrote the paper with input from all co-authors.

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Figures

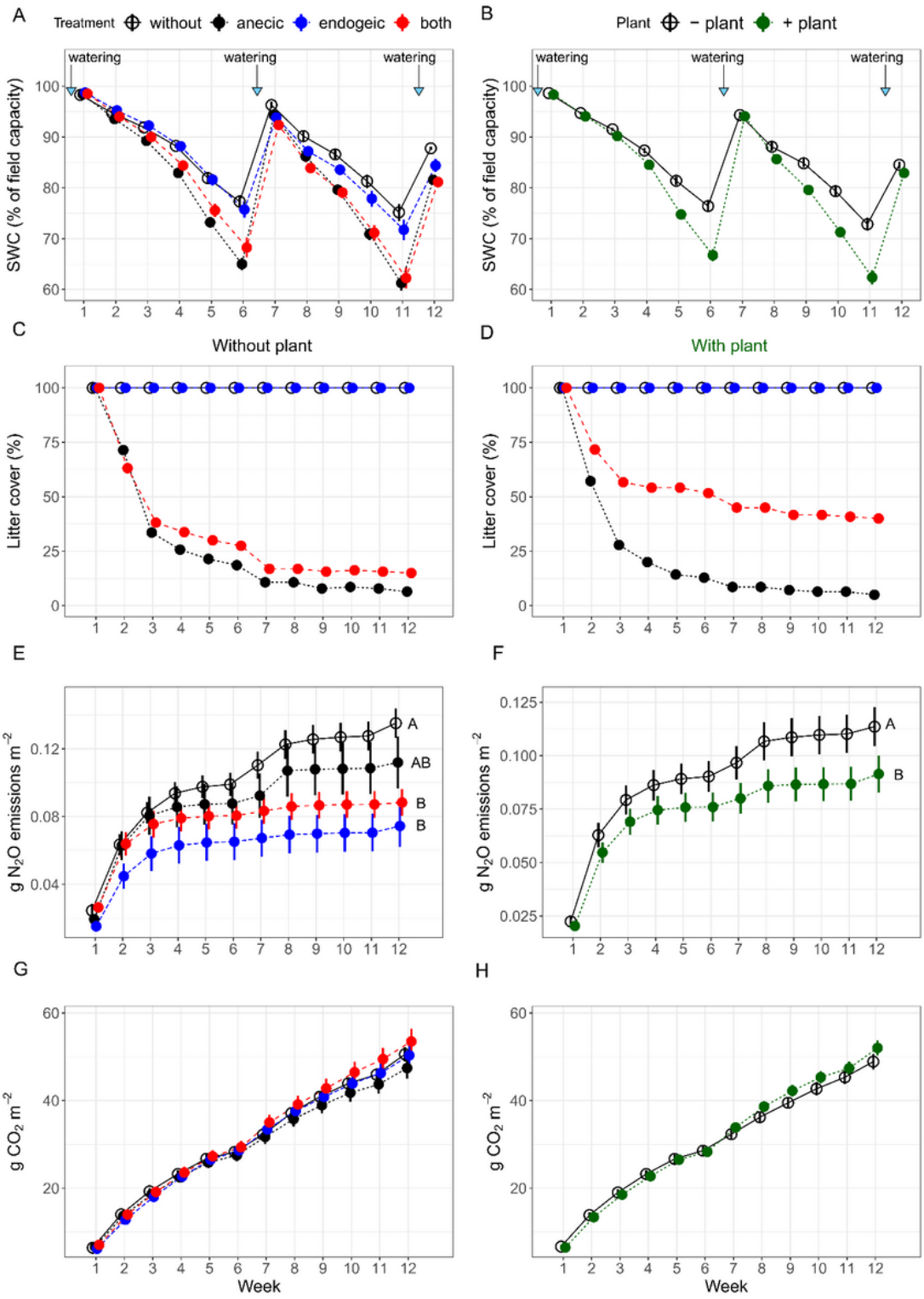

Figure 1

Temporal dynamic of (A,B) soil water content (SWC expressed as percentage of field capacity), (C,D) percentage of surface covered by litter, cumulative N_2O (E,F) and CO_2 (G,H) emissions, as affected by the earthworm treatment and plant treatment. Error bars represent ± 1 SEM. Different letters represent significantly different levels as estimated by Tukey multicomparison posthoc test. Effect of earthworm independently of plant, and vice-versa, is displayed when the Ew:Plant interaction was not significant (A,B, E-H), i.e. for all response variables except litter cover.

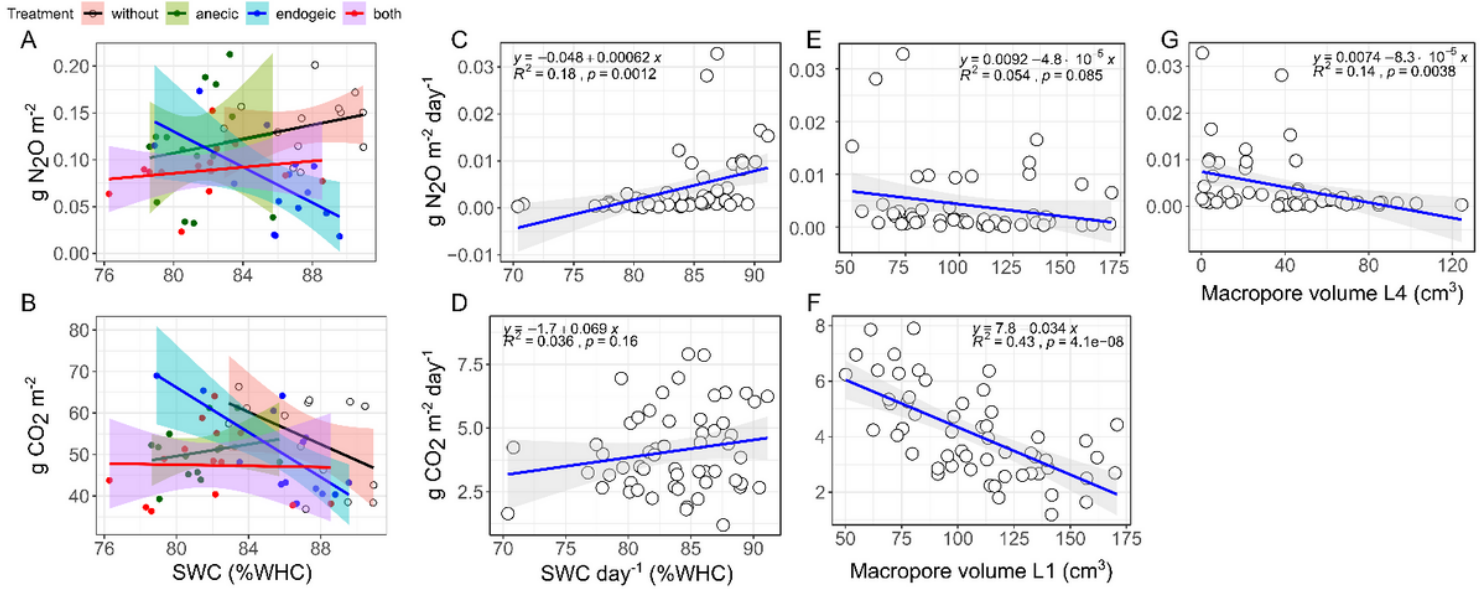

Figure 2

Cumulative N_2O (A) and CO_2 (B) emissions as affected earthworm × SWC interaction, where SWC represents the 3-month average SWC and the emissions the total cumulative gas emissions. Relationships between N_2O and CO_2 emissions measured in the last experimental week (week 12) and soil water content (C, D), X-ray tomography estimated volume of macropores in the top-soil layer (E,F) and in the bottom layer for N_2O (G). In C to F, linear regression line and 95% confidence intervals are displayed along with line equation, coefficient of determination (R^2) and p-value. Note that this relationship will differ from the glm models results (Table 1 and 4).

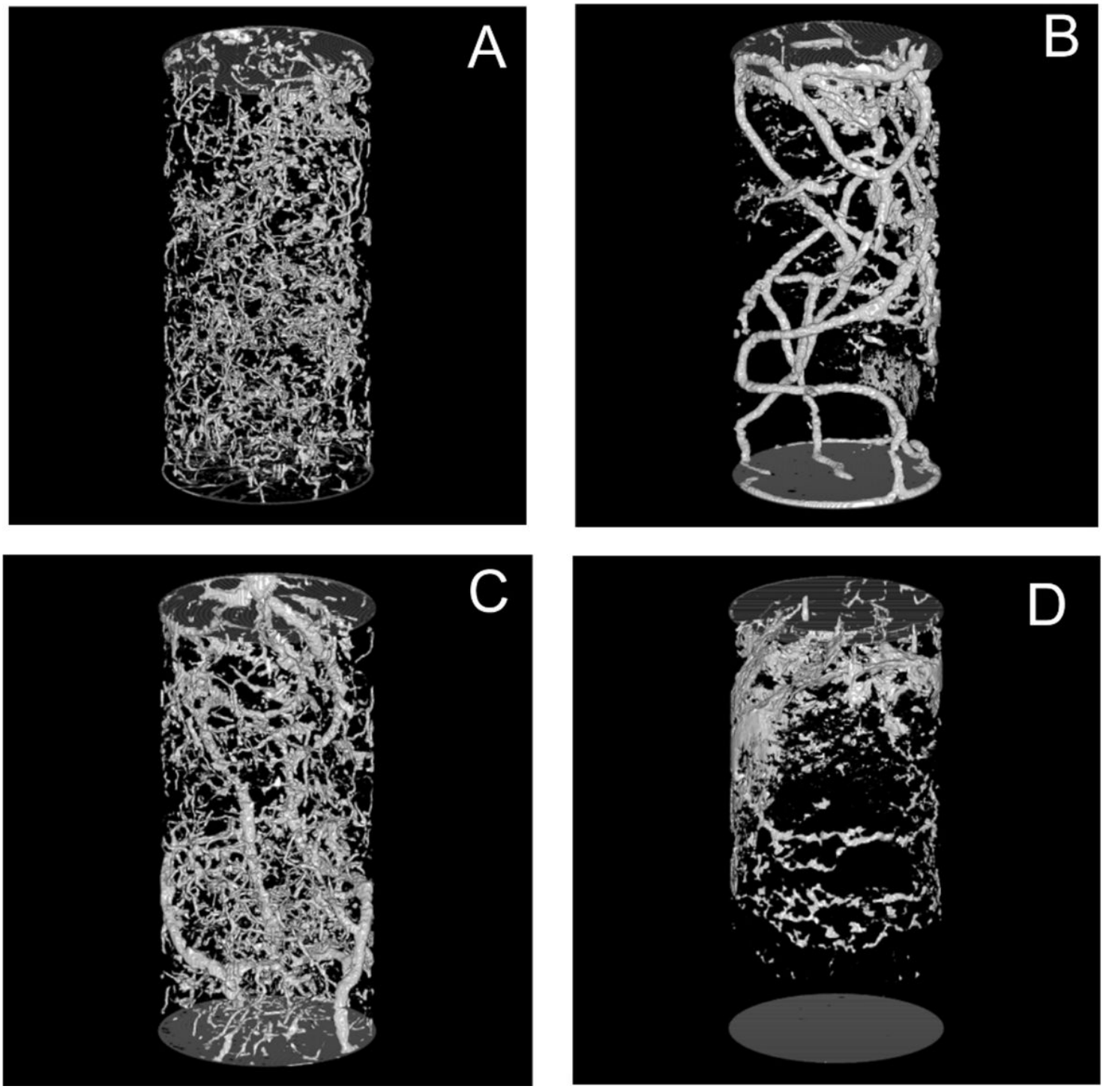

Figure 3

Examples of 3D reconstruction of the macroporosity within the soil cores: (A) *A. icterica* (endogeic) only, (B) *L. terrestris* (anecic) only, (C) both species and (D) control without earthworms (cracks can be observed as the less circular porosity)

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