Earthworms do not increase greenhouse gas emissions (CO2 and N2O) in an ecotron experiment simulating a realistic three-crop rotation system

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Article

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Abstract

Earthworms are known to stimulate soil greenhouse gas (GHG) emissions, but previous studies have used simplified models or had limited measurements. To address this, we conducted a two-year study using large lysimeters in an ecotron facility, continuously measuring ecosystem-level CO_2, N_2O, and H_2O fluxes. We investigated the impact of endogeic and anecic earthworms on GHG emissions and ecosystem water use efficiency (WUE) in an agricultural setting. Although we observed transient stimulations of carbon fluxes in the presence of earthworms, cumulative fluxes over the study indicated no significant increase in CO_2 emissions. Endogeic earthworms marginally reduced N_2O emissions during the wheat culture (-44.6%), but this effect was not sustained throughout the experiment. No consistent effects on ecosystem evapotranspiration or WUE were found. Our study suggests that earthworms do not significantly contribute to GHG emissions over a two-year period in experimental conditions that mimic an agricultural setting. These findings highlight the need for realistic experiments enabling continuous GHG measurements.

Introduction

Earthworms are important decomposers in many ecosystems as they help to break down organic matter and release nutrients that can be used by plants and other organisms[1]. Thus, they are crucial for the functioning of many ecosystems and there is evidence that they play a vital role in supporting soil fertility and plant growth[2,3], with the exception of ecosystems in which they are not native[4,5]. However, their activity can also lead sometimes to the release of greenhouse gases (GHG), such as carbon dioxide (CO_2) and nitrous oxide (N_2O)[6]. This is particularly concerning given the crucial role of soil in mitigating climate change through carbon (C) sequestration[7,8] and N_2O regulation[9,10].

According to the latest meta-analysis[6], earthworms can increase soil CO_2 and N_2O emissions by 33 and 42%, respectively, with emissions being significantly stimulated in the presence of anecic earthworms. Furthermore, earthworms are considered ecosystem engineers due to their ability to modify soil structure and interact with soil microorganisms and plants through their feeding, burrowing, and casting activities[11]. They can be divided into three ecological categories based on their feeding and burrowing habits: (1) anecic species that feed on fresh litter from the soil surface and create mainly permanent burrows, (2) epigeic species that live on the soil surface and feed on surface litter without creating permanent burrows, and (3) endogeic species that live and feed on mineral soil and associated organic matter below the surface, and that create non-permanent burrows without preferential orientation[12]. However, some field and laboratory studies included in the Lubbers et al.[6] meta-analysis as well as more recent ones[13–19] have reported results that are not in line with the general conclusion that earthworms increase greenhouse gas emissions.

The high number of studies reporting inconsistent results indicates that the mechanisms by which earthworms affect CO_2 and N_2O emissions are complex and not fully understood and likely depend on a

variety of factors, including the earthworm ecological category, the type of soil, the amount and type of organic matter present and the experimental setup[6]. Earthworms can affect the soil CO_2 emissions directly as the result of breaking down the soil and litter organic matter through digestive processes, releasing CO_2 as a by-product, but also indirectly by incorporating plant residues into the soil, modulating the microbial-controlled decomposition of organic matter through changes in soil moisture dynamics, nutrient status, soil aggregation and CO_2 diffusivity[1]. In addition to these effects that mainly stimulate the CO_2 release from the soils, earthworms have also been suggested to induce long-term stabilization of soil C in casts by enhancing the stabilization of C relative to mineralization[20], but contrasting effects have also been found[21]. Concerning the earthworm impact on the N_2O emissions, the proposed mechanisms are both direct, such as the stimulation of denitrifier activity in the earthworm gut due to favorable conditions for denitrifying bacteria such as anaerobic conditions, availability of nitrogen (N) and C at favorable moisture levels[22], as well as indirect, including the stimulation of denitrifiers communities in the soil (as well as in the burrows, casts and middens) which can be further modulated by earthworms through incorporating plant residues in the soil and enhancing N and C mineralization[16] as well as through burrowing effects on soil water infiltration and gas diffusivity[23]. These later two effects could also reduce N_2O emissions[14] if they lead to less anaerobic microsites and increased soil aeration, which is detrimental to denitrifiers[24].

Considering the complexity and context-dependence of the mechanisms discussed above, it is not surprising that the literature contains a wide range of reports on the impacts of earthworms on GHG, which may be influenced by the specific conditions of the experimental setups. A possible explanation for the lack of significant GHG stimulation in the few field studies included in the Lubbers et al.[6] meta-analysis is that the results obtained from highly simplified laboratory experiments, which were conducted using model systems that lacked important features of field conditions such as plants, natural light, larger containers, surface litter layer, and earthworm burrows that affect water drainage and soil moisture fluctuations, cannot be generalized to field conditions. Other common drawbacks of many experiments include limited duration and the use of only point measurements of fluxes, which may fail to capture the full range of phenomena and processes in the system. These conjectures are supported by experimental results conducted in field conditions or based on laboratory setups trying to mimic natural conditions and in which two main naturally occurring factors were found to mitigate earthworm mediated CO_2 and N_2O emissions, namely: (i) growing plants and (ii) soil water content fluctuations due to drainage or drying and rewetting cycles[13–17]. Plants, as primary producers, play a crucial role in biogeochemical cycles because they determine the amount and quality of carbon that enters the soil system[25]. Additionally, they can stimulate microbial activity through the release of root exudates, which can create sub-oxic microsites in the rhizosphere due to the influx of root-derived carbon substrates. This leads to an increased demand for oxygen, favoring denitrification[26]. On the other hand, plants compete with microbes for nitrogen acquisition[27], decrease soil water content through transpiration, and modify soil porosity through root growth, which can alter the dominant processes that produce N_2O emissions

(nitrification, denitrification, and nitrate ammonification)[24,28]. This is in line with the results from a mesocosm experiment with plants and earthworms[15], which found that the presence of plants lowered N_2O emissions by 19.8%, in correlation with a 43% and 20% decrease in nitrate and ammonium respectively.

Soil water status is a known factor that plays a significant role in the production of greenhouse gases in soil[29,30]. Studies have shown that soil moisture can explain up to 95% of GHG emissions[31]. The availability of oxygen, nitrates, ammonium, and carbon in the soil is determined by the moisture content, which in turn affects the activity of microorganisms. Furthermore, anoxic conditions under high soil water content can stimulate N_2O emissions, primarily through the process of denitrification, while nitrification is more likely to occur in aerobic conditions with unsaturated soil moisture. Naturally occurring fluctuations in soil moisture and drying-rewetting cycles, can affect the proportion of denitrified nitrogen that is converted to N_2O or N_2, ultimately modulating the N_2O/N_2 ratio that is emitted into the atmosphere[32,33]. Therefore, experimental setups aiming for constant soil moisture, as used in many laboratory incubations, are likely to lead to biased conclusions. Moreover, as earthworms are known to be able to affect the water infiltration in the field[34], in order to assure the transferability of the earthworm effects on CO_2 and N_2O emissions from controlled to field conditions, it is crucial to use experimental setups that include the earthworm engineering effects on soil water infiltration and aeration in a realistic field-like way.

In this study, we addressed the need for realistic long-term experiments to evaluate the impact of earthworms on CO_2 and N_2O emissions using a relatively large model system (5 m^2 of surface, and 1.5m depth lysimeters) simulating and agricultural context, and using an advanced controlled environment facility that has the capability to automatically and continuously measure the net ecosystem exchange of CO_2, N_2O and H_2O fluxes[35,36]. We followed the impact of earthworm communities belonging to two ecological categories (endogeic and anecic separately) versus a control with reduced earthworm abundance and biomass over a two-year crop rotation with three crops (wheat-mustard-maize) and two fallow intercrops (see Fig. 1 for the experimental timeline). We hypothesized that, under simulated field-like conditions and in the presence of plants, higher anecic and endogeic earthworm biomass would not result in an increase in cumulative CO_2 and N_2O emissions or H_2O loss as evapotranspiration, compared to a control with very low earthworm biomass.

Table 1: Experimental details of the crop sequence: sowing and harvest dates, as well as sowing density, for each crop.

Crop	Sowing date	Harvest/destruction date	Sowing density
Winter wheat	2017-11-29	2018-07-09	184 ind. m^{-2}
Intercrop 1		2018-11-21	Bare ground with spontaneous weeds
Mustard	2018-11-21	2019-03-29	1g m^{-2} (10 kg ha^{-1})
Intercrop 2		2019-05-16	Bare ground with spontaneous weeds
Maize	2019-05-16	2019-09-21	11 ind. m^{-2}

Results

Wheat crop

NEE followed the wheat growing stages (Fig. 2A) as shown by the Week explanatory variable retained in the minimum adequate model (P-value < 0.001; Table 2), but no significant effect of the earthworm treatment (EW_T) nor of the amount of earthworm biomass (EW_BM) was found (Table 2). Cumulative NEE fluxes over the whole wheat crop (Fig. 2B; Table 2) reached 4646.63 (± 66.62), 4347.13(± 67.47) and 4491.32 (± 105.01) g CO_2 m^{-2} in the control, anecic and endogeic earthworm treatment levels, respectively, and showed a marginally significant effect of EW_T, with lower cumulative values (-6.44%, Table 2; Fig. 2B) in the anecic earthworm treatment level relative to control (P-value = 0.086). Unlike NEE, weekly GPP fluxes (Fig. 2C) showed an EW_BM effect, GPP fluxes slightly increasing with EW_BM (P-value = 0.027; Table 2), and a strong Week effect (P-value < 0.001; Table 2). The cumulative GPP fluxes reached 6585.20 (± 72.42), 6304.43 (± 79.51) and 6344.18 (± 141.18) g CO_2 m^{-2} in the control, anecic and endogeic earthworm treatment levels, respectively. However, although the cumulative GPP was found to be slightly increasing with EW_BM (irrespective of the earthworm ecological category; Table 2), it was also marginally significantly lower in the anecic (-4.26%) and endogeic (-3.66%) earthworm treatment levels relative to control (P-value = 0.065; Table 2; Fig. 2D). The later result combined with the simultaneous positive EW_BM effect on GPP indicates that higher biomass within each treatment level also led to a slight increase in GPP. A similar relationship between EW_BM and EW_T was occasionally observed in other response variables.

Table 2
Minimal adequate models (F-values) for (i) **weekly time series** as affected by the sampling week (Week), earthworm biomass (EW_B), treatment (EW_T) and their interactions, and (ii) **cumulative emissions** as affected by the earthworm biomass (EW_B) and treatment (EW_T) **in the wheat crop**. "NA" stands for non-applicable. ***P < 0.001; **P < 0.01; *P < 0.05; +P < 0.1.

Weekly time series						
Source	NEE	GPP	Reco	N_2O	ET	WUE
Week	**$F_{32/352}$= 895.45*** **	**$F_{32/352}$= 1330.22*** **	**$F_{32/288}$= 642.24*** **	**$F_{28/308}$= 30.79*** **	**$F_{32/352}$= 299.31*** **	**$F_{32/352}$= 1624.52*** **
EW_BM	NA	**$F_{1/10}$= 6.68*** **	NA	NA	NA	NA
EW_T	NA	NA	**$F_{2/9}$= 5.19*** **	F2/9 = 2.54	NA	NA
EW_T:Week	NA	NA	**$F_{64/288}$= 1.73*** **	NA	NA	NA
Cumulative						
EW_BM	NA	**$F_{1/8}$= 7.99*** **	**$F_{1/8}$= 41.41*** **	$F_{1/8}$ = 1.59	NA	NA
EW_T	$F_{2/9}$ = 3.38+	$F_{2/8}$ = 3.91+	**$F_{2/8}$= 12.18*** **	$F_{2/8}$ = 3.06	NA	NA

Reco weekly emissions increased with wheat development until the beginning of May and slowly decreased thereafter with the senescence of the plants until the harvest (Fig. 2E). Reco weekly fluxes were significantly affected by the EW_T×Week interaction (P-value = 0.001; Table 2), with several weeks where the anecic earthworms stimulated Reco at the beginning of the crop, however this changed in the middle of the crop where Reco values were higher in the endogeic treatment level relative to control and during the last four weeks of the experiment where Reco values were higher both in the anecic and endogeic earthworm treatment levels relative to control. The cumulative Reco fluxes generally increased with earthworm biomass (P-value < 0.001; Table 2) and reached − 2491.38 (± 14.98), -2490.78 (± 97.91) and − 2382.04 (± 59.33) g CO_2 m^{-2} in the control, anecic and endogeic earthworm treatment levels respectively. The cumulative Reco was also affected by the EW_T (P-value = 0.004; Table 2) with significantly lower fitted model parameter estimates for the endogeic treatment level relative to control (-4.4%), however, the Tukey's HSD test used in Fig. 2F does not capture this difference.

The analyses of the weekly dynamics of N_2O emissions showed a significant Week effect (P-value < 0.001; Fig. 2G; Table 2) and a stimulation of emissions after the addition of fertilizer (digestate of methanisation in April and May). A tendency for an EW_T effect (P-value = 0.133; Table 2; Fig. 2G) was also found, with the endogeic earthworms marginally reducing N_2O emissions (-19.8%) relative to control.

The cumulative N_2O fluxes reached 20.53 (± 4.38), 22.10 (± 4.71) and 11.36 (± 2.69) g m^{-2} in the control, anecic and endogeic earthworm treatment levels, respectively. Analysis of the cumulative N_2O confirmed that the N_2O emissions were statistically marginally significantly lower in the endogeic earthworm treatment level relative to control (P-value = 0.109), however the effect size was notable (-44.6%; Table 2, Fig. 2H).

ET was not affected by either the earthworm treatment or earthworm biomass (Table 2). ET significantly varied with the Week following the crop development (Fig. 2I) and was about 2 kg m^{-2} day^{-1} at the start of the wheat growing season until mid-April and then reached to ~ 4.5 kg m^{-2} day^{-1} for the rest of the season. Cumulative evapotranspiration fluxes reached 562.47 (± 19.03), 572.78 (± 7.06) and 572.15 (± 5.41) kg H_2O m^{-2} in the control, anecic and endogeic levels, respectively and showed no statistically significant effects of the earthworm treatment nor earthworm biomass (Table 2; Fig. 2J).

WUE weekly means increased for all treatment levels from around − 0.1 to 19 g CO_2 kg H_2O m^{-2} day^{-1} and then decreased, following wheat development (Fig. 2K) and were only influenced by Week (P-value < 0.001; Table 2). WUE cumulative values reached 2423.10 (± 96.05), 2279.78 (± 67.78) and 2286.10 (± 35.41) g CO_2 kg H_2O $^{-1}$ m^{-2} in the control, anecic and endogeic earthworm treatment level, respectively, and showed no statistically significant effects of EW_T or EW_BM (Table 2; Fig. 2L).

Mustard crop

The weekly NEE (Fig. 3A) only varied with time (P-value < 0.001) and no significant effect of EW_BM nor EW_T was found (Table 3). The cumulative NEE emissions reached 1242.63 (± 71.66), 1132.70 (± 82.43) and 1154.18 (± 109.96) g CO_2 m^{-2} in the control, anecic and endogeic earthworm treatment levels, respectively, with no significant differences between the three earthworm treatment levels (Table 3; Fig. 3B). However, the cumulative NEE emissions were found to increase with EW_BM (P-value < 0.001; Table 3).

Table 3
Minimal adequate models (F-values) for (i) **weekly time series** as affected by the sampling week (Week), earthworm biomass (EW_B), treatment (EW_T) and their interactions, and (ii) **cumulative emissions** as affected by the earthworm biomass (EW_B) and treatment (EW_T) **in the mustard crop**. "NA" stands for non-applicable. ***P < 0.001; **P < 0.01; *P < 0.05; +P < 0.1.

Weekly time series						
Source	*NEE*	GPP	Reco	N_2O	ET	WUE
Week	**$F_{18/198}$= 322.35*** **	**$F_{18/162}$= 1326.67*** **	**$F_{18/162}$= 280.5*** **	**$F_{18/162}$= 85.55*** **	**$F_{18/198}$= 307.84*** **	**$F_{18/198}$= 214.94*** **
EW_BM	$F_{1/8}$ = 0.65	$F_{1/8}$ = 0.1	NA	NA	NA	$F_{1/10}$ = 2.37
EW_T	$F_{2/8}$ = 1.84	$F_{2/8}$ = 0.96	$F_{2/9}$ = 2.12	$F_{2/9}$ = 2.71	NA	NA
EW_T:Week	NA	**$F_{36/162}$= 1.76*** **	$F_{36/162}$ = 0.71	**$F_{36/162}$= 2*** **	NA	NA
Cumulative						
EW_BM	**$F_{1/8}$= 40.52*** **	**$F_{1/8}$= 18.47*** **	NA	NA	$F_{1/10}$ = 3.78+	NA
EW_T	$F_{2/8}$ = 1.38	$F_{2/8}$ = 0.62	NA	NA	NA	**$F_{2/9}$= 4.66*** **

Weekly GPP fluxes (Fig. 3C) were significantly affected by the EW_T×Week interaction (P-value = 0.009; Table 3), with several weeks where the GPP was significantly lower in the endogeic treatment level in the middle of the growing season. The cumulative GPP fluxes reached 1619.70 (± 103.59), 1508.68 (± 87.85) and 1548.54 (± 123.90) g CO_2 m^{-2} in the control, anecic and endogeic earthworm treatment levels, respectively, and were also found to increase with EW_BM (P-value = 0.003; Table 3).

Reco weekly emissions fluctuated with the changes in environmental conditions and the development of the mustard canopy (Fig. 3E) and showed transient tendencies of higher Reco under anecic and endogeic treatment levels in the first four weeks of the culture as indicated by the EW_T×Week interaction (P-value = 0.883; Table 3) which was retained in the minimal adequate model despite not having a significant P-value. The cumulative Reco fluxes reached − 553.12 (± 50.32), -540.21 (± 41.06) and − 562.57 (± 27.58) g CO_2 m^{-2} in the control, anecic and endogeic earthworm treatment levels, respectively and showed no statistically significant effects either of EW_T or EW_BM (Table 3; Fig. 3F).

The weekly N_2O emissions (Fig. 3G) were significantly affected by the EW_T×Week interaction (P-value < 0.001; Table 3), with marginally higher N_2O the last two weeks of December in the endogeic treatment level relative to control. However, these effects proved to be transient, as the cumulative N_2O fluxes of 14.8 (± 3.33), 14.22 (± 1.05) and 13.43 (± 0.56) g m^{-2} in the control, anecic and endogeic earthworm

treatment levels, respectively showed no statistically significant effects of EW_BM nor EW_T (Table 3; Fig. 3H).

Weekly ET fluxes followed the crop development and increased progressively from about 1 kg m^{-2} day^{-1} from the start of the mustard growing season to ~ 2 kg m^{-2} day^{-1} before the crop harvest (Fig. 3I), and only a significant Week effect was found (P-value < 0.001; Table 3). Cumulative ET fluxes reached 204.6 (± 6.97), 217.74 (± 6.02) and 205.72 (± 10.44) kg H_2O m^{-2} in the control, anecic and endogeic earthworm treatment levels, respectively, and were not affected by EW_BM nor EW_T (Table 3; Fig. 3J).

WUE started to steadily increase for all earthworm treatment levels one month after sowing, going from around 0.8 g CO_2 kg H_2O m^{-2} day^{-1} at the beginning of December to 12 g CO_2 kg H_2O m^{-2} day^{-1} at the end of January, then progressively decreasing to 6 g CO_2 kg H_2O m^{-2} day^{-1} at the end of the mustard growing season (Fig. 3K). WUE weekly means varied with Week (P-value < 0.001; Table 3). The cumulative WUE reached 1014.15 (± 34.31), 891.54 (± 40.29) and 1009.00 (± 17.29) g CO_2 kg H_2O^{-1} m^{-2} in the control, anecic and endogeic earthworm treatment levels, respectively, and was found to be affected by the EW_T with statistically significantly lower (-12%) values in the anecic treatment level relative to control (P-value = 0.041; Table 3; Fig. 3L).

Maize crop

NEE and GPP mustard weekly fluxes followed the maize growing stages, with a steep and fast increase over the month of June, followed by a plateau until mid-July and a progressive and long decrease during maize senescence (Fig. 4A and 4C). The NEE weekly fluxes were influenced by the EW_T×Week interaction (P-value = 0.002; Table 4), with higher NEE the second week of June 2019 and in the last two weeks of the experiment for the endogeic treatment level relative to control. The cumulative NEE emissions reached 2379.21 (± 95.99), 2327.87 (± 103.88) and 2237.60 (± 64.70) g CO_2 m^{-2} in the control, anecic and endogeic earthworm treatment levels, respectively and were not influenced by EW_BM or EW_T (Table 4; Fig. 4B). Weekly GPP fluxes showed similar pattern and effects as NEE (Fig. 4C), and were influenced by the EW_T×Week interaction (P-value = 0.001; Table 4), with transient stimulation (e.g., weeks 21, 24 and 38) or dampening (week 32; second week of August) of GPP fluxes by the endogeic earthworms relative to the control (Fig. 4C). The cumulative GPP fluxes reached 3577.9 (± 121.50), 3410.82 (± 152.84) and 3435.09 (± 48.68) g CO_2 m^{-2} in the control, anecic and endogeic earthworm treatment levels, respectively, and, like cumulated NEE fluxes, were not influenced by the earthworm treatment (Table 4; Fig. 4D).

Table 4
Minimal adequate models (F-values) for (i) weekly time series as affected by the sampling week (Week), earthworm biomass (EW_B), treatment (EW_T) and their interactions, and (ii) cumulative emissions as affected by the earthworm biomass (EW_B) and treatment (EW_T) **in the maize crop**. "NA" stands for non-applicable. ***P < 0.001; **P < 0.01; *P < 0.05; +P < 0.1.

Weekly time series						
Source	NEE	GPP	Reco	N_2O	ET	WUE
Week	**$F_{18/162}$= 235.87*** **	**$F_{18/162}$= 231.97*** **	**$F_{18/198}$= 94.35*** **	**$F_{14/154}$= 11.84*** **	**$F_{18/198}$= 623.3*** **	**$F_{18/198}$= 1719.96*** **
EW_BM	NA	NA	NA	NA	**$F_{1/8}$= 3439.95*** **	**$F_{1/8}$= 1457.34*** **
EW_T	$F_{2/9}$ = 1.26	$F_{2/9}$ = 2.75	NA	NA	**$F_{2/8}$= 395.68*** **	**$F_{2/8}$= 47.09*** **
EW_T:Week	**$F_{36/162}$= 1.96*** **	**$F_{36/162}$= 2.12*** **	NA	NA	NA	NA
Cumulative						
EW_BM	NA	$F_{1/10}$ = 2.75	NA	NA	**$F_{1/8}$= 34.02*** **	NA
EW_T	NA	NA	NA	NA	$F_{2/8}$ = 1.51	NA

Reco weekly emissions followed quite similar dynamics to NEE and GPP, following maize development, however with a less steep decrease in absolute value during maize senescence (Fig. 4E). Reco weekly fluxes were not significantly affected by EW_BM or EW_T, but they significantly varied with Week (P-value < 0.001; Table 4). Cumulative Reco fluxes reached − 1464.63 (± 34.22), -1338.85 (± 65.01) and − 1452.10 (± 56.98) g CO_2 m^{-2} in the control, anecic and endogeic earthworm treatment levels, respectively, and showed no statistically significant effects of EW_BM or EW_T (Table 4; Fig. 4F).

The weekly N_2O emissions ranged globally for all earthworm treatment levels from 0.25 g m^{-2} day^{-1} at the beginning of the growing season to 0.35 g m^{-2} day^{-1} at the end, showing a high peak (0.7 g m^{-2} day^{-1}) in mid-June (Fig. 4G), and were only significantly influenced by Week (P-value < 0.001; Table 4). The cumulative N_2O fluxes reached 40.77 (± 12.26), 36.52 (± 3.61) and 34.09 (± 12.92) g m^{-2} in the control, anecic and endogeic earthworm treatment levels respectively and showed no EW_BM or EW_T statistically significant effect (Table 4; Fig. 4H).

Weekly ET fluxes followed the crop development (Fig. 4I) and were significantly affected by Week (P-value < 0.001), EW_T (P-value < 0.001) and EW_B (P-value < 0.001; Table 4). The EW_T effect indicated higher ET under both the anecic and endogeic treatment combination whereas the EW_BM indicated a decrease of ET with EW_BM. Cumulative ET fluxes reached 394.13 (± 6.23), 403.99 (± 2.40) and 405.94

(± 6.45) kg H_2O m^{-2} in the control, anecic and endogeic earthworm treatment levels. No earthworm treatment effect on ET was found (Fig. 4J), but overall ET decreased with EW_BM (P-value < 0.001; Table 4).

Weekly WUE rates followed the crop development (Fig. 4K) and were significantly affected by Week (P-value < 0.001; Table 4), EW_BM and EW_T. It increased with EW_BM (P-value < 0.001; Table 4) and was lower in the endogeic and anecic earthworms relative to control (P-value < 0.001; Table 4. The cumulative WUE reached 1076.82 (± 59.14), 991.68 (± 33.71) and 1023.53 (± 53.76) g CO_2 kg H_2O^{-1} m^{-2} in the control, anecic and endogeic earthworm treatment levels, respectively, and showed no statistically significant effect of the earthworm treatment (Table 4; Fig. 4L).

Refer to the Supplementary Information file for the weekly dynamics and cumulative fluxes obtained during the two intercropping periods (i.e., between wheat and mustard, and between mustard and maize).

Whole crop rotation

The cumulative values over the whole crop rotation including the three main cultures and the two intercrop periods (see Supplementary Information section on the results of the two intercrop periods) and approximately 24 months of experimentation are shown for each variable on Fig. 5. Of all six response variables, none showed any significant EW_T nor EW_BM effects.

Discussion

Earthworm effects on carbon fluxes (NEE, GPP, Reco)

In line with our hypothesis, our findings suggest that the earthworm treatment had only a minor and transient effect on carbon fluxes during certain periods of the experiment. However, when considering the entire two-year period, neither the earthworm treatment nor the earthworm biomass had a significant impact on carbon fluxes. Although Lubbers et al.[6] reported a 33% increase in soil CO_2 emissions in the presence of earthworms, it remains unclear whether this translates into ecosystem-level carbon losses. Our results suggest that this is not the case. It should be noted that our measurements were taken at the ecosystem-level and included both plant and soil fluxes, which differ from those reported by Lubbers et al.[6] that only considered soil CO_2 emissions. Nonetheless, we observed short-term stimulation of ecosystem respiration (Reco) during experimental periods with minimal plant contribution to CO_2 fluxes (e.g., before and immediately after sowing or during intercrop periods after aboveground biomass harvest). For instance, we observed such transient stimulations during the first few weeks of mustard growth and during intercrop_2 (between mustard and maize). However, these temporary increases in soil CO_2 emissions did not result in ecosystem-level carbon losses over the course of our experiment. Although there are numerous studies conducted in artificial and simplified setups that do not align with our findings, our results are consistent with several literature reports based on field experiments that demonstrate the short-lived impacts of earthworms on soil CO_2 emissions. For example, Borken et al.[37]

conducted an earthworm inoculation experiment in a beech forest and observed that the presence of earthworms (*L. terrestris*) led to a 16–28% increase in CO_2 emissions during the initial 3–4 weeks, but subsequently, the emissions were reduced after 11 weeks. Similarly, Schindler Wessels et al.[38] in a two-year field experiment (corn agroecosystem), found that CO_2 emissions were stimulated by earthworms, but only in the second year during the period going from June to August and not during the first year due to a severe drought. In a more recent field study (upland rice), John et al.[39] showed that earthworms did not affect CO_2 emissions over the whole rice growing season but that they did have an effect depending on the rice growth stage. Last, but not least, the results are in line with the findings of Ganault et al.[15], performed on the same soil and using a subset of the same earthworm species in a mesocosm experiment with plants, which showed no significant effect of earthworms on soil CO_2 emissions.

Other transient responses worth noting are a marginal decrease in GPP in the presence of anecic and endogeic earthworms for the wheat and mustard cultures, simultaneously with a stimulation of GPP with increasing earthworm biomass. These effects are not straightforward to interpret. The GPP is a measure of the amount of carbon fixed by plants and is controlled by several environmental factors, including the availability of light, CO_2, temperature, water availability, and nutrients. The last two factors can be directly affected by earthworms, although some hormone-like effects of earthworms on plant growth have also been proposed[40,41].

Earthworm effects on N_2O emissions

Consistent with our hypothesis, we observed a weak impact of earthworms on N_2O emissions when considering the cumulative emissions across the entire experiment. Moreover, the presence endogeic earthworms let to a statistically marginal reduction of N_2O emissions in the wheat (-48.6%) and intercrop_1 period (i.e., between wheat and mustard) and a similar trend was noted for the mustard and maize crops. These findings are in agreement with Ganault et al.[15] study, which reported a significant reduction in N_2O emissions in mesocosm experiments with the same soil type when the endogeic species *A. icterica* was present. Similar to Ganault et al.[15], we also identified transient N_2O stimulation during some periods of the experiment, but overall, these effects were not significant when integrating over the entire duration of the experiment. It's worth noting that the inhibitory impact of endogeic earthworms on N_2O emissions was more prominent (both statistically and in terms of effect size) following fertilizer application during the wheat cultivation and subsequent intercrop, but less apparent during other crop cycles without nitrogen fertilization. These results suggest that this phenomenon may be more pronounced under conditions conducive to N_2O emissions, such as higher soil N availability. While no data on soil porosity was included in this study, it is possible that a mechanism similar to the one proposed by Ganault et al.[15] could be at play, whereby endogeic earthworms promote soil aeration, leading to reduced denitrification and N_2O emissions. This effect has been previously suggested by a number of studies[16,18]. The aforementioned effect is expected to be comparatively weaker for anecic

earthworm species, since although they are known to create larger burrows, the total number of burrows and macropores generated per unit volume of soil is anticipated to be lower[42].

Earthworm effects on H_2O fluxes (ET, WUE)

Similar to the C fluxes, while punctual effects of our earthworm treatment on ET were observed in some periods, no significant impact on cumulated fluxes was found over the entire experimental period. However, it is worth noting that during maize, intercrop_1 and intercrop_2, ET significantly decreased with earthworm biomass. Since ET is the combination of soil evaporation and plant transpiration, the decrease in ET with EW_BM in intercrop periods with minimal vegetation suggests that the effect mainly resulted from reduced evaporation from the topsoil. This reduction is likely due to the faster infiltration of water into the deeper soil layers, consistent with previous studies[43]. The presence of anecic earthworms in the mustard treatment also resulted in a 12% decrease in WUE, while the presence of endogeic earthworms led to a 28% increase during intercrop_2. While a mechanistic explanation of these effects is not straightforward, it is possible to speculate that the reduction of WUE in the anecic treatment could be attributed to their feeding behavior. Anecic earthworms are known to be able to consume small seeds, such as mustard seeds, which could have impacted the density of the established plants[44], thus reducing the GPP. Regarding the stimulation of WUE in the presence of endogeic earthworms, this could be a weed biomass mediated effect as a trend of higher biomass of weeds was observed in the endogeic earthworms.

Caveats and limits of this experiment

While our study benefits from the advantages of a realistic long-term and large-scale ecosystem sample in controlled environment conditions, there are several caveats that must be acknowledged. One crucial limitation is that since only one soil type was used, we cannot be sure that our findings are transferable to other soil types. Another limitation is that, despite our efforts to achieve an earthworm-free control by removing all sampled earthworms during two extractions before each culture, some earthworms were still present in the control, with an estimated biomass in the controls at the end of the culture ranging from 9.56 g FW m^{-2} in maize to 21.16 g FW m^{-2} in wheat. However, to account for this effect, the sampled earthworm biomass at the end of each culture was used a covariable in all statistical analyses. Furthermore, although our targeted minimum earthworm biomass of 100 g FW m^{-2} was on the lower range of the field-sampled biomass at the reference site, it is possible that the low organic matter content in our soil and the lack of regular organic fertilizer applications may have limited the capacity of our soil to sustain a higher earthworm biomass[45]. Morevoer, the ecotron facilities have a limited number of experimental units, which prevented us from including a combined endogeic and anecic earthworm treatment combination, and we chose to investigate the individual effects of the two main earthworm ecotypes separately. Finally, the relatively low level of replication (n = 4) may limit the statistical power necessary to detect impacts with a lower effect size, although we argue that the frequent and continuous measurements available in the ecotron facility partially compensate for this limitation.

In this study, we address the gaps in knowledge that exist in the current literature on the impact of earthworms on greenhouse gases (CO_2 and N_2O). Most previous studies in this area have been conducted in simplified and artificial model systems or in the field, but without continuous high-frequency measurements of fluxes over a large surface area[6,46]. In contrast, we conducted a two-year experiment in an advanced controlled environment facility, an ecotron[36], that was specifically designed for continuous measurements of ecosystem fluxes over replicated large model ecosystems (5 m^2 area and 1.5 m soil depth) in a context mimicking agricultural management over a two-year three-crop rotation. Our findings indicate that earthworms do not stimulate the ecosystem-level emissions of greenhouse gases (CO_2 and N_2O), and that in certain conditions, endogeic earthworms may even reduce N_2O emissions, a result also reported by Ganault et al.[15]. However, our results are in line with those of Lubbers et al.[6] in that we found transient stimulations of soil CO_2 and N_2O emissions under certain conditions (in the first weeks after sowing and during the intercrop periods), although these effects were offset by periods of low emissions over the duration of the entire experiment.

In conclusion, our study highlights the importance of realistic experimental setups under controlled environment conditions that allow for continuous high-frequency measurements. Furthermore, the findings emphasize the importance of experimental designs that include plants and allow for the earthworm engineering effect on soil water status and aeration to take place in a realistic way. Drawing on our findings and an expanding body of research demonstrating that under realistic conditions, earthworms do not result in elevated greenhouse gas emissions[14,15,39], we recommend updating the meta-analysis performed by Lubbers et al.[6]. This includes incorporating more recent studies and assigning additional weight to studies that adhere to realistic experimental conditions.

Methods

Macrocosms and soil

The experiment was conducted in the European Ecotron of Montpellier (Montferrier-sur-Lez, France, www.ecotron.cnrs.fr), an advanced controlled-environment experimental infrastructure developed by the Centre National de la Recherche Scientifique (CNRS) to study the response of ecosystems to global environmental changes. The Macrocosms platform used in this experiment consists of twelve identical and independent experimental units, each being composed of an ~ 30 m^3 aboveground compartment enclosed by a highly transparent material to light and UV radiation (250 µm thick Teflon-FEP film, DuPont, USA) and a belowground compartment containing a 5 m^2 stainless steel lysimeter hosting 14 t of soil (volume of ~ 7.5 m^3); for additional information on the Macrocosms platform see Milcu et al.[47] and Roy et al.[36].

The soil was excavated from field margins adjacent to the SOERE-PROs EFELE agricultural experimental site (Brittany, North West of France, 8°05'35.9”N, 1°48'53.1”W). According to the analyses performed by the Soil Analysis Laboratory, INRAE Arras, the upper 30 cm layer of this loamy soil (luvisol-redoxisol) is

composed of 14.6% clay, 72.1% silt and 13.3% sand, with a pH of 6.14. It contains 1.5% total organic matter, 0.84% carbon, 0.1% nitrogen, with a C:N ratio of 8.4. The soil was excavated in three layers (0-0.3, 0.3–0.7, 0.7–1.5 m), transported to the ecotron where it was homogenised and reconstructed layer by layer in lysimeters outdoors. The aim of this process was to obtain the same soil density as in the original field, i.e., 1.35, 1.4 and 1.55 g cm^{-3} in the 0-0.3, 0.3–0.7 and 0.7–1.4 m soil layers respectively. The lysimeters were introduced in the Macrocosms platform in April 2017 and left as a fallow until October 2017 when the first culture was sown after weeding any spontaneous vegetation and a superficial manual tillage (upper 5 cm) of the soil.

Experimental setup - earthworm treatment and crop management

The experimental setup consisted in applying an earthworm treatment with three levels (i) anecic earthworms (A), (ii) endogeic earthworms (E), and (iii) a control (Ctr) with very low earthworm biomass. In the two levels with earthworms (A and E), three species for each ecological group were used to avoid an identity effect: *Aporrectodea nocturna* Evans, *Lumbricus terrestris* L. and *Scherotheca gigas* Dugès. for the anecic level and *Aporrectodea chlorotica* Savigny, *Aporrectodea caliginosa* Savigny and *Aporrectodea icterica* Savigny for the endogeic level. As the soil excavation, transport and recompacting to field density in lysimeters severely reduced the survival of living earthworms, at the onset of the experiment (April 2017), a total of 100 g FW m^{-2} earthworm biomass (with roughly equal biomass per species) originating from the EFELE site were inoculated/added in the lysimeters containing earthworms, and this biomass was kept as the minimal target earthworm biomass for the whole experiment. The target biomass is within the range of the earthworm biomass values at the EFELE site sampled in 2016, which ranged from 98 to 135 g FW m^{-2} (unpublished data).

To stimulate earthworm development and to prevent the excessive drying of the bare ground topsoil during the summer after the lysimeter filling, a total of 3.3 kg of dry plant residues (a mix of 0.25 kg of maize leaves, 1.36 kg of wheat straws and 1.72 kg of hay) was applied homogeneously at the surface of the soil at the end of June 2017. Earthworms were first sampled in October 2017 and thereafter at the end of each culture, and if the sampled earthworm biomass was lower than the target (100 g FW m^{-2}), at each sampled point additional earthworms were added to reach the target biomass. Any earthworms sampled in the Ctr were removed, while the earthworms sampled in the A and E treatment levels were added back to their corresponding lysimeter following biomass evaluation. To ensure a maximal removal of earthworms, the Ctr lysimeters were subjected to dual sampling at each designated point, spaced several days apart. Earthworm sampling was conducted utilizing the non-invasive octet electric method[48], which enabled sampling of a 1 m^2 surface area for a duration of 50 minutes using a customized version of the octet device manufactured by Electrotechnik Schuller (Darmstadt, Germany). To achieve coverage of the entire 5 m^2 surface area, five devices were simultaneously deployed (see Fig. 1 for the experimental timeline showing a picture of the device and Fig. S1 showing the earthworm FW biomass sampled at each extraction).

The experiment simulated a three plant species crop rotation that is used at the reference EFELE site from where the soil originated, and which is composed of a succession of *Triticum aestivum* - *Sinapsis alba* - *Zea mays* (i.e., winter wheat - winter white mustard - maize). The periods between the wheat and mustard as well as between mustard and maize are henceforth called intercrop_1 and intercrop_2, respectively (see Table 1 for the crop dates, sowing and harvesting information). During these periods, after the aboveground biomass was harvested, any unwanted plants/weeds were removed, and the soil surface was maintained as much as possible as bare ground, however some weed growth still occurred despite weeding (see Table S1 showing the weed biomass). Before sowing each crop, a manual and simplified superficial soil tillage (upper 5 cm only) was conducted to prepare a suitable seedbed for the next crop. All crops were manually sown, in rows for the wheat and maize crop, and broadcasted for the mustard crop; for the latter, the soil was pressed down with a rattle after sowing and 1.5 kg of wheat straw was added as top soil mulching. For the maize crop, one dose of téfluthrin (10 kg ha^{-1} active substance, Force 1.5, Syngenta AG, Switzerland) was applied in the soil around the seeds at the time of sowing as a protection against wireworms. Fertilization was done only once during the wheat growing season through addition of methane digestate slurry supplied by Schiesslhof GbR farm (Neunburg, Bavaria). The digestate was applied using a watering can at the beginning of April (4.5 kg) and in mid May 2018 (5 kg), dates corresponding to tillering and flowering/anthesis phenological stages, respectively, and amounting to an equivalent of 87 kg N ha^{-1} (Table S2 for the physico-chemical properties of the digestate).

Simulated climatic conditions

The experiment simulated the climatic conditions (air temperature, air humidity, and precipitation; see Figs. S4-S8) recorded in year 2017 at the EFELE experimental site and the conditions was recreated in the experimental years 2018–2020, with setpoints at 1 h intervals. To account for the differences in solar radiation between Montpellier and the reference site, a shading black mesh was mounted on top of the transparent domes form October to March to bring the integrated solar radiation to similar levels; no difference was observed in summer when the 15% attenuation due to the Teflon-FEP film was sufficient. Global radiation (Rg) was measured every 20 s with a BF5 Sunshine Sensor (Delta-D device) located in a weather station close to the Macrocosms platform. Air temperature and relative humidity were measured every 20 s by a DT269 Duct Mount Digital Relative Humidity and Temperature Transmitter (MICHELL Instruments) located in the middle of each macrocosm in a solar shelter at 1.5 m aboveground. Volumetric soil moisture and soil temperature were measured every 5 min. with TDR sensors (Trime PICO 32, IMKO manufacturer) located at 0.13, 0.25 and 0.8 m depth and at three distances from the western edge of the lysimeter (0.65, 1.25 (lysimeter center) and 1.85m). Irrigation was performed at night and the amount of water applied with sprinkler systems was defined so as to mimic the amount of rainfall of the EFELE experimental site from which the soil was extracted. In addition, TDR soil moisture values at 0.13 and 0.25 m depth were used in comparison with counterpart measurements at the reference site to inform whether compensation irrigation would occasionally be needed in order to compensate for the increased evapotranspiration that can occur in confined conditions due to continuous air flow in the dome.

Ecosystem CO_2, N_2O and water fluxes

The CNRS Ecotron was designed to continuously measure CO_2 net ecosystem exchange (NEE) by sequentially measuring the CO_2 concentration at the inlet and outlet of each dome (every 12 min) using a multiplexer system coupled with two LI-7000 CO_2/H_2O analysers (LI-COR Biosciences, Lincoln, NE, USA). We used the Reichstein et al.[49] C flux partitioning algorithm to estimate the daytime ecosystem respiration based on an exponential regression model[50]. This allowed for the estimation of ecosystem respiration over 24 h (Reco = Reco_night + Reco_day) and gross primary production (GPP = NEE_day – Reco_day).

Ecosystem-level N_2O fluxes were measured continuously as an open system using a TILDAS Compact Single analyser (N_2O Aerodyne Research, Inc., USA). The analyzer was coupled to a multiplexer system allowing N_2O fluxes measurement every 72 min for each Macrocosm. Evapotranspiration (ET) was computed as the lysimeter weight difference between two consecutive days. Four shear beam load cells per lysimeter (CMI-C3, Precia-Molen, Privas CEDEx France), with an accuracy of ± 200 g, were used to measure the changes in mass. Ecosystem WUE was estimated as the ratio of GPP to ET derived from measurements by lysimeter weight changes over 24h.

Data treatment and statistical analyses

Data processing and statistical analyses were performed using R version 4.2.1 (R Development Core Team, 2015) in Rstudio version 2023.03.0 Build 386 (RStudio Team, 2015). Data was screened for outliers before statistical analyses and values that were lower or higher than 2 × IQR for each replicate were considered to be outliers due to measurement errors or perturbations (e.g. when entering the domes, etc.). The C flux partitioning and gap-filling was performed within the “REddyProc (v. 1.3.2)” package[51]. Missing values from the N_2O time series were replaced with the predicted values from a loess regression, with a 0.05 span.

We conducted three distinct but complementary statistical analyses, each aimed at discerning specific aspects of the ecosystem fluxes: (i) an analysis of the treatment effect on weekly averaged fluxes to capture potential differences in temporal dynamics, (ii) a per crop analysis of cumulated ecosystem fluxes and (iii) a whole crop sequence cumulated analysis including the data from the whole experiment.

For the weekly mean analysis, a mixed linear model of the form “response_variable ~ EW_BM + EW_T*Week” with the macrocosm ID as a random effect (random = ~ 1|mac) was used for each of the six response variables (NEE, GPP, Reco, N_2O, ET and WUE) using the lme() function from the nlme package[52] (version 3.1–160). Following the guidelines suggested by Zuur et al.[53], in a first step we identified the most appropriate random structure using a restricted maximum likelihood approach by testing five different models for each response variable and then selecting the random structure with the lowest Akaike information criterion (AIC): Model 0 (reference), Model 1 (reference with autocorrelation structure “correlation = corAR1(form = ~ 1|mac)”), Model 2 (Model 1 with weighting structure for variances between

weeks, “weights = varIdent(form = ~ 1|Week)”), Model 3 (Model 1 with weighting structure for variances between EW_T levels, “weights = varIdent(form = ~ 1|EW_T)”), and Model 4 (Model 1 with weighting structure for variances between the interaction of EW_T*Week, “weights = varIdent(form = ~ 1|EW_T*Week)”). In a second step, model simplification to reach the minimal adequate model was performed on the model with the lowest AIC selected during the first step. This was done using the stepAIC() function from the MASS package[54,55] (version 7.3–58.1). For the per crop cumulated analysis, the statistical procedure resembled the one followed for the weekly means, with the notable differences that the Week predictor is no-longer pertinent and was removed, all variables were log transformed to reduce the sensitivity of the models to data scarcity (less data points since values were cumulated), and that a generalised least squares model (gls) of the form gls(log(response_variable) ~ EW_T + EW_BM) was used for each of the six response variables. Only two models were compared using the same comparison procedure: (i) Model 1, gls(log(Response_variable) ~ EW_T + EW_BM) and (ii) model 2, model 1 with the addition of a weighting structure taking into account the possible differences in variances between EW_T levels (weights = varIdent(form = ~ 1|EW_T)). For the whole crop sequence analysis, the same models and procedure as for the per crop cumulated analysis were used, this time on the whole crop sequence thus including the cumulated values from wheat, intercrop_1, mustard, intercrop_2 and maize crops. To denote differences between the means of cumulative values, Tukey’s post-hoc honest significant difference (HSD) test was used[56]. This test is commonly used in situations where multiple pairwise comparisons are made, and helps to control the overall false positive rate. However, as this test is not capturing all of the relevant sources of variation in the data, occasional discrepancies between the mixed-effects fitted coefficients and the Tukey test can occur.

Declarations

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Author contributions

AM designed the experiment with input from JR and JN. All authors provided methods and carried out specific measurements. OF analyzed the data with input from AM. OF and AM wrote the paper with input

from all co-authors.

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Figures

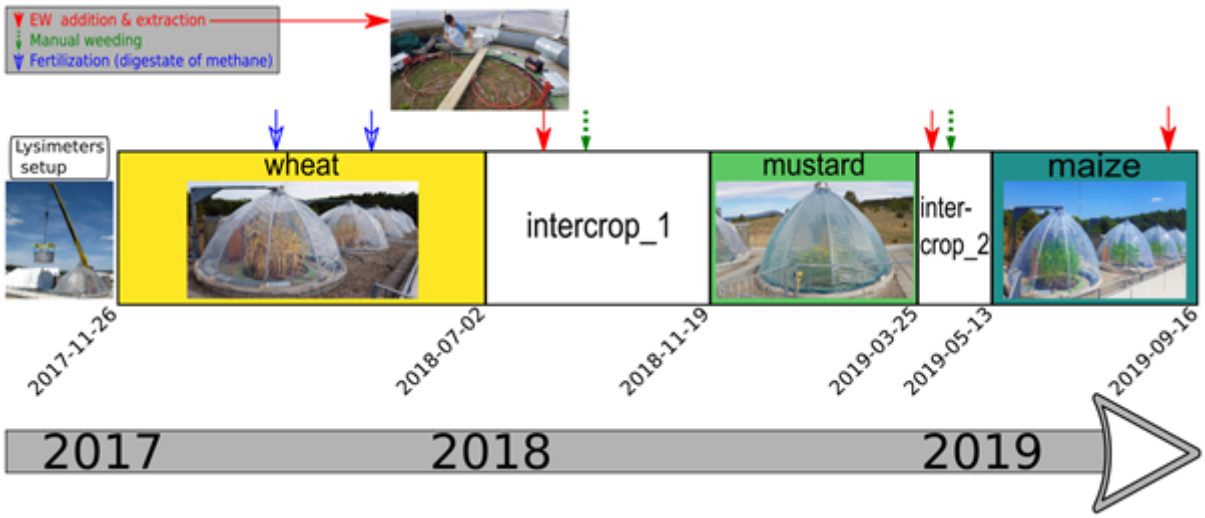

Figure 1

Experimental timeline showing sowing and harvest/destruction dates along with earthworm (EW) extraction, manual weeding and fertilization events. The crop intervals are scaled to the number of days of each crop.

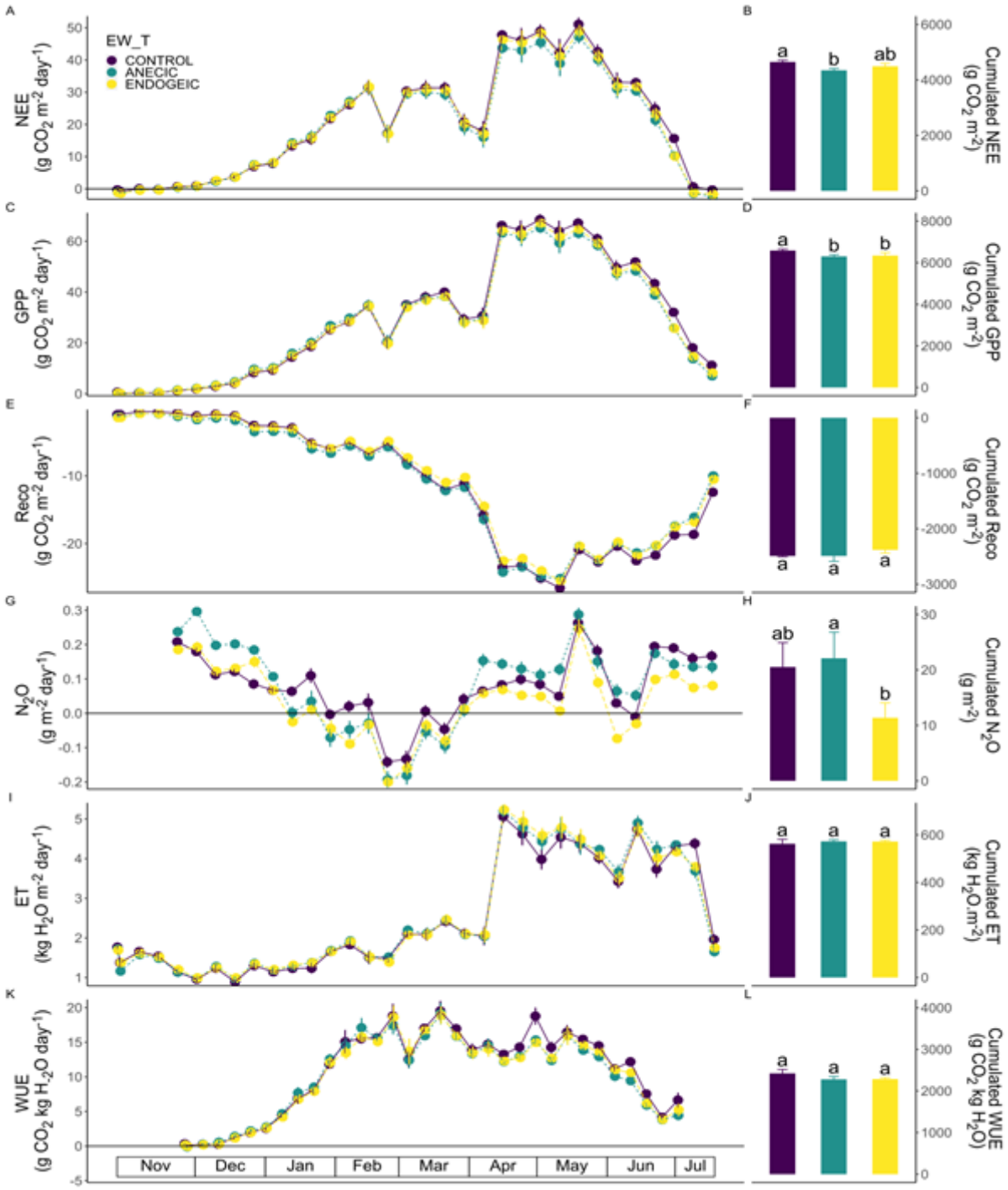

Figure 2

Weekly dynamics (left) and cumulative fluxes (right) of carbon, N_2O and water fluxes as affected by the earthworm treatment (EW_T) **in the wheat crop**. (A,B) NEE. (C,D) GPP. (E,F) Reco. (G,H) N_2O. (I,J) ET. (K,L) WUE. Data represent means ± SEM of four replicates. Different letters above bars denote significant differences between means according to Tukey's HSD post-hoc test.

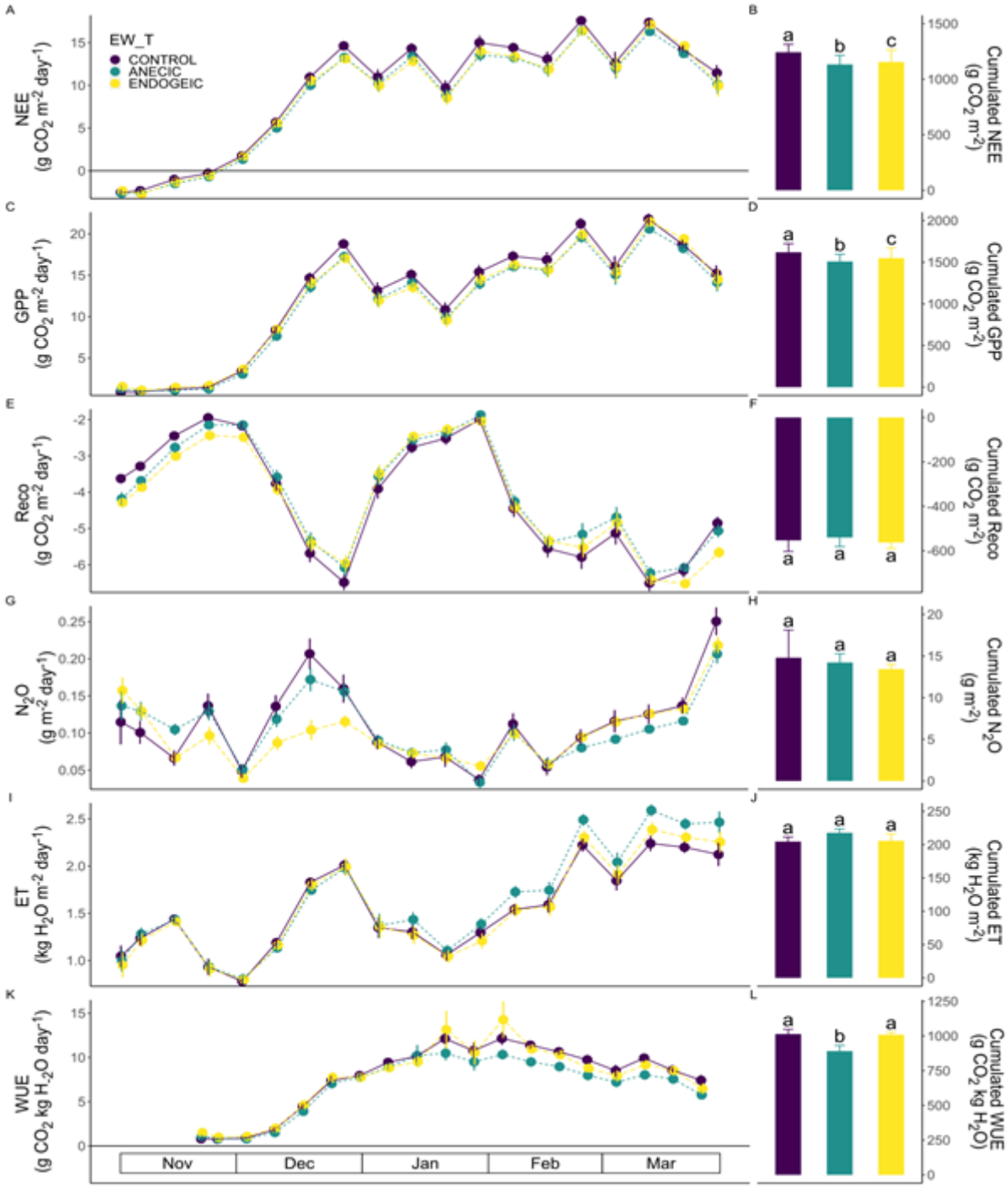

Figure 3

Weekly dynamics (Left, lines) and cumulative fluxes (Right, bars) of carbon, N_2O and water fluxes as affected by the earthworm treatment (EW_T) **in the mustard crop**. (A,B) NEE. (C,D) GPP. (E,F) Reco. (G,H) N_2O. (I,J) ET. (K,L) WUE. Data represent means ± SEM of four replicates. Different letters above bars denote significant differences between means according to Tukey's HSD post-hoc test.

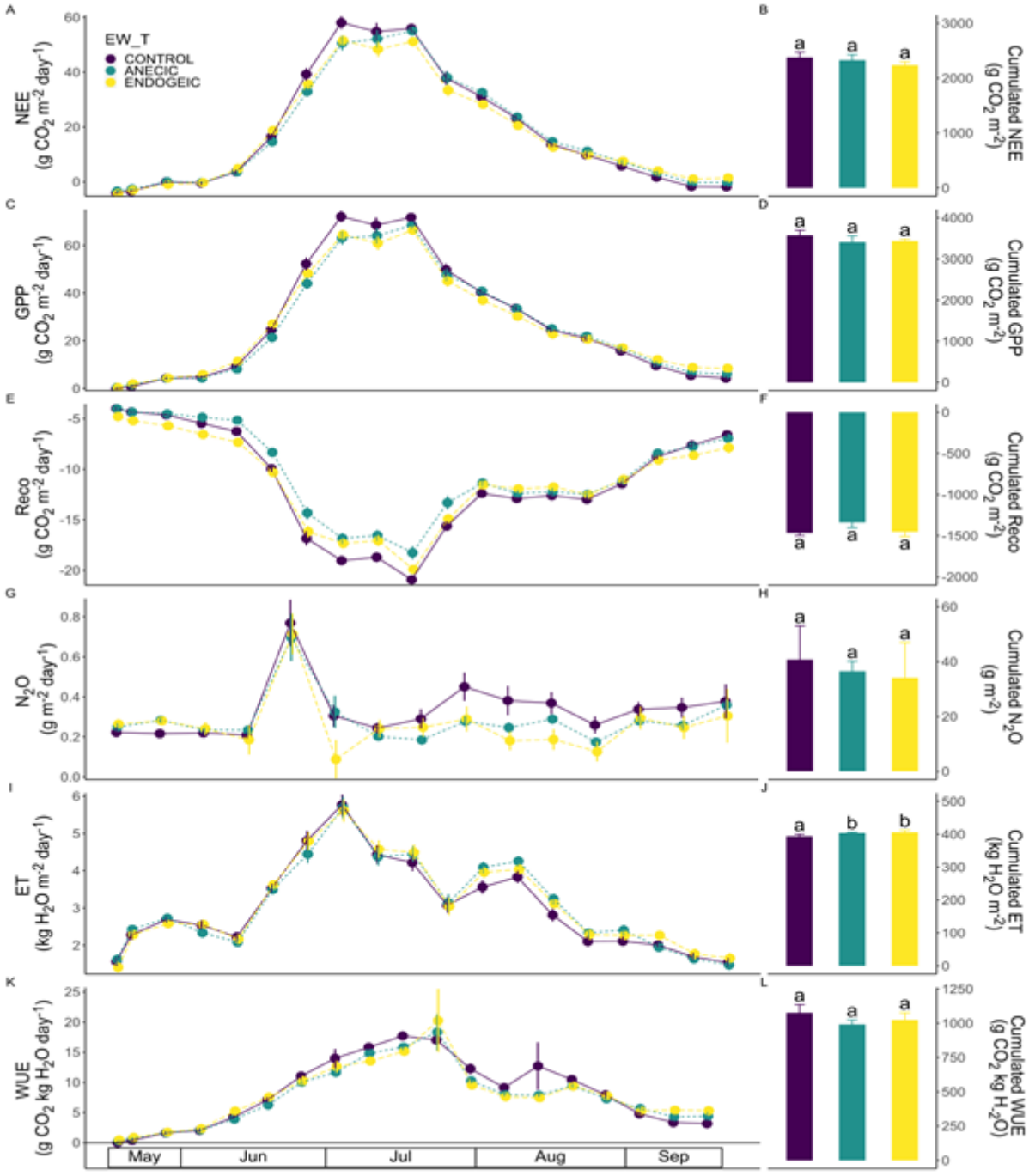

Figure 4

Weekly dynamics (Left, lines) and cumulative fluxes (Right, bars) of carbon, N_2O and water fluxes as affected by the earthworm treatment (EW_T) **in the maize crop**. (A,B) NEE. (C,D) GPP. (E,F) Reco. (G,H) N_2O. (I,J) ET. (K,L) WUE. Data represent means ± SEM of four replicates. Different letters above bars denote significant differences between means according to Tukey's HSD post-hoc test.

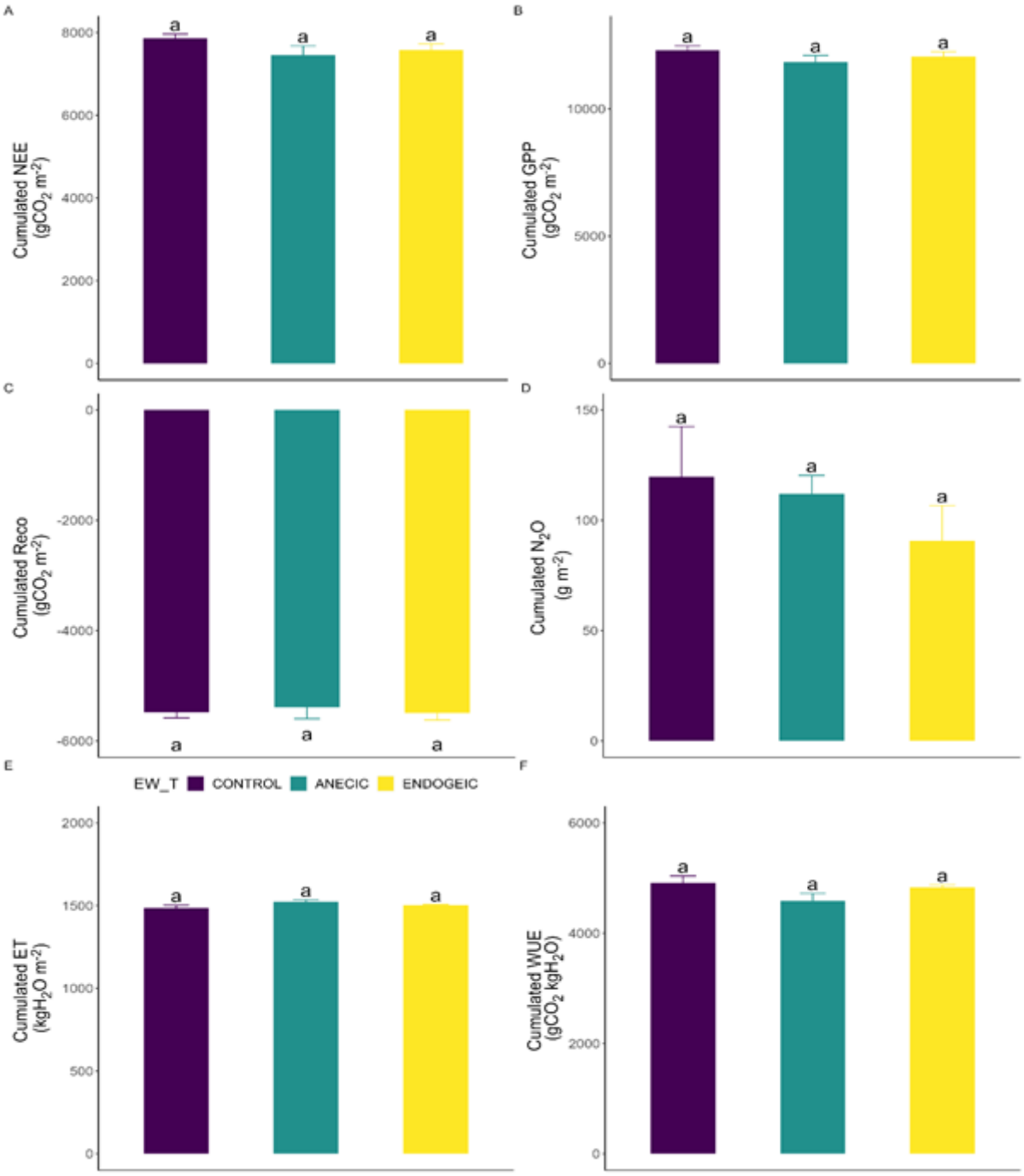

Figure 5

Cumulative fluxes of carbon, N_2O and water fluxes as affected by the earthworm treatment (EW_T) **over the whole crop rotation**. (A) NEE. (B) GPP. (C) Reco. (D) N_2O. (E) ET. (F) WUE. Data represent fluxes cumulated over 640 days ± SEM of four replicates. Different letters above bars denote significant differences between means according to Tukey's HSD post-hoc test.

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