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Earthworms regulate ability of biochar to mitigate CO_2 and N_2O emissions from a tropical soil

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ABSTRACT

Soils account for > 80% and 20% of the total agricultural N_2O and CO_2 emissions respectively. Soil management activities that target improved soil health, such as enhancing earthworm activity, may also stimulate further emissions of CO_2 and N_2O. One recommended strategy for mitigating these soil emissions is biochar amendment. However greater clarity on the interaction between earthworm activity and biochar, and subsequent impact on CO_2 and N_2O are needed to evaluate the environmental impacts of management practice. We measured N_2O and CO_2 emissions from a kaolinitic Acrisol in the presence or absence of earthworms, with and without application of two different biochars in a microcosm study. The two biochars were derived from indigenous trees; *Zanthoxylum gilletii* and *Croton megalocarpus*, and were tested at three application rates of 5 Mg ha^{-1}, 10 Mg ha^{-1} and 25 Mg ha^{-1}. Emissions of CO_2 and N_2O increased by 26% and 72% respectively in the presence of earthworms. In microcosms with biochar and earthworms however, emissions depended on type of biochar and rate of application. With *C. megalocarpus*, CO_2 emission increased with increasing rates of biochar application with 25 Mg ha^{-1} resulting in higher CO_2 fluxes compared to no-biochar control ($p = 0.002$), while no change was observed with *Z. gilletii* at the same rate. Nitrous oxide emissions were suppressed at 25 Mg ha^{-1} for both *C. megalocarpus* ($p = 0.009$) and *Z. gilletii* ($p = 0.011$). Reduction in N_2O flux was however not consistent across biochar types. No change in N_2O was observed with 5 Mg ha^{-1} and 10 Mg ha^{-1}of *C. megalocarpus*. Biochar from *Z. gilletii* at 5 Mg ha^{-1} however led to increase in N_2O emissions ($p < 0.001$). Our findings suggest that earthworms may moderate the effect of biochar, with suppression of N_2O emissions occurring at only high biochar application rates, which may occur at the cost of increasing CO_2 emissions. These findings contrast with biochar suppressing effect on N_2O emissions even at moderate biochar rates of (10 Mg ha^{-1}) when in absence of earthworms, an outcome typical of many laboratory experiments. These findings highlight new interactions among application rate, source of biochar (and hence properties) and earthworms.

1. Introduction

Agriculture directly contributes approximately 12% of the annual anthropogenic greenhouse gas (GHGs) emissions (IPCC, 2014); 39% of which is directly from soils (FAOSTAT, 2014). Soils account for 20% and 87% of the total global anthropogenic emissions of CO_2 and N_2O respectively (FAOSTAT, 2014; Rastogi et al., 2002). At regional level, agriculture account for 42% of the total anthropogenic emissions of N_2O in Africa (excluding savannah and grassland burning), 15% of which is directly contributed by soils, and this is expected to double by 2050 (Hickman et al., 2011).

GHG emissions are mediated by microbial processes in soil. Generation of CO_2 within soils occurs mainly through biological processes namely: microbial, root or faunal respiration. These processes usually occur at or near the soil surface where most of organic materials are found (Hanson et al., 2000). The main factors affecting emission of

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CO_2 include available C, moisture, pH, temperature, soil texture and N content (Rastogi et al., 2002). Soil nitrous oxide (N_2O) is generated through both nitrification and denitrification (Baggs, 2011; Wrage et al., 2001), with nitrification an aerobic process and denitrification an anaerobic process. Most soil N_2O losses result from denitrification and therefore tend to be related to soil water content and gas diffusivity (Balaine et al., 2013), labile C concentrations (Pelster et al., 2012) and C:N ratios (Chantigny et al., 2013), which can deplete soil oxygen (Davidson et al., 2000) and decrease the N_2:N_2O ratio.

Field management options that increase soil macrofauna, including earthworms, are some of the most promising strategies for improving soil productivity (Kamau et al., 2017a; Pauli et al., 2011; Van Groenigen et al., 2014). Earthworms enhance soil structure and biological productivity (e.g. aggregate stability, aeration, nutrient availability and mineralization rates) through feeding, burrowing and casting activities (Barrios, 2007; Jouquet et al., 2008; Six et al., 2004). Unfortunately, the ability of earthworms to enhance soil functions lies primarily in the very same abilities that can increase GHG emissions, a paradox referred to as the "earthworm dilemma" (Lubbers et al., 2013). Earthworms accelerate decomposition of organic material, enhance mineralization and produce casts characterized by higher N, C, pH, moisture and density and decreased porosity, all of which have been shown to favor CO_2 and N_2O production (Jouquet et al., 2008; Lubbers et al., 2011; Speratti and Whalen, 2008). However, not all earthworm effects are indirect. Some studies have suggested that earthworms might have a direct role in emission of N_2O through the involvement of gut-associated denitrifying bacteria (Horn et al., 2006; Matthies et al., 1999). In a meta-analysis involving 36 CO_2 and 13 N_2O studies, Lubbers et al. (2013) estimated that earthworms can increase N_2O and CO_2 emissions from soils by as much as 42% and 33%, respectively. Furthermore, the majority of soil management practices targeting enhanced earthworm populations (e.g. agroforestry, organic residue and compost management, no-tillage management) accomplish this by increasing soil organic matter (SOM) concentrations (Fonte et al., 2010a; Mbau et al., 2015; Six et al., 2004), which as previously highlighted, can stimulate CO_2 and N_2O emissions. Management options that can influence dynamics of labile C and N are therefore needed to mitigate emissions of CO_2 and N_2O from soils.

Biochar, a product of the pyrolysis of biomass residues, has been suggested as an amendment for mitigating soil GHG emissions, especially N_2O (Cayuela et al., 2014; Lehmann et al., 2006). A recent meta-analysis found that biochar suppressed N_2O emissions by 28% under field conditions and 54% under laboratory conditions, (Cayuela et al., 2015). Further, reduction in N_2O production due to biochar was directly related with application rates. Average laboratory application rates almost double field rates by weight basis and have been shown to lead to more N_2O suppression.

There are several mechanisms through which biochar may influence N_2O emissions from soils. Biochar may increase the soil pH which enhances the induction of enzymes responsible for complete denitrification and hence preventing N_2O production (Obia et al., 2015). Furthermore, biochar increases porosity and aeration of soil, which may inhibit denitrification, although this impact may be minimal and offset as biochar can also increase soil moisture, which enhances N_2O emissions (Case et al., 2012; Saarnio et al., 2013; Yanai et al., 2007). Biochar can also reduce N_2O emissions by adsorbing mineral N onto its surface or because it's high C/N ratio ($>$ 60) causes immobilization of soil N (Feng and Zhu, 2017). Finally, some studies have suggested that biochar may reduce N_2O emission through substances retained during production that inhibit soil nitrifying/denitrifying organisms: e.g. phenolic compounds and polycyclic aromatic hydrocarbons (PAHs) (Z. Wang et al., 2013).

However, with respect to CO_2 fluxes, results of biochar application are mixed. While some studies report increased CO_2 emissions due to biochar application (e.g. Zimmerman et al., 2011), others report a reduction but only with high rates of application (e.g. Shen et al., 2017). Increased CO_2 emissions are usually attributed to microbial decomposition and mineralization of additional labile C, priming effect of biochar on soil and abiotic release of inorganic C (Luo et al., 2011). Reductions are thought to be due to adsorption of CO_2 to the biochar surface as carbonates, inhibition of C-mineralizing enzymes; shifting of microbial community composition such as fungal:bacterial ratio, and negative priming (Augustenborg et al., 2012; Cross and Sohi, 2011; Lehmann and Joseph, 2015; Rittl et al., 2015).

The effects of biochar application on earthworm behavior have equally varied outcomes. Some studies have found earthworms avoid biochar while others suggest an earthworm preference for soil-biochar mixtures. (Topoliantz and Ponge, 2003; Elmer et al., 2015). The varied responses are mostly attributed to the chemical composition of biochar, which tends to be controlled by the source material and production conditions. Studies where earthworms avoided the biochar attributed this behavior to toxic elements in biochar, dryness of biochar leading to physical injury, or alteration of soil pH (Gomez-Eyles et al., 2011; Li et al., 2011; Liesch et al., 2010; Schmidt et al., 1999). On the other hand, studies that showed earthworm attraction to biochar attributed this to an increased presence of ash or increased minerals such as Ca, Mn, and Si, or an increase in pH to a preferred range (Beesley and Dickinson, 2011; Elmer et al., 2015; Van Zwieten et al., 2010).

A better understanding of the interaction between earthworms and biochar is needed given their role in soil fertility and their impact on CO_2 and N_2O emissions. So far, only Augustenborg et al. (2012) have investigated the relationship between earthworms and biochar on GHG emissions. They concluded that biochar reduced CO_2 emission only in the absence of earthworms, and that biochar moderated earthworm-induced increases in N_2O emissions. Our study includes a different type of endogeic earthworm, *Pontoscolex corethrurus*, and three application rates of biochar derived from two native woody species. Unlike Augustenborg et al. (2012), our study focuses on a highly weathered, low C content soil typical of Sub-Saharan Africa. Furthermore, we use biochar pyrolyzed under local conditions hence mimicking conditions under which biochar would typically be used in the African context. We hypothesized that: (1) biochar applications would reduce N_2O and increase CO_2 emissions regardless of source material and these changes will be linearly related to application rate, (2) presence of earthworms would cause an increase in both CO_2 and N_2O emissions, and (3) earthworms plus biochar would have lower emissions of both gases compared to earthworms alone.

2. Material and methods

2.1. Study site

The experiment was conducted at the World Agroforestry Center (ICRAF) Soil Ecology Facility greenhouse in Nairobi, Kenya (1^0 14′ 08″ S, 36^0 49′ 11″ E; altitude 1703 m). Topsoil (0 to 0.10 m depth) for the experiment was obtained from several randomly selected farms in Kapchorwa, Nandi county, Kenya (0^0 10′ 00″ N, 35^0 00′ 00″ E; altitude 1600–1900 m) and composited. The soils are classified as clayey kaolinitic Acrisols (FAO/UNESCO classification; Kimetu et al., 2008). Further details about the site are described by Kamau et al. (2017a). The soils were thoroughly mixed, dried and manually ground using a wooden rolling pin before being passed through a 2 mm sieve. To obtain finely ground homogenous soil mixture for chemical analysis, the soil was then passed through a ball-mill grinder (The Mortar Grinder RM 200).

Soil chemical properties were analyzed by standard procedures. Total C and N were determined by FLASH 2000 NC Analyser (ThermoFisher Scientific, Cambridge, UK), while soil pH was determined using a pH meter using a soil-water ratio of 1:2.5 (Anderson and Ingram, 1993). Soil texture was determined using Bouyoucos hydrometer after pre-treatment with hydrogen peroxide to remove organic matter (Okalebo et al., 2002). The soil chemical properties were:

pH of 5.6, Total C 36.2 g kg^{-1}, Total N 3.19 g kg^{-1} and C/N ratio 11.34. Texture analysis showed 69% clay, 11% sand and 20% silt content.

2.2. Biochar

The biochar was produced by pyrolysis of biomass from two tree species: *Zanthoxylum gilletii* (De Wild.) P.G. Waterman and *Croton megalocarpus* Hutch. These tree species were selected because of their high relative abundance in the Kapchorwa landscape (Kamau et al., 2017a). The trees biomass was pyrolyzed using traditional earth-mound kilns commonly used locally. Wood from each tree were pyrolyzed separately at temperatures of about 500 °C for four days. We used traditional earth-mound kilns because (i) they reflect conditions under which biochar could be produced locally, and (ii) these production temperatures are not out of range of biochar examined by Cayuela et al. (2015) in their meta-analysis. Biochar produced at low temperatures typically has higher cation exchange capacity (CEC), total C and N content but decreased C/N ratio, ash, pH, total P, porosity and surface area (Chan et al., 2008; Enders et al., 2012; Paz-Ferreiro et al., 2015; Y. Wang et al., 2013).

The biochars were then crushed and passed through a 2 mm sieve. Subsequent analysis followed standard procedures and methods outlined by Rayment and Higginson (1992). Briefly, C and N concentrations were measured by Dumas combustion; pH was determined in a 1:5 biochar:water slurry; available P was measured using Bray's method by flow injection analysis (FIA); effective CEC using the compulsive exchange method; and NH_4^+ and nitrate by extracting 20 g subsamples soils with 100 mL of 2 M KCl solution and automated procedures using continuous flow injection colorimeter (QuickChem 8000 FIA+; Lachat Instruments, Loveland, CO). Table 1 shows the main properties of the biochar used in this experiment.

2.3. Experimental design

A total of 40 open-top microcosms (11-cm-diameter by 30-cm-height) were arranged in a randomized complete block design. In total, there were 10 treatments replicated four times. Treatments without earthworms included: 1) soil only (S); 2) soil +10 g kg^{-1} of *Z. gilletti* biochar (S-Z^{10}); 3) soil +10 g kg^{-1} of *C. megalocarpus* biochar (S-C^{10}). Treatments with earthworms included: 4) soil + earthworm only (S-E); and 5–10) soil + earthworm + each biochar at 3 application rates of 5 g kg^{-1} 10 g kg^{-1} and 25 g kg^{-1} (S-E-Z^5, S-E-C^5, S-E-Z^{10}, S-E-C^{10}, S-E-Z^{25}, S-E-C^{25}) (Table 2).

The S, S-C^{10}, S-Z^{10} and S-E treatments resulted in an unbalanced design. The treatments with biochar applied at 5, 10, and 25 g kg^{-1} (S-E-Z^5, S- E-C^5, S-Z^{10}, S-C^{10}, S-E-Z^{10}, S-E-C^{10}, S-E-Z^{25}, S-E-C^{25}) correspond to rates of 5, 10 and 25 Mg ha^{-1} respectively assuming a bulk density of 1 Mg m^{-3} and a depth of 0.1 m. The 5 Mg ha^{-1} rate is within the lower range at which biochar can reduce CO_2 emissions. 10 Mg ha^{-1} represents the point at which CO_2 emissions are expected to increase due to biochar application while 25 Mg ha^{-1} is a reasonable application rate at which both CO_2 and N_2O should be suppressed (Song et al., 2016). Biochar and soil (≈1.5 kg) were both transferred into plastic buckets and mixed thoroughly using a trowel, and subsequently packed into the microcosms. Inert sand was used at the base of the microcosm to allow for capillary wetting. The base was fitted with a muslin cloth to prevent loss of soil and/or earthworms. The mixture was moistened to 65% field capacity through capillary wetting (Fig. 1).

Table 1
Properties of biochar from *C. megalocarpus* and *Z. gilletii* trees species.

	C. megalocarpus	*Z. gilletii*
pH (H_2O)	9.61	8.83
Total C (%)	84	78
Total N (%)	0.68	0.67
KCl extractable NH_4-N (mg kg^{-1})	0.46	23
KCl extractable NO_3-N (mg kg^{-1})	0.88	0.85
Effective CEC (cmol(+) kg^{-1})	13	97
Bray P (mg kg^{-1})	5.1	0.99

Two mature pre-weighed endogeic earthworms (*Pontoscolex corethrurus*), collected from the Kenya Agricultural and Livestock Research Organization (KALRO) fields in Embu, Kenya (00 30′10″ S, 370 27′30″ E; altitude 1500 m) were introduced from the top to the appropriate microcosms immediately following re-wetting of the soils. This species was used because it is commonly found in tropical soils and it actively feeds on soil (Fonte et al., 2010b; Pauli et al., 2010). The earthworm density used in this study is described as low density according to Lubbers et al. (2013). However, earlier study across different land management practices in the Kapchorwa area found an average of earthworm density of 103 individuals m^{-2}. Thus, the density used in the current experiment is higher than the observed levels in Kapchorwa.

2.4. Gas sampling and analysis

The experiment ran for 27 days after earthworm addition. Sampling was conducted every day for the first 4 days and every other day thereafter, for a total of 14 sampling days. During sampling, microcosms were closed for 15 min and four gas samples (15 mL each) were taken from the head space at five-minute intervals using 60 mL propylene syringes with Luerlocks (i.e. immediately after the closure and after 5, 10, and 15 min). Gas samples were immediately transferred into 10 mL pre-evacuated glass vials fitted with crimp seals. The vials were over-pressurized in order to reduce the likelihood of contamination with ambient air. Analysis of gas samples was conducted using an SRI gas chromatograph (model 8610C; SRI) with a methanizer in conjunction with a flame ionization detector (FID) (CO_2) and a ^{63}Ni electron capture detector (ECD) (N_2O). The gas chromatograph was operated with Hayesep D packed columns (3 m, 1/8″) with oven temperature of 65 °C, ECD detector and methanizer temperature of 350 °C, and flow rates of 25 mL min^{-1} with N_2 as the carrier gas on both FID and ECD lines. Soil N_2O-N and CO_2-C fluxes (herein referred to as N_2O and CO_2) were calculated using equations proposed by Rochette and Bertrand (2008).

2.5. Data analysis

When sampling was done every other day, we calculated daily N_2O and CO_2 flux rates for dates between samplings using linear interpolation. We calculated the cumulative N_2O and CO_2 fluxes as the sum of all the daily fluxes for the entire experimental period (27 days). One of the 600 N_2O flux measurement from the third replicate for Soil only (control) treatment was identified as an outlier using Grubb's test (Grubbs, 1969), and subsequently excluded from analysis. We filled this excluded value using linear interpolation between sampling events.

Due to the unbalanced nature of the experiment we divided analysis into two parts to test treatment effects on cumulative N_2O and CO_2 fluxes, and soil parameters (C, N and pH). (i) First, we sought to test the impact of earthworms and biochar application on cumulative emissions and soil characteristics. Here, we conducted analysis of variance (ANOVA) with earthworms (with and without) and biochar type (none, *Z. gilletii* at 10 Mg ha^{-1}, and *C. megalocarpus* at 10 Mg ha^{-1}) as fixed factors and block as a random factor involving soil only, soil + earthworm, soil + earthworm +10 Mg ha^{-1} of *Z. gilletii* and soil + earthworm +10 Mg ha^{-1} of *C. megalocarpus* (S, S-E, S-Z^{10}, S-C^{10}, S-E-Z^{10} and S-E-C^{10}). (ii) In the second part of analysis, we evaluated the effects of biochar application rates when earthworms are present. This involved comparing the effects of the three biochar application rates in the presence of earthworms. Therefore, we included only the treatments containing earthworms i.e. S-E, S-E-Z^5, S-E-C^5, S-E-Z^{10}, S-E-C^{10}, S-E-Z^{25} and S-E-C^{25}. For this part we conducted ANOVA involving S-E-Z^5, S-E-C^5, S-E-Z^{10}, S-E-C^{10}, S-E-Z^{25} and S-E-C^{25} treatments. Biochar

Table 2
Treatment descriptions. Treatment number 1, 2, 3, 4, 7 and 8 were involved in the first part of data analysis testing the impact of earthworms and biochar application on cumulative emissions and soil characteristics. The second part of analysis involved treatment numbers 4, 5, 6, 7, 8, 9 and 10 evaluating effects of biochar rates in presence of earthworms.

Treatment no.	Earthworm	Biochar applied	Biochar type		Application rate ($g\,kg^{-1}$)	Acronym
			C. megalocarpus	*Z. gilletii*		
1	−	−	−	−		S
2	−	+	−	+	10	S-Z^{10}
3	−	+	+	−	10	S-C^{10}
4	+	−	−	−		S-E
5	+	+	−	+	5	S-E-Z^{5}
6	+	+	+	−	5	S-E-C^{5}
7	+	+	−	+	10	S-E-Z^{10}
8	+	+	+	−	10	S-E-C^{10}
9	+	+	−	+	25	S-E-Z^{25}
10	+	+	+	−	25	S-E-C^{25}

− absent; + present.

Fig. 1. Microcosms with muslin cloth fitted at the base on water filled plates to allow for wetting by capillary action.

type (*Z. gilletii* or *C. megalocarpus*) and rates of application were the fixed factors while block was a random factor. All ANOVA was determined using the generalized linear mixed model from the R package 'lme4' (Bates et al., 2014). Where the effect of a variable was significant, we conducted the Tukey post hoc test and extracted pairwise comparisons using the 'cld' function from the 'lsmeans' package in R (Lenth and Hervé, 2016). Finally, to test effects of the different biochar rates in presence of earthworms relative to the earthworm only control (S-E), we performed contrasts between the no-biochar treatment against the individual levels of both biochar types. Differences were considered statistically significant at $p < 0.05$, although higher significance level ($p < 0.1$) was also noted for the fluxes. All analyses were carried out using R version 3.3.3 (R Core Team, 2018).

3. Results

3.1. Soil characteristics

In the absence of earthworms, biochar application rates of $10\,Mg\,ha^{-1}$, *Z. gilletii* (S-Z^{10}) resulted in higher soil N ($p = 0.009$) than the control (S), although the addition of earthworms resulted in similar soil N concentrations between the control and *Z. gilletii* biochar treatments (S-E-Z^{10}) (Table 3). While *Z. gilletii* application (S-E-Z^{10}) showed no differences, *C. megalocarpus* biochar in the presence of earthworms (S-E-C^{10}) resulted in lower soil N concentrations compared to the earthworm only treatment (S-E) ($p = 0.013$) (Table 3). S-E-Z^{10} had lower soil N concentrations compared to S-Z^{10} ($p = 0.047$); however, there was no significant difference in soil N between the no-earthworm and earthworm treatments when *C. megalocarpus* biochar was applied (S-E-C^{10} and S-C^{10}). In the presence of earthworms, the different biochar application rates had no measurable effect on soil N concentrations when *Z. gilletii* biochar was applied, while the addition of $25\,Mg\,ha^{-1}$ of *C. megalocarpus* biochar resulted in lower soil N ($p < 0.001$) compared to the no biochar treatment.

Biochar and their rates of application influenced soil C. Biochar application at $10\,Mg\,ha^{-1}$ increased soil C in the absence of earthworms ($p < 0.001$ and $p = 0.005$ for *Z. gilletii* and *C. megalocarpus* respectively). In the presence of earthworms, only *Z. gilletii* at $10\,Mg\,ha^{-1}$ had higher soil C ($p < 0.\ 001$) compared to earthworm only control (S-E) (Table 3). Soil C concentrations were higher with greater biochar application rates, with increased soil C compared with the S-E measured at S-E-Z^{10} ($p = 0.004$) and S-E-Z^{25} ($p < 0.001$) or when $25\,Mg\,ha^{-1}$ of *C. megalocarpus* (S-E-C^{25}) ($p < 0.001$) was added (Table 4). Earthworm presence or absence had no measurable effect on soil C:N ratio, while S-E-C^{10}, S-E-C^{25} and S-E-Z^{25} led to increased C: N ratios compared to the S-E ($p < 0.001$). No differences were observed between the biochar types (Table 3) except at the highest application rate when the C:N ratio of the S-E-C^{25} was greater than the S-E-Z^{25} ($p < 0.001$) (Table 4).

3.2. Greenhouse gas emissions

Soil CO_2 fluxes peaked initially, declined over the first 12 days and then leveled off for the final 15 days (Fig. 3a). We observed a 26% increase in cumulative CO_2 emission due to earthworm addition ($p < 0.001$), while the addition of $10\,Mg\,ha^{-1}$ of *C. megalocarpus* biochar increased CO_2 fluxes by 9% compared to control ($p = 0.019$) (Fig. 2a). In the presence of earthworms though, there was an

Table 3

Soil properties in the presence (+) or absence (−) of earthworms with *C. megalocarpus* (*Cm*) and *Z. gilletii* (*Zg*) biochar application at a rate equivalent to 10 Mg ha^{-1}. Within rows, means followed by different letters in superscript are significantly different at $p < 0.05$. Lowercase letters indicate the differences due to interaction effects of earthworm presence/absence and biochar type. Uppercase letters indicate differences due to main effects of earthworm presence/absence and biochar type (when either biochar treatments (rows) or earthworm treatments (columns) are aggregated). Means were separated based on Tukey's honest significant difference (HSD) test. Numbers in brackets indicate standard error. [1]Aggregate mean effect of earthworm. [2]Aggregate mean effect of each biochar type.

Soil property	N (g kg^{-1})				C (g kg^{-1})			
Biochar	−	*Cm*	*Zg*	Mean[1]	−	*Cm*	*Zg*	Mean[1]
Earthworm								
−	3.38 ± (0.011)^{bc}	3.32 ± (0.011)^{bc}	3.58 ± (0.029)^{a}	3.42 ± (0.011)^{A}	39.9 ± (0.18)^{e}	44.1 ± (0.09)^{bc}	46.5 ± (0.21)^{a}	43.5 ± (0.24)^{A}
+	3.45 ± (0.037)^{ab}	3.26 ± (0.001)^{c}	3.42 ± (0.029)^{bc}	3.37 ± (0.011)^{A}	41.5 ± (0.68)^{de}	43.3 ± (0.08)^{cd}	45.6 ± (0.21)^{ab}	43.5 ± (0.19)^{A}
Mean[2]	3.41 ± (0.04)^{A}	3.29 ± (0.00)^{B}	3.5 ± (0.017)^{A}		40.7 ± (0.25)^{C}	43.7 ± (0.07)^{B}	46.1 ± (0.12)^{A}	
ANOVA								
Earthworm			$p < 0.001$				$p = 0.91$	
Biochar			$p < 0.001$				$p < 0.001$	
Earthworm * biochar			$p = 0.001$				$p = 0.049$	

Soil property	C:N ratio				pH			
Biochar	−	*Cm*	*Zg*	Mean[1]	−	*Cm*	*Zg*	Mean[1]
Earthworm								
−	11.84 ± (0.067)^{a}	13.30 ± (0.061)^{a}	12.99 ± (0.073)^{a}	12.71 ± (0.058)^{A}	5.57 ± (0.003)^{b}	5.53 ± (0.003)^{c}	5.54 ± (0.005)^{c}	5.55 ± 0.002^{B}
+	12.01 ± (0.127)^{a}	13.31 ± (0.026)^{a}	13.36 ± (0.074)^{a}	12.89 ± (0.06)^{A}	5.54 ± (0.003)^{c}	5.63 ± (0.005)^{a}	5.54 ± (0.005)^{c}	5.57 ± 0.004^{A}
Mean[2]	11.92 ± (0.048)^{B}	13.3 ± (0.022)^{A}	13.17 ± (0.042)^{A}		5.55 ± 0.002^{B}	5.58 ± 0.007^{A}	5.54 ± 0.002^{C}	
ANOVA								
Earthworm			$p = 0.175$				$p = 0.001$	
Biochar			$p < 0.001$				$p < 0.001$	
Earthworm * biochar			$p = 0.555$				$p < 0.001$	

Table 4

Soil properties of soils in presence of earthworms with *C. megalocarpus* (*Cm*) and *Z. gilletii* (*Zg*) biochars at application rates equivalent to 5 Mg ha^{-1}, 10 Mg ha^{-1} and 25 Mg ha^{-1}. Within rows, means followed by different letters in superscript are significantly different at $p < 0.05$. Lowercase differences due to interaction effects of rate and biochar type. Uppercase letters indicate significant differences due to main effects of either biochar type or rates (when either biochar types (rows) or rates (columns) are aggregated). Means were separated based on Tukey's honest significant difference (HSD) test. Numbers in brackets indicate standard error. [1]Aggregate mean effect of each rate of biochar application. [2]Aggregate mean effect of each biochar type.

Soil property						
	N ($g\,kg^{-1} g\,kg^{-1}$)			C ($g\,kg^{-1} g\,kg^{-1}$)		
Rate	*Cm*	*Zg*	Mean[1]	*Cm*	*Zg*	Mean[1]
5	3.22 ± $(0.015)^{ab}$	3.35 ± $(0.011)^{ab}$	3.29 ± $(0.011)^{A}$	41.0 ± $(0.83)^{a}$	42.9 ± $(0.42)^{a}$	42.0 ± $(0.33)^{C}$
10	3.26 ± $(0.001)^{ab}$	3.42 ± 0.029^{a}	3.34 ± $(0.014)^{A}$	43.3 ± $(0.08)^{a}$	45.6 ± $(0.21)^{a}$	44.5 ± $(0.17)^{B}$
25	2.55 ± $(0.02)^{c}$	3.17 ± 0.068^{b}	2.86 ± $(0.048)^{B}$	47.7 ± $(0.18)^{a}$	(49.2) ± $(0.42)^{a}$	48.5 ± $(0.18)^{A}$
Mean[2]	3.01 ± $(0.029)^{B}$	3.31 ± $(0.016)^{A}$		44.0 ± $(0.29)^{B}$	45.9 ± $(0.25)^{A}$	
ANOVA						
Biochar		$p < 0.001$			$p = 0.012$	
Rate		$p < 0.001$			$p < 0.001$	
Biochar * rate		$p = 0.001$			$p = 0.878$	

Soil property						
	C: N ratio			pH (H_20)		
Rate	*Cm*	*Zg*	Mean[1]	*Cm*	*Zg*	Mean[1]
5	12.74 ± $(0.202)^{c}$	12.79 ± $(0. 103)^{c}$	12.76 ± $(0.074)^{B}$	5.55 ± $(0.007)^{b}$	5.52 ± $(0.004)^{b}$	5.54 ± $(0.003)^{B}$
10	13.31 ± $(0.026)^{c}$	13.36 ± $(0. 074)^{c}$	13.33 ± $(0.026)^{B}$	5.63 ± $(0.005)^{a}$	5.53 ± $(0.005)^{b}$	5.58 ± $(0.007)^{A}$
25	18.74 ± $(0.08)^{a}$	15.59 ± $(0. 344)^{b}$	17.17 ± $(0.241)^{A}$	5.63 ± $(0.007)^{a}$	5.53 ± $(0.002)^{b}$	5.58 ± $(0.007)^{A}$
Mean[2]	14.93 ± $(0.239)^{A}$	13.91 ± $(0.123)^{B}$		5.61 ± $(0.004)^{A}$	5.53 ± $(0.001)^{B}$	
ANOVA						
Biochar		$p = 0.001$			$p < 0.001$	
Rate		$p < 0.001$			$p = 0.001$	
Biochar * rate		$p < 0.001$			$p = 0.011$	

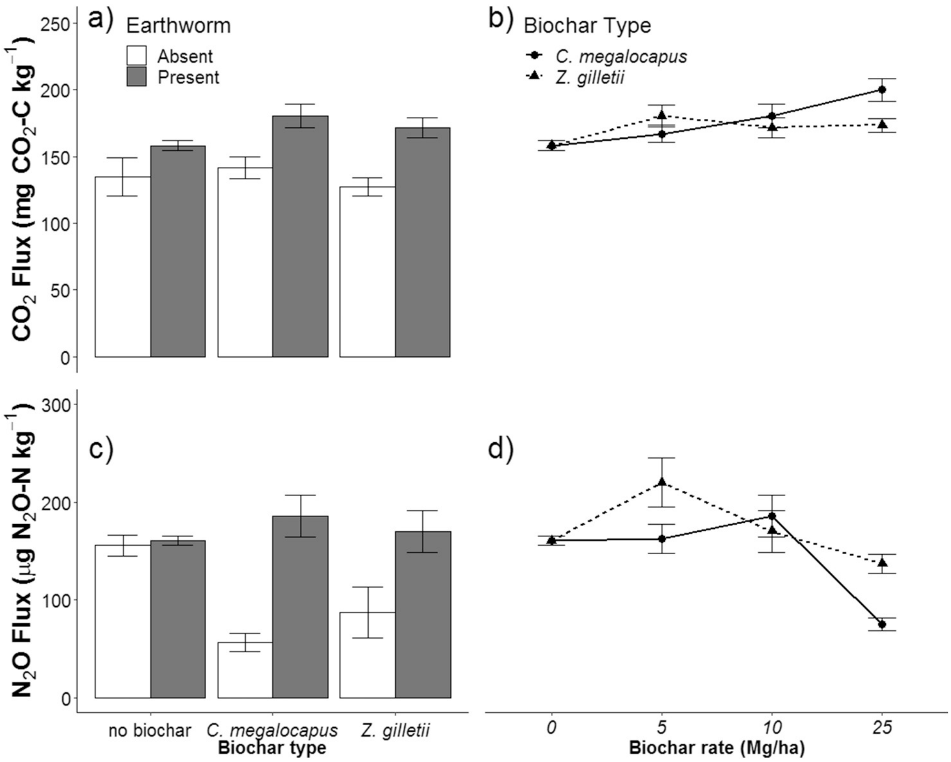

Fig. 2. (a) Cumulative 27-day CO_2 and (c) N_2O after biochar application at $10\,g\,kg^{-1}$ with earthworms present and absent.
(b) Cumulative CO_2 and (d) N_2O emission from biochar treated soils in presence of earthworm. Symbols represent differences from results of contrast analysis (differences between 0 rate and three levels of *Croton megalocarpus* and *Zanthoxylum gilletii* biochar application). Error bars indicate ± 1 SED.

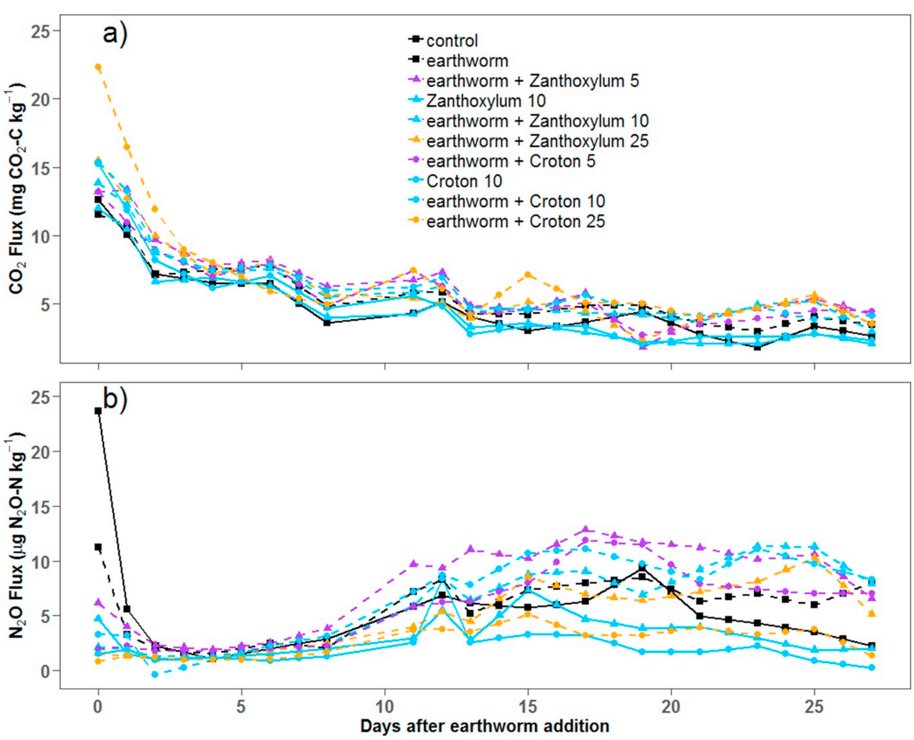

Fig. 3. (a) Times series of carbon dioxide (mg CO_2-C kg^{-1}) and (b) nitrous oxide (μg N_2O-N kg^{-1}) emission from soils with or without earthworms, and with or without two biochar types (*Croton megalocarpus* and *Zanthoxylum gilletii*) over 27 days of the experiment. Different line types represent presence/absence of earthworms, different symbols represent type of biochar, and different colors represent rate of biochar application.

interaction effect of application rate and biochar type on cumulative CO_2 flux ($p = 0.018$) (Fig. 2b). Cumulative CO_2 emissions were greater at *C. megalocarpus* application rates of $25\,Mg\,ha^{-1}$ compared to the $5\,Mg\,ha^{-1}$ application ($p = 0.017$) and no-biochar control (S-E) ($p = 0.002$) (Fig. 2b), while the emissions were similar across all the three *Z. gilletii* application rates tested.

After the initial peak in N_2O emissions immediately following earthworm addition, there was a decline in N_2O fluxes for a few days, before gradually increasing again, peaking midway through the experiment at around day 17 (7.70 ± 3.48 μg N_2O kg^{-1}; Fig. 3b). There were significant interaction effects of earthworm presence and biochar application rates on cumulative N_2O fluxes ($p = 0.018$). Without earthworms, both S-C^{10} ($p = 0.001$) and S-Z^{10} ($p = 0.034$) reduced N_2O emissions by 54% relative to the control (S). However, when earthworms were present, no reduction in N_2O emissions was observed regardless of biochar type (Fig. 2c). When averaged across treatments, we observed a 72% increase in N_2O losses as a result of the introduction of earthworms. In microcosms with earthworms, biochar application rates of at least 10 Mg ha^{-1} generally resulted in a reduction of N_2O emissions, with the lowest cumulative losses from soils where biochar was applied at 25 Mg ha^{-1} (Fig. 2d). Both S-E-C^{25} ($p = 0.003$) and S-E-Z^{25} ($p = 0.005$) resulted in lower fluxes compared to S-E-C^5 and S-E-Z^5 respectively.

Cumulative N_2O emissions were generally lower with application of the *C. megalocarpus* biochar compared to the *Z. gilletii* biochar. Application of 25 Mg ha^{-1} of *C. megalocarpus* suppressed N_2O more than *Z. gilletii* at 25 Mg ha^{-1} ($p = 0.046$), while application rates of 5 Mg ha^{-1} *C. megalocarpus* indicated a trend ($p = 0.073$) towards lower emissions compared to 5 Mg ha^{-1} of *Z. gilletii*. There were however no differences between *C. megalocarpus* and *Z. gilletii* at 10 Mg ha^{-1}. In the presence of earthworms, only the highest application rate of both biochar types (S-E-Z^{25}, $p = 0.011$ and S-E-C^{25}, $p = 0.009$) reduced N_2O emissions compared to the S-E control. While *C. megalocarpus* at both 5 Mg ha^{-1} and 10 Mg ha^{-1} had no significant effect, S-E-Z^5 led to increased N_2O emission relative to the S-E control ($p < 0.001$).

4. Discussion

4.1. Effect of earthworm and biochar application on CO_2 emission

The observed pattern of CO_2 fluxes over the experimental period (Fig. 3a) was likely due to increased labile C after biochar addition and wetting of the soils that is known to increase labile C availability due to destabilization of soil aggregates. Labile C can also result from accumulation of microbial decomposition products during the dry phase, the death and lysis of microbes and subsequent release of microbial products upon rewetting, and the release of osmolytes by soil microbes (Lundquist et al., 1999; Ruser et al., 2006; Warren, 2014). Our findings that earthworms led to a 26% increase in CO_2 emissions was within the range (7–58%) noted in previous studies (Caravaca et al., 2005; Speratti and Whalen, 2008), and similar to the mean CO_2 increase (34%) from earthworm addition estimated in a meta-analysis of lab studies (Lubbers et al., 2013). This increase has been attributed to earthworm respiration. However, earthworms also break down soil organic matter, burrow, feed on soil and produce casts that provide ideal microhabitats for soil microbes that may have also contributed to the increase in CO_2 emissions (Caravaca et al., 2005; Chapuis-Lardy et al., 2010; Speratti and Whalen, 2008).

Wood-based biochar is generally expected to result in increased CO_2 emissions (Song et al., 2016). In our study, we also observed an increase in CO_2 emissions under *C. megalocarpus* biochar application, likely due to increased concentrations of labile C from inclusion of the biochar as reported Kinney et al. (2012) and Chan et al. (2008). Biochar with high labile C can lead to high overall emissions of CO_2 in the short term, especially when combined with earthworms that use soil organic matter, including biochar and its derivatives, as an energy source (Ameloot et al., 2013; Shan et al., 2013; Van Zwieten et al., 2010). Furthermore, the increased CO_2 emissions in presence of earthworms at increasing *C. megalocarpus* biochar application rates supports our explanation that greater CO_2 emissions were mainly the result of increased labile C. However, there are other possible causes of increased emissions following *C. megalocarpus* application including mineralization of native SOM (i.e. positive priming) and changes to the soil physical environment (e.g. improved aeration) that support aerobic microbes and thus enhancing decomposition (Lehmann et al., 2011; Luo et al., 2011).

Contrary to our expectation, *Z. gilletii* application did not follow the same pattern as *C. megalocarpus*, as there were no changes in CO_2 emissions when application rates increased from 5 to 25 Mg ha^{-1}. This suggests that additions of labile C were not responsible for the increased CO_2 emissions compared to the no biochar control (S-E). One possible explanation for the initial increase in CO_2 fluxes with the addition of 5 Mg ha^{-1} of *Z. gilletii* is that the application caused improvements to the soil physical environment, which enhanced soil respiration. The lack of further increases in CO_2 emissions from the higher application rates suggests that further improvements in soil structure could not be achieved with increased application rates. However, another scenario is that further decomposition of C was inhibited at high *Z. gilletii* application rates through mechanisms mediated by earthworm and chemical properties of the biochar. Biochar from *Z. gilletti* had distinctly higher CEC than the biochar derived from *C. megalocarpus* (Table 1). According to the Microbial Efficiency-Matrix Stabilization framework by Cotrufo et al. (2013), CEC plays a critical role in the ability of soil to stabilize C. We suggest that addition of the high CEC *Z. gilletii* biochar, especially at rates > 5 Mg ha^{-1}, could increase the soil CEC providing greater matrix stabilization potential, and hence greater protection of C within the soil matrix. This stabilization could be further enhanced by the presence of earthworms as they facilitate movement of organic substances into the soil mineral matrix.

The contribution of earthworms in our study may be particularly important as Kamau et al. (2017b) found *Z. gilletii* to be attractive to earthworms, an observation supported by Elmer et al. (2015) who also noticed preference of earthworms to certain biochar-soil mixtures. Earthworms can influence microbial abundance and activity (Curry and Schmidt, 2007; Edwards, 2004) which could therefore affect matrix stabilization. Stabilization also occurs as materials are mixed with mucus in the earthworm guts thus enhancing micro-aggregation, and cast formation (Bossuyt et al., 2005). However, it has been pointed out that earthworm mediated stabilization takes place over longer time scales (usually > 200 days) (Martin, 1991; Six et al., 2004). Future research needs to evaluate whether the relatively smaller soil mass used in this experiment (≈1.5 kg) may have magnified the influence of earthworms thus reducing the time scale for earthworm mediated SOC stabilization.

4.2. Effect of earthworm and biochar application on N_2O emission

Our observation that N_2O emissions were unexpectedly high immediately after earthworm addition was likely related to initial wetting of the soil. The subsequent pattern of a slow increase in N_2O emissions (Fig. 3b) followed a trend reported by Flessa et al. (2002) and Singh et al. (2010) who attributed it to nitrification and subsequent denitrification of the initial readily available N. Sharp increases in N_2O emissions after wetting have been observed by Gelfand et al. (2015) and LaHue et al. (2016) and are thought to be the result of increased nitrification due to release of readily available N and C (Birch, 1960) from dead microbial biomass and from the release of labile C compounds that are used by soil microbes to cope with water deficits (Warren, 2014). Increased consumption of the labile C by heterotrophs creates anaerobic microsites that can promote denitrification. Additionally, soil NO_2^- builds up when soil is dry, which can subsequently be reduced to N_2O upon wetting (Davidson, 1992; Ruser et al., 2006).

The 54% reduction in N_2O emissions after application of 10 Mg ha^{-1} of biochar was consistent with a previous meta-analysis that reported the exact same reduction (Cayuela et al., 2014). Biochar suppresses N_2O emissions through microbial immobilization of N, and modifies the microbial community structure of the soil which alters respiration rates (Ameloot et al., 2013; Feng and Zhu, 2017; Paz-Ferreiro et al., 2015). Biochar may also suppress N_2O emissions by improving aeration hence reducing denitrification; adsorbing NO_3^- to its surface; and increasing pH which favors the further reduction of N_2O to N_2 (Song et al., 2016). In our study, however, changes in pH were unlikely to have played a major role since pH did not vary much with biochar application.

The lack of any reductions in N_2O emissions with incorporation of earthworms suggests that earthworms played a role in increasing emissions from biochar treated soils. Earthworms may contribute to increased emissions of N_2O through several processes including direct emissions from their gut (Matthies et al., 1999), and production of casts with higher nutrient and moisture content than surrounding soil.

Earthworms' burrowing activity also produces compacted drillosphere soil (Orgiazzi et al., 2016), resulting in compacted spaces and higher moisture contents that result in conditions favorable for denitrification. Additionally, casts and mucus associated with earthworms tend to stimulate the enzymes responsible for N mineralization resulting in higher mineral N concentrations, and which can be further enhanced by biochar application (Parkin and Berry, 1994; Paz-Ferreiro et al., 2014). Higher mineral N concentrations can then lead to increased production of N_2O.

Similar to our study, suppression of N_2O with increasing rates of biochar was also observed by Cayuela et al. (2013) and Song et al. (2016). They suggested that this could result from increased immobilization and by the ability of biochar to adsorb mineral N to its surface. This reduction with increasing application rates in presence of earthworms however, was not consistent across biochar types in our study as the lower application rates of *Z. gilletii* actually increased fluxes while low application rates of *C. megalocarpus* had no detectable impact. These differences in N_2O emissions between biochar types have previously been demonstrated by Feng and Zhu (2017), who found that the ratio of total carbon to inorganic nitrogen (TC/IN) was crucial in determining the ability of biochar to mitigate N_2O emissions. *C. megalocarpus* biochar had more labile C but presumably little available N as we observed no change in N between S-E-C^{10} and S-C^{10} (Table 3) which meant that only native soil N was being utilized by earthworms as they avoided soil with biochar from *C. megalocarpus* (see Kamau et al., 2017b). Mineralization of the additional labile C likely led to immobilization of N which reduced N_2O fluxes when biochar was applied at 25 Mg ha^{-1}.

It is also feasible that reduced N_2O emission at 25 Mg ha^{-1} of *C. megalocarpus* may not be due to reduced denitrification, but rather because of an increase in the amount of N_2O reduced to N_2. High respiration rates can create highly anaerobic conditions. At the same time, biochar reduced the availability of NO_3^-. These two conditions i.e. low NO_3^- and highly anaerobic conditions, favor the final step of denitrification to N_2 (Cayuela et al., 2013; Richardson et al., 2009), which aligns with findings by Obia et al. (2015) who observed increased N_2 production along with reduced N_2O and NO fluxes following biochar application.

In contrast to biochar from *C. megalocarpus*, biochar from *Z. gilletii* had high labile C as well as high mineral N concentrations. Therefore, the 5 Mg ha^{-1} application rate probably introduced enough NH_4^+ to enhance production of N_2O. Also, as noted above, earthworm mucus and casting tends to increase mineralization of soil N, which would have increased the NH_4^+ and NO_3^- content of the soil + *Z. gilletii* mixture. This observation supports the findings from Lubbers et al. (2011) who found that N_2O emissions from soil with earthworms occur only when extra N is supplied. Further increases in *Z. gilletii* application rates, however, led to reductions in N_2O emissions, which supports our hypothesis that high concentrations of labile C and N, along with the high CEC of *Z. gilletii*, combined with the earthworms contributed to the matrix stabilization of the soil leading to a reduction in fluxes (both CO_2 and N_2O).

Despite different mechanisms involved, it is clear that in presence of earthworms, only high application rates of *Z. gilletii* and *C. megalocarpus* suppressed N_2O fluxes. Cayuela et al. (2015) also noted contrasting results between laboratory and field experiments with the reduction in N_2O emissions following biochar application in field experiments being about half of that in laboratory experiments. Field experiments by Case et al. (2014) similarly found that biochar application of at least 49 Mg ha^{-1} had no impact on N_2O fluxes. We suggest that earthworms could play a critical role in these apparent differences in N_2O fluxes between lab and field experiments. Under controlled conditions, there is certainty regarding absence of earthworms, thus the impacts on N_2O are purely due to biochar application. In the field, differences in spatial distribution of earthworms may confound N_2O emissions from soil. Where they are present however, we speculate that earthworms are vital to increased emissions despite biochar application, a factor that is likely excluded from microcosm studies.

5. Conclusion

Biochar application has been proposed as an effective treatment for mitigating GHG emissions from agricultural soils. Our results show that biochar suppresses N_2O at moderate levels only when earthworms were not present, and that cumulative N_2O fluxes in the presence of earthworms depend on biochar type and application rates. The implications here are that under field conditions (i.e. including earthworms), suppression of N_2O may only be achieved under very high application rates of biochar (25 Mg ha^{-1} or more) although this may come at the cost of increasing CO_2 emissions and are unlikely to be feasible at scale. The differential effect of the two biochar types highlights the need for better understanding of the impacts of biochar quality and quantity on soil functions driven by soil biota. In practice, these differences suggest that recommendations for biochar use, especially those produced under local conditions, may only apply for certain types of biochar produced from certain organic resources and not from others. Further investigations into how soil and biochar properties interact with type of earthworm are needed to understand the best pathways to mitigate emissions of GHGs from tropical soils.

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