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Endogeic earthworm densities increase in response to higher fine-root production in a forest exposed to elevated CO_2

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ABSTRACT

Net primary productivity (NPP) influences soil food webs and ultimately the amount of carbon (C) inputs in ecosystems. Earthworms can physically protect organic matter from rapid mineralization through the formation of soil aggregates. Previous studies at the Oak Ridge National Laboratory (ORNL) Free Air CO_2 Enrichment (FACE) experiment showed that elevated [CO_2] (e[CO_2]) increased fine-root production and increased soil C through soil aggregation compared to ambient [CO_2] (a[CO_2]) conditions. Our first objective was to study the response of earthworms to increased leaf and root-litter inputs caused by increased atmospheric [CO_2] exposure. We also took advantage of the CO_2 shutdown at the ORNL FACE site to track the shift of the $\delta^{13}C$ signal in leaf-litter, fine roots, earthworms, earthworm casts, and bulk soil. Densities of the most abundant endogeic earthworm, *Diplocardia* spp., were positively correlated with the previous-year production of leaf litter ($r = 0.66$, $P = 0.02$) and fine roots ($r = 0.62$, $P = 0.03$); and with the leaf-litter production ($r = 0.63$, $P = 0.03$) and fine-root production ($r = 0.59$, $P = 0.05$) two years before earthworms were sampled. Within two years after the CO_2 fumigation ceased, the $^{13}C/^{12}C$ ratio increased in leaf litter ($P = 0.01$) and in fine roots ($P = 0.05$), showing an ecosystem legacy effect on soil C inputs. However, the C isotopic composition of soil, endogeic earthworms and casts had not changed the two years after the CO_2 fumigation ended. The positive response of earthworms to increased root NPP, caused by elevated [CO_2], is consistent with the increased soil aggregate formation and increased soil C at the ORNL FACE in the e[CO_2] treatment.

1. Introduction

One of the most pronounced global effects of human activity is the sharp increase in atmospheric CO_2 concentration (Schulze, 2006). Elevated atmospheric CO_2 concentration has a direct impact on global vegetation by increasing productivity, with potentially important cascading effects on soil organisms (Gonzalez-Meler et al., 2004; Norby and Zak, 2011). Many studies have evaluated plant responses to elevated [CO_2] (e.g. Norby and Zak, 2011; Iversen et al., 2012), but very few have examined effects on the belowground macrobiota. One of the biggest uncertainties is how the soil macrofauna respond to ecosystems exposed to elevated [CO_2], despite the recognized role of the macrofauna in processing plant litter, and incorporating and stabilizing organic matter in soils. Knowing the feedbacks and interactions among biotic and edaphic processes that determine the strength of an ecosystem to capture and maintain carbon (C) becomes critical for increasing the predictive capability of models of global change. The soil macrofauna may be a substantial player in these feedbacks.

Research at the Oak Ridge National Laboratory (ORNL) Free-Air CO_2 Enrichment (FACE) site has shown that forest net primary productivity (NPP), particularly the fraction of NPP attributable to fine-root production, increases in response to elevated atmospheric CO_2 concentrations (e[CO_2]) (Matamala et al., 2003; Norby et al., 2004; Iversen et al., 2008). However, the CO_2-induced enhancement of NPP was not sustained as nitrogen availability in the forest declined (Norby et al., 2010a). Changes in NPP can directly affect the quantity and

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quality of organic inputs to the soil, potentially leading to cascading effects on the soil food web and ultimately on C cycling. Despite the known influence of the soil macrofauna on soil processes, the effects of elevated atmospheric [CO_2] on the macrofauna and feedbacks between the soil macrofauna and biogeochemical cycles in forest ecosystems are largely unknown (Scullion et al., 2014).

Because activities of the soil macrofauna can influence biogeochemical processes (Lavelle et al., 2006; Coleman, 2008), neglecting responses of the soil macrofauna to changes in atmospheric [CO_2] overlooks a potentially critical component of the ecosystem response. Earthworms, which are major constituents of the soil macrofauna, can impact soil processes disproportionately to their density or biomass (Lavelle et al., 2006; Brussaard et al., 2007). Through their feeding, casting and burrowing behavior, earthworms can influence decomposition, C and nutrient cycling, and the maintenance of soil structure (Blair et al., 1995; Groffman and Bohlen, 1999; Lavelle et al., 2006). Earthworm species have been placed in one of three major ecophysiological groups (epigeic, anecic and endogeic) based primarily on their soil microhabitat and burrowing and feeding behaviors (Bouché, 1977; Blair et al., 1995; Lavelle, 2002; Coleman et al., 2004). Epigeic earthworms are litter feeders that live in the litter layer. Anecic earthworms, which are litter feeders that live primarily in mineral soil, form deep vertical burrows and feed mainly on surface litter that they transport deeper into the soil profile. Endogeic earthworms are soil feeders that live in the mineral soil. Intermediate categories exist for species that do not completely fit within one of the major ecological categories; for example, epi-endogeic earthworms inhabit the surface soil and consume plant litter (Coleman et al., 2004).

Earthworms can increase soil-C sequestration by producing casts (Lee, 1985; Edwards and Bohlen, 1996; Frelich et al., 2006), which become soil aggregates that protect organic C from microbial decomposition (Bossuyt et al., 2005; Sánchez-de León et al., 2014). The ORNL experiment was one of only a few FACE studies to have documented increased soil-surface C under elevated [CO_2] (Jastrow et al., 2005; Iversen et al., 2012), including an increase in microaggregated C (Jastrow et al., 2005). Thus, the ORNL FACE site offered an almost unique opportunity to investigate whether increased earthworm densities could help explain the increased C seen in surface soils under elevated [CO_2].

Our first objective was to study how changes in the production of plant litter affects earthworms. We hypothesized that because of increased belowground production resulting from higher rates of organic-matter input under elevated [CO_2] (e[CO_2]) conditions, endogeic earthworms would be more abundant in e[CO_2] than ambient [CO_2] (a[CO_2]) treatments. We also hypothesized that because of time lags in the endogeic earthworm response, there would also be a correlation between previous rates of plant litter production and current earthworm abundance. Our second objective was to establish a connection between increased belowground production and earthworm densities by tracking C from plant litter to earthworm tissues and casts using the stable isotope ^{13}C tracer (Pataki et al., 2003). We were able to accomplish this because the e[CO_2] plots had a unique $\delta^{13}C$ signature after 12 years exposure to a ^{13}C-depleted CO_2 source. In addition, Lynch et al. (2013) found that new fine roots started to show "relaxation" (i.e. loss) of the $\delta^{13}C$ signal as early as 6 months after the cessation of the CO_2 fumigation at the Oak Ridge FACE site. We tracked the transfer of the ^{13}C allocated to leaf litter and fine roots to earthworms and then to soil organic matter (SOM) via earthworm casts. We hypothesized that earthworm isotopic composition would shift in a manner reflecting the turnover rate of its source of C. Thus, the $\delta^{13}C$ signal of epigeic, anecic and epi-endogeic earthworms – all of which consume plant litter – would shift after one year of the cessation of CO_2 fumigation. For endogeic earthworms, which feed upon SOM, we hypothesized that the $\delta^{13}C$ signal would shift after incorporation of plant litter into SOM, which would require at least 2 years.

2. Materials and methods

2.1. Study site and experimental design

The ORNL FACE site was located in a sweetgum (*Liquidambar styraciflua* L.) plantation that had been established in 1988 in the Oak Ridge National Environmental Research Park in Roane County, Tennessee, USA (35°54′ N; 84°20′ W). The soils are classified as Aquic Hapludult with silty clay loam texture (Soil Survey Staff, 2018). The FACE site consisted of two 25 m diameter plots that experienced air with e[CO_2] from April 1998 until September 2009 (12 growing seasons), and two 25 m diameter control plots with similar infrastructure and ambient CO_2 concentrations (a[CO_2]) (Norby et al., 2006; Riggs et al., 2009). An additional control plot without the FACE infrastructure was not used in this analysis. The CO_2 enrichment was achieved using the Brookhaven National Laboratory design (Hendrey et al., 1999) as described by Norby et al. (2001). Pure CO_2 was released through vertical vent pipes surrounding the plots, with the rate of release dependent on wind speed and a feedback control. The atmospheric CO_2 concentration of the e[CO_2] treatment averaged 547 ppm during the experiment, and [CO_2] in the ambient plots averaged 395 ppm (Riggs et al., 2009; Walker et al., 2014). The elevated [CO_2] treatment was continuous during daylight hours during each growing season (April through November) until September 2009.

The CO_2 used in the e[CO_2] treatment was $\delta^{13}C$-depleted with an $\delta^{13}C$ signature of approximately −50‰ (Norby et al., 2006) which is incorporated into tissues and fluxes during the experiment (Matamala et al., 2003; Lynch et al., 2013; Gonzalez-Meler et al., 2014). Other ecological data, such as root productivity, litter fall, root mortality and tissue nitrogen concentration were periodically collected (Ledford et al., 2008; Norby et al., 2010b).

2.2. Estimating earthworm densities

Earthworm sampling was conducted in September 2007, May 2008, July 2008, October 2008, May 2010, October 2010 and May 2011, when moist and warm soil conditions promote earthworm activity. We used a combination of hand sorting and extraction with a solution of allyl isothiocyanate (AITC) in four sub-samples within each of the four plots (i.e. two e[CO_2] plots and two a[CO_2] plots). The AITC was selected over formalin (most commonly used extractant) because of AITC's documented efficacy (Zaborski, 2003) and the fact that it is not toxic to earthworms, researchers or the soil environment (Valckx et al., 2011). We first irrigated the soil with a solution of AITC (100 mg L^{-1}), isopropanol and water (Zaborski, 2003) over an area of 0.06 m^2 (25 cm × 25 cm) to drive worms to the surface. After 10 min, we manually excavated the 25 cm × 25 cm area to a depth of 10 cm, placed the soil over a plastic sheet and sorted earthworms by hand. We then did a second application of the AITC solution in the soil pit to bring to the surface earthworms that were below the 10 cm depth. Earthworms were taken to the laboratory, classified to morphospecies, counted, and their fresh weight was measured. Selected adult specimens were preserved in a formalin solution (formaldehyde 1:10 solution) (James, 1999) for taxonomic identification. Thus, earthworm density was expressed both in terms of numbers and a measure of biomass (fresh weight).

2.3. Preparation of earthworm tissue and casts for isotope analysis

Methods for preparation of earthworm tissues and casts for isotope analysis were adapted from Schmidt (1999). Earthworms that were not used for taxonomic identification were left in Petri dishes with glass-fiber filters (Fisherbrand® Glass Fiber Filter Circles G6), moistened with a diluted 1:8 solution of frog Ringer's Solution for 3 days to allow them to empty their guts (Schmidt, 1999). The filter paper was removed daily and dried at 60 °C for 15 min or until the casts were dry enough to be

gently scraped from the filter paper. After 3 days of cast collection, the earthworms were frozen and freeze dried. Earthworm tissue and earthworm casts were ground and stored dry until isotopic analysis at the University of Illinois at Chicago.

2.4. Leaf litter, fine roots and soil sampling

Leaf litter was collected from the same sampling area each time earthworms were collected. Loose surface litter from the 25 cm × 25 cm area was placed in paper bags and dried at 60 °C for 48 h or until constant dry weight was obtained. Samples were stored dry, ground and homogenized for isotopic analysis.

Soil and fine-root samples at 0–10 cm depth were collected with a 4.8 cm diameter soil corer in areas adjacent to the areas used for earthworm sampling. Fine roots (< 1 mm diameter) and other materials were removed manually from the soil samples. The soil samples were air-dried, ground to pass a 2 mm mesh sieve and homogenized for isotopic analysis. Fine roots were gently washed with distilled water and dried at 60 °C for 48 h. After drying, fine-root samples were ground and homogenized for isotopic analysis.

2.5. Stable carbon isotope analysis

Analyses of the stable C isotope ratio ($^{13}C/^{12}C$) of earthworm tissues, casts, sweetgum leaf litter, fine roots and soil were performed at the Ecology Stable Isotope Laboratory, University of Illinois at Chicago. We used a Thermo Finnigan Delta-Plus XL isotope ratio mass spectrometer coupled to a Costech Elemental Analyzer. Each sample was ground to a fine powder before isotopic analysis. The $^{13}C/^{12}C$ isotope ratios are reported in delta (δ) notation, where $\delta^{13}C$ represents sample units per mil relative to the standard reference material VPDB (Coplen, 1996) according to the convention of $\delta^{13}C$, in ‰, = $[(R_{sample}/R_{VPDB}) - 1] \times 1000$, where R is the atomic ratio $^{13}C/^{12}C$ and VPDB is the standard reference material (Coplen, 1996).

2.6. Data analysis

Linear mixed-effect models were used to analyze effects of e[CO_2] on earthworm densities (expressed as numbers and fresh weight of all species combined) and stable isotope ratios across sampling events. Date was considered a categorical variable rather than continuous variable to maintain symmetry with season (*i.e.*, spring or fall) and phase (*i.e.*, during CO_2 fumigation or post CO_2 fumigation). Data collected in July 2008 was not included in the isotopic analyses because we were only able to sample one summer and did not have multiple years as with the fall and spring seasons. We transformed earthworm numbers and fresh weights using the fourth-root transformation to fulfill normality assumptions.

Separate Pearson linear correlation analyses were performed to evaluate relationships between production of fine-roots and leaf litter with numerical densities and biomass-densities of the endogeic *Diplocardia* spp. Fine-root and leaf-litter production data were taken from Norby et al. (2010b). Correlations were performed with fine-root and leaf-litter production from the same year, the previous year and two-years previously. Fine-root and leaf-litter NPP data were not collected after 2009 (Norby et al., 2010b), and earthworm data were not collected before 2007. Therefore, earthworm densities came from 2007, 2008, 2010 and 2011; and data on production of fine roots and litter came from 2005, 2006, 2007, 2008 and 2009. All statistical analyses were performed using the R statistical software (The R Foundation for Statistical Computing).

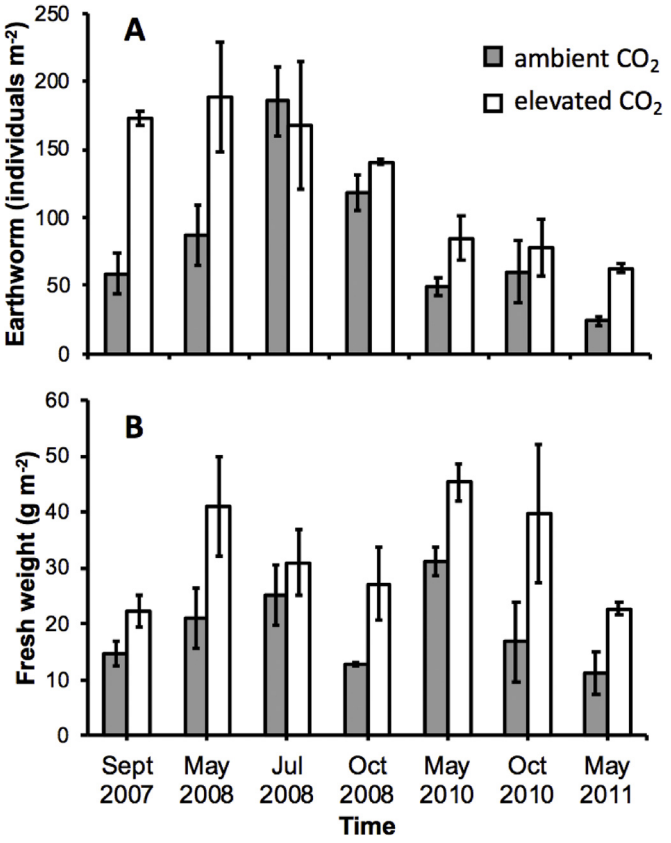

Fig. 1. Mean (± SE) earthworm (A) numerical density and (B) biomass (fresh weight) density in elevated [CO_2] and ambient [CO_2] treatments (n = 2) across time.

3. Results

3.1. Earthworm densities

Over 94% of the earthworms collected belonged to the genus *Diplocardia,* which is native to the eastern and southeastern United States (James, 1999). Most *Diplocardia* are classified as endogeic (Kalisz and Wood, 1995; Sánchez-de León et al., 2014). We found the native species *D. singularis* and two potentially undescribed *Diplocardia* species. We also found three exotic (non-native) earthworm species: *Aporrectodea rosea* (endogeic), *Amynthas corticis* (epigeic; García and Fragoso, 2003) and *Lumbricus rubellus* (epi-endogeic). Our sampling did not retrieve any anecic species.

Over the course of the study, there was a trend of more earthworms (numerical density of all species combined) in the plots exposed to elevated [CO_2] than in ambient plots (Fig. 1a; $F_{1,\ 2} = 7.70$; P = 0.11). Similarly, a trend of greater earthworm biomass (all species) was found in the e[CO_2] treatment than in the ambient one over the course of the study (Fig. 1b; $F_{1,\ 2} = 5.49$; P = 0.14).

Endogeic earthworm numerical densities showed positive correlations with production of fine roots and leaf litter only from one or two years previously (Figs. 2 and 3). Numerical densities of the endogeic *Diplocardia* spp. were not correlated with current-year production of fine roots (Fig. 2A, r = 0.21, P = 0.62) or leaf litter (Fig. 3A, r = 0.47, P = 0.24). However, numerical densities of *Diplocardia* spp. were positively correlated with previous-year production of fine roots (Fig. 2B, r = 0.62, P = 0.03) and leaf litter (Fig. 3B, r = 0.66, P = 0.02). Numerical densities were also correlated with production from two years before, for both fine roots (Fig. 2C, r = 0.59, P = 0.05) and leaf litter (Fig. 3C, r = 0.63, P = 0.03).

In contrast to this pattern, there was no correlation between

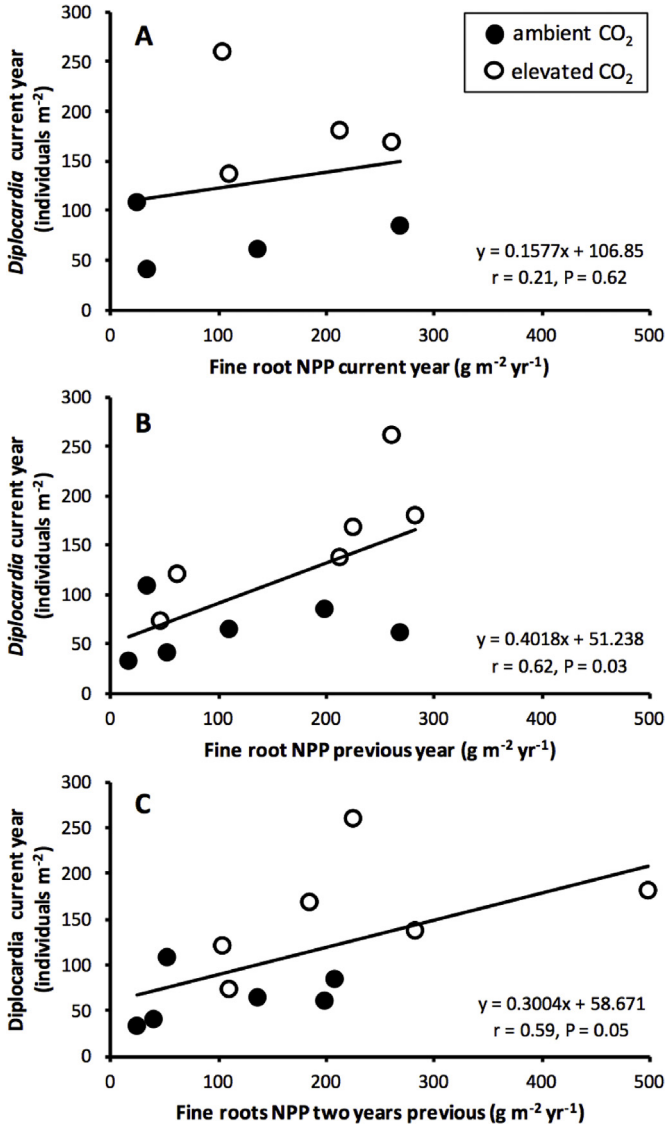

Fig. 2. Relationship between *Diplocardia* spp. numerical density and fine-root production of (A) the current year, (B) previous year and (C) two previous years. Closed circles (●) correspond to ambient [CO_2] and open circles (○) correspond to elevated [CO_2]. NPP was available as a yearly rate; thus estimates of earthworm abundance were averaged within each year. Earthworms were not sampled in 2009, and NPP values were not available after 2009. Thus, for (A) there are eight data points [2 years (2007 and 2008) x 4 plots]. For (B) there is an additional year of earthworm densities – 2010 – so there are 12 points (3 years × 4 plots). Earthworm data for 2011 are not included because there was no NPP data available for 2010. Data for 2011 were also excluded for (C) to keep the design balanced, i.e. same earthworm samples for (B) and (C). Values are by treatment plot (n = 2) and r is the Pearson correlation coefficient.

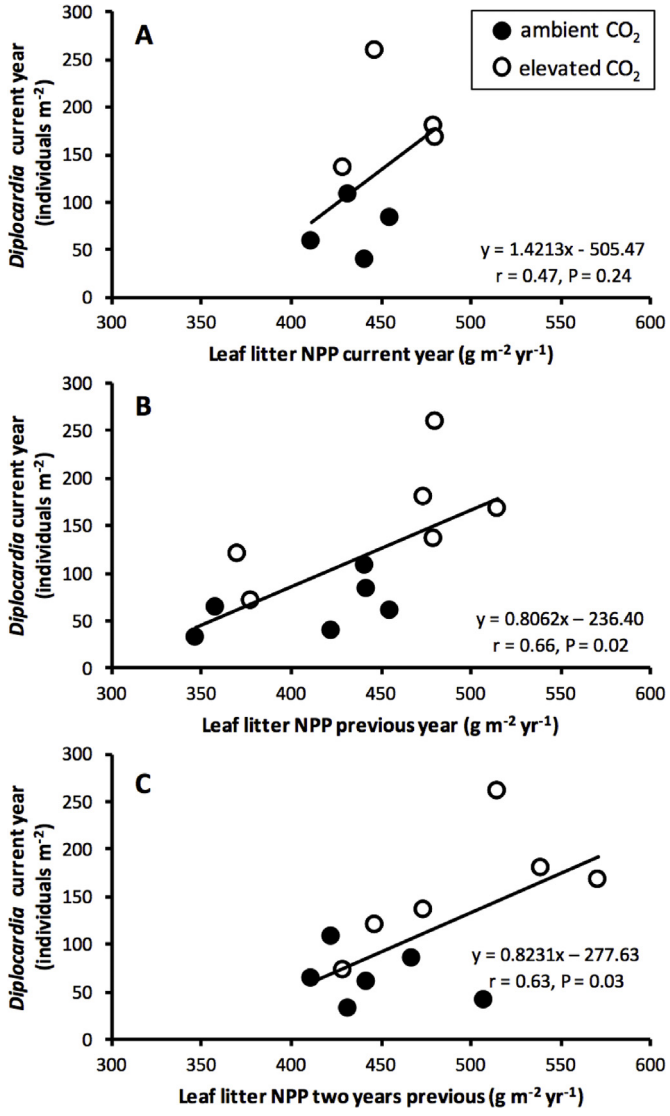

Fig. 3. Relationship between *Diplocardia* spp. numerical density and leaf litter production of (A) the current year, (B) previous year and (C) two previous years. Same comment as Fig. 2.

Diplocardia spp. fresh-weight mass-density and production of fine-roots or leaf litter from the current year, previous year or two years before (Figs. 4 and 5). Correlation analysis with the other earthworm species were not performed due to their low densities.

3.2. Stable isotope patterns

Relaxation of the $\delta^{13}C$ signal occurred in sweetgum leaf litter and fine roots after CO_2 fumigation ceased in 2009 (Table 1; P(Trt x Phase) ≤ 0.05 in Tables 3 and 4). The $\delta^{13}C$ signal of leaf litter was constant from 2007 to 2011 in the ambient treatment, and in the e[CO_2] plots was lower than ambient values from 2007 through Spring 2010 (Table 1). However, by ~12 months after CO_2 shutdown in the e[CO_2] plots in September 2009, the $\delta^{13}C$ signal of leaf litter in e[CO_2] plots had increased noticeably, and by Spring (2011) was close to that of the a[CO_2] treatment (−31.68 ± 0.53‰ versus −29.83 ± 0.12‰, respectively; Table 1). The relaxation pattern for fine roots was roughly similar to that of leaf litter, except that the convergence between treatments was less than that observed for leaf litter (Table 1). The $\delta^{13}C$ signal in e[CO_2] plots had also increased by a year after CO_2 shutdown, but by Spring (2011) was still noticeably lower than in ambient plots (−36.36 ± 0.07‰ versus −29.5 ± 0.09‰, respectively; Table 1).

In contrast to the pattern for leaf litter and fine roots, there was no relaxation of the $\delta^{13}C$ signal in earthworm tissues, earthworm casts, or soil (P(Trt x Phase) = 0.87, 0.19, and 0.65, respectively; Table 5). For all three, the $\delta^{13}C$ signal was consistently lower in the e[CO_2] treatment (P(Trt) ≤ 0.05; Table 5). The difference in $\delta^{13}C$ signal between a[CO_2] and e[CO_2] plots was greatest for earthworm tissue (~7.5‰) and least for soil (~4‰); the signal for earthworm casts was intermediate (~5.5‰) (Table 2).

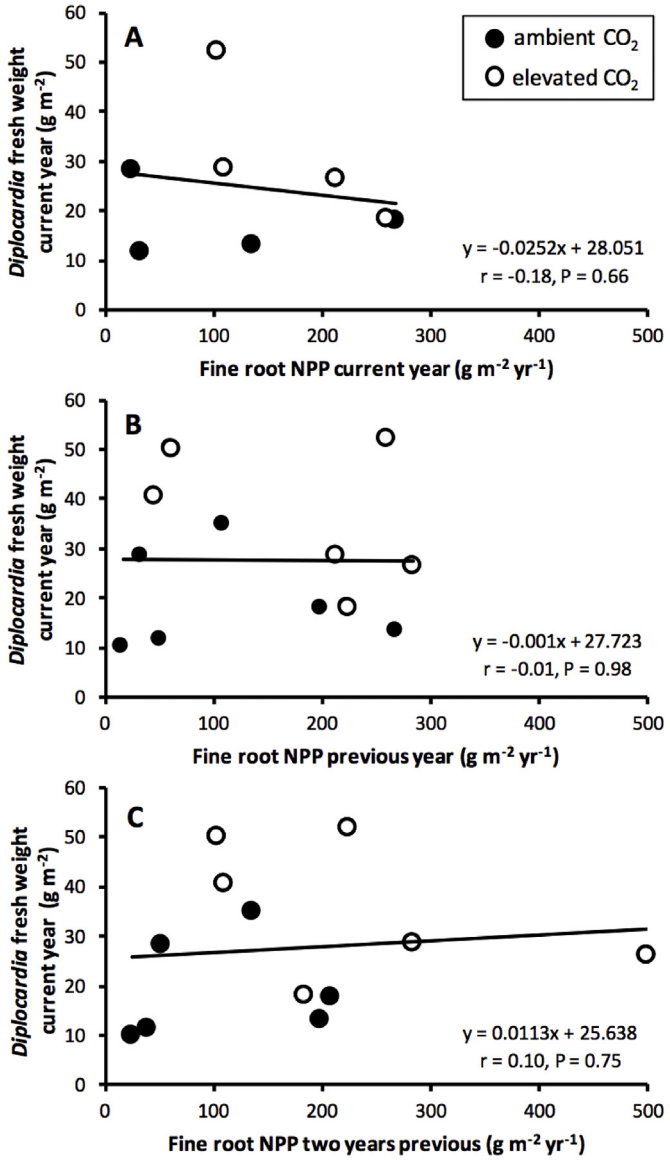

Fig. 4. Relationship between *Diplocardia* spp. Biomass-density (measured as fresh weight) and fine-root production of (A) the current year, (B) previous year and (C) two previous years. Same comment as Fig. 2.

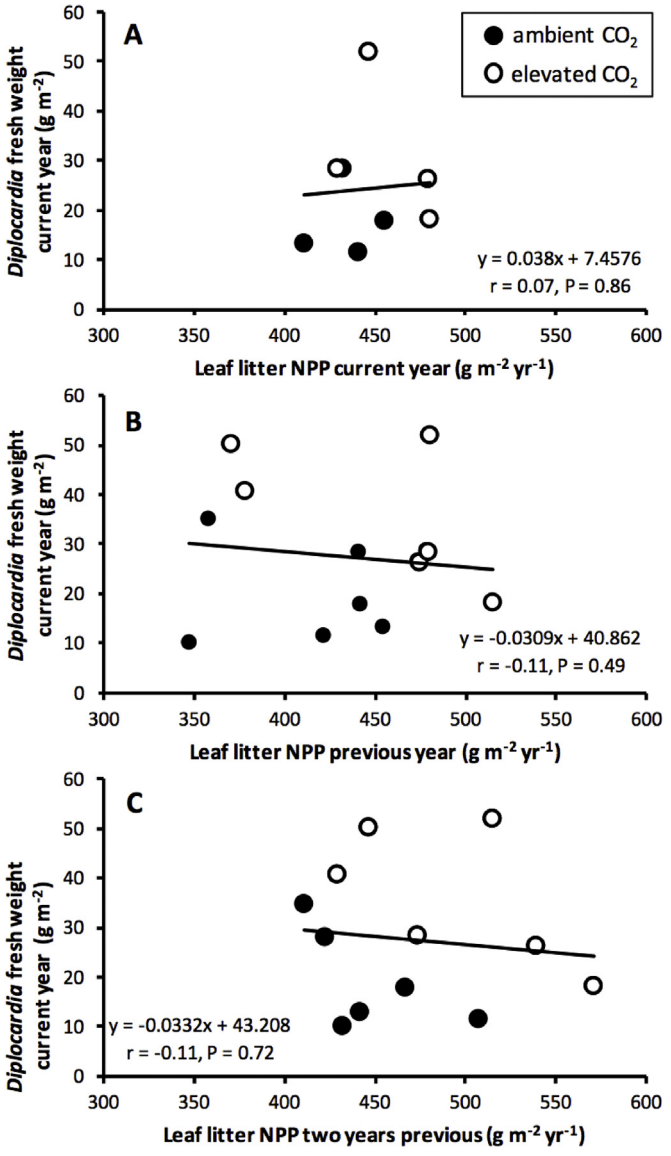

Fig. 5. Relationship between *Diplocardia* spp. Biomass-density (measured as fresh weight) and leaf litter production of (A) the current year, (B) previous year and (C) two previous years. Same comment as Fig. 2.

4. Discussion

4.1. Endogeic earthworm densities and SOM

Our first hypothesis was that earthworm numerical and biomass (fresh weight; all species) densities would be higher in plots with e[CO_2] because of increased higher rates of organic matter input under elevated [CO_2]. Our results support the postulated effect of e[CO_2] on densities and the mechanism underlying the response. Total earthworm numbers and fresh weight tended to be higher in the e[CO_2] treatments, although the evidence for these observations is suggestive but not strong (Fig. 1; $0.10 < P\text{'s} < 0.15$). Stronger support for our first hypothesis comes from the positive correlation between the overall plant litter production from the previous years and current densities of the endogeic *Diplocardia* spp. ($0.01 < P\text{'s} < 0.05$). The latter evidence is consistent with the findings that SOM is the main source of food for *Diplocardia* spp. (Sánchez-de León et al., 2014). Thus, the trends of higher earthworm densities in the e[CO_2] treatments, and the positive correlations of *Diplocardia* spp. numerical density with rates of previous-year inputs of organic matter, which would lead to elevated SOM, support our first hypothesis.

Table 1
Mean (± SE) $\delta^{13}C$ signal in sweetgum leaf litter and fine-roots (< 1 mm) in ambient [CO_2] (a[CO_2]) and elevated [CO_2] (e[CO_2]) treatments (n = 2) across time. Exposure to elevated CO_2 was terminated in 2009.

Time	Leaf litter $\delta^{13}C$ (‰)		Fine roots $\delta^{13}C$ (‰)	
	a[CO_2]	e[CO_2]	a[CO_2]	e[CO_2]
Fall 2007	−29.63 (0.02)	−37.75 (0.84)	not collected not collected	not collected not collected
Spring 2008	−29.14 (0.23)	−37.85 (0.04)	−28.86 (0.20)	−38.82 (0.28)
Fall 2008	−29.32 (0.17)	−38.48 (0.08)	−28.86 (0.03)	−39.74 (0.46)
Spring 2010	−29.97 (0.01)	−38.16 (0.17)	−29.24 (0.16)	−39.92 (0.41)
Fall 2010	−29.84 (0.19)	−32.70 (0.01)	−29.16 (0.29)	−36.59 (0.10)
Spring 2011	−29.83 (0.12)	−31.68 (0.53)	−29.50 (0.09)	−36.36 (0.07)

Table 2
Mean (± SE) $\delta^{13}C$ signal in earthworm tissues, earthworm casts and bulk soil (0–10 cm) ambient [CO_2] (a[CO_2]) and elevated [CO_2] (e[CO_2]) treatments (n = 2) across time. Exposure to elevated CO_2 was terminated in 2009.

Time	Earthworm tissues $\delta^{13}C$ (‰)		Earthworm casts $\delta^{13}C$ (‰)		Soil (0–10 cm) $\delta^{13}C$ (‰)	
	a[CO_2]	e[CO_2]	a[CO_2]	e[CO_2]	a[CO_2]	e[CO_2]
Fall 2007	−23.69 (0.27)	−32.17 (0.83)	−26.87 (0.56)	−31.57 (0.83)	−24.97 (0.01)	−28.74 (0.01)
Spring 2008	−23.75 (0.25)	−31.48 (0.10)	−26.79 (0.01)	−31.42 (0.71)	−25.25 (0.15)	−28.69 (0.09)
Fall 2008	−23.25 (0.12)	−31.43 (0.25)	−26.05 (0.06)	−30.57 (0.05)	−25.08 (0.09)	−29.83 (0.20)
Spring 2010	−23.79 (0.28)	−32.67 (0.10)	−26.14 (0.00)	−31.66 (0.49)	−24.96 (0.15)	−29.16 (0.42)
Fall 2010	−23.48 (0.57)	−31.42 (0.48)	−27.58 (0.57)	−33.61 (1.47)	−24.95 (0.01)	−28.66 (0.31)
Spring 2011	−23.30 (0.19)	−30.24 (0.14)	−26.22 (0.22)	−32.02 (0.40)	−25.77 (0.09)	−30.12 (0.39)

Table 3
Statistics of ANOVA for the $\delta^{13}C$ signal of sweetgum leaf litter. Degrees of freedom for numerator (numDF) and denominator (denDF) are presented. Independent variables (Source) tested were: Treatment (Trt) (aCO_2 or eCO_2), Phase (during CO_2 fumigation or post CO_2 fumigation) and Season (spring or fall).

Source	numDF	denDF	F-value	P-value
Trt	1	2	53.17	0.02
Phase	1	14	0.06	0.81
Season	1	14	0.05	0.82
Trt:Phase	1	14	7.93	0.01
Trt:Season	1	14	< 0.01	0.98
Phase:Season	1	14	0.038	0.85
Trt:Phase:Season	1	14	0.52	0.48

Table 4
Statistics of ANOVA for the $\delta^{13}C$ signal of fine-roots (< 1 mm). Same comment as Table 3.

Source	numDF	denDF	F-value	P-value
Trt	1	2	101.61	0.01
Phase	1	10	0.08	0.78
Season	1	10	0.00	1.00
Trt:Phase	1	10	5.12	0.05
Trt:Season	1	10	0.37	0.56
Phase:Season	1	10	0.02	0.89
Trt:Phase:Season	1	10	1.25	0.29

Correlations between the input of organic matter and *Diplocardia* spp. numbers, but not biomass, suggests that the impact of increased SOM is primarily upon reproductive rates (e.g. hatching rate) and juvenile survival. One possible explanation is that because large earthworms weigh much more than juveniles, sampling variation in the number of large individuals, which were relatively uncommon in the samples, could obscure differences between treatments in total fresh weight (i.e. juveniles plus adults). With only two replicates per CO_2 treatment statistical power is low.

4.2. Ecosystem C legacy in the isotopic composition of earthworms

We hypothesized that earthworm isotopic composition would shift in a way that reflected their feeding behaviors and the turnover rates of various C sources after CO_2 fumigation stopped in September 2009. The observed differences in $\delta^{13}C$ signal between treatments were expected because the CO_2 used for the fumigation was ^{13}C depleted (Norby et al., 2006). Our focus was evaluating how shutting down CO_2 fumigation affected differences in earthworm parameters between e[CO_2] and a [CO_2] treatments, as has been done for other ecosystem variables (Pataki et al., 2003; Taneva et al., 2006; Hopkins et al., 2013; Lynch et al., 2013; Kim et al., 2017). The $\delta^{13}C$ signal in leaf litter and fine roots from the e[CO_2] had relaxed (i.e. had moved closer to a[CO_2] values) within two years after CO_2 shut down. Furthermore, the leaf litter showed a faster relaxation rate than the fine roots. This result suggests that leaf litter had turned over completely within two years, but that fine roots had a slower turnover rate (see Lynch et al., 2013). However, after two years the $\delta^{13}C$ signal in soil, earthworms and casts had not yet shown signs of isotope relaxation. Lynch et al. (2013) showed that new fine roots produced after the CO_2 shutdown at the ORNL FACE site were composed of the "new" ambient C (i.e., after CO_2 shutdown), while the "old" C (i.e., incorporated into plant tissues during CO_2 fumigation) was maintained in existing root tissues and was used to partly support root respiration. Lynch et al. (2013) also discovered that turnover of newly produced fine roots takes more than two years under ambient conditions (see also Matamala et al., 2003). Thus, not finding relaxation of the $\delta^{13}C$ signal in the tissues and casts of the endogeic *Diplocardia* spp. (Table 2; Table 5) is consistent with the time that it will take for new fine root-derived C to be incorporated into the SOM, since these earthworm species derive most of their nutrition from SOM (Sánchez-de León et al., 2014). Thus, our results support our second hypothesis (that earthworm isotopic composition would shift in a manner reflecting the turnover rate of its C source), but with the caveat that more than two years would have been required to observe the predicted signal shift in the tissues and casts of endogeic earthworms.

It is still surprising that *Diplocardia* numerical density was also correlated with previous rates of production of leaf litter, which unlike fine roots had turned over almost completely within two years, yet earthworm tissues, casts and soil showed no evidence of relaxation of the $\delta^{13}C$ signal. Thus, it at first seems contradictory that *Diplocardia*, whose main source of C is SOM (Sánchez-de León et al., 2014), responded to inputs of leaf litter and fine-root NPP earlier than it took for these inputs to have been completely converted into SOM. In an incubation study earthworms favored the incorporation of root litter into SOM at the expense of leaf litter (Sánchez-de León et al., 2014). The results showed that *Lumbricus rubellus* (epi-endogeic) consumed leaf and root litter, preferring root litter, and incorporating the plant litter C into

Table 5
Statistics of ANOVA for the $\delta^{13}C$ signal of earthworm tissues, earthworm casts and soil (0–10 cm). Same comment as Table 3.

			Earthworm tissues		Earthworm casts		Soil (0–10 cm)	
Source	numDF	denDF	F-value	P-value	F-value	P-value	F-value	P-value
Trt	1	2	325.62	< 0.01	59.51	0.02	237.31	< 0.01
Phase	1	14	< 0.01	0.99	2.36	0.15	0.14	0.71
Season	1	14	0.92	0.35	0.20	0.66	1.01	0.33
Trt:Phase	1	14	0.03	0.87	1.87	0.19	0.22	0.65
Trt:Season	1	14	0.63	0.44	< 0.01	0.98	1.90	0.19
Phase:Season	1	14	0.23	0.64	2.62	0.13	0.32	0.58
Trt:Phase:Season	1	14	0.11	0.74	0.07	0.79	0.69	0.42

new soil macroaggregates. These and other results from the site suggest that most C in SOM are likely derived from belowground C inputs and not leaf litter. We propose that *L. rubellus* and other members of the detrital food web consume and incorporate the root and leaf litter into SOM. Afterwards, it is consumed by *Diplocardia* spp. Needless to say that we cannot exclude the influence of C inputs from other sources, such as rhizodeposits or other specific pools of soil C (e.g. microbial biomass C, particulate organic matter). Because at the ORNL FACE site NPP is mostly influenced by fine-root production (Matamala et al., 2003; Norby et al., 2004; Iversen et al., 2008), we propose that the correlations with inputs of leaf litter and fine-root NPP found in this study are reflecting changes in NPP indirectly as a function of the time it takes for plant litter to become part of SOM that is preferably consumed by *Diplocardia* spp.

4.3. Implications of our findings for soil C at high [CO_2]

The increase of endogeic earthworms associated with higher NPP is directly relevant to understanding mechanisms of C incorporation into soils. At the ORNL FACE plantation, both Jastrow et al. (2005) and Norby and Zak (2011) reported an increase in soil C within the first 0–5 cm, the key mechanism being the formation of soil microaggregates. In a microcosm study, Sánchez-de León et al. (2014) found that the presence of *Diplocardia* spp. Increased the production of soil aggregates two to three-fold when compared to soils with no earthworms or no endogeic earthworms. We suggest that the positive response of *Diplocardia* spp. to increased fine-root and, to lesser extent, leaf litter productions caused by elevated [CO_2] in the ORNL FACE is a major cause of the increased C accrual resulting from increased soil aggregate formation. Our results suggest that earthworms can have a significant influence in promoting the C accrual observed at the ORNL FACE site through promoting physical protection in soil aggregates.

5. Conclusions

In this study we documented the response of earthworms to e[CO_2] conditions. We found an indirect response of native endogeic *Diplocardia* spp. densities to increased NPP promoted by e[CO_2] conditions. In addition, we found that plant tissues showed a partial "relaxation" of the ^{13}C signal towards the values of the a[CO_2] treatment, whereas soil and earthworms and their casts did not. Endogeic earthworms from *Diplocardia* spp. dominated the earthworm populations at the ORNL FACE site, and no significant representation of other ecological categories was found. Our results show the densities of endogeic earthworms respond to variations on belowground NPP over multiple years, which in turn can impact soil physical properties and the C transfer from detritus to soil.

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References

Blair, J.M., Parmelee, R.W., Lavelle, P., 1995. Influences of earthworms on biogeochemistry. In: Hendrix, P.F. (Ed.), Earthworm Ecology and Biogeography in North America. Lewis Publisher, Florida, pp. 127–158.

Bossuyt, H., Six, J., Hendrix, P.F., 2005. Protection of soil carbon by microaggregates within earthworm casts. Soil Biology and Biochemistry 37, 251–258.

Brussaard, L., Pulleman, M.M., Ouedraogo, E., Mando, A., Six, J., 2007. Soil fauna and soil function in the fabric of the food web. Pedobiologia 50, 447–462.

Bouché, M.B., 1977. Stratégies lombriciennes. In: In: Lohm, U., Persson, T. (Eds.), Soil Organisms as Components of Ecosystems Ecological Bulletins 25. pp. 122–133 Stockholm.

Coleman, D.C., 2008. From peds to paradoxes: linkages between soil biota and their influences on ecological processes. Soil Biology and Biochemistry 40, 271–289.

Coleman, D.C., Crossley Jr., D.A., Hendrix, P.F., 2004. Fundamentals of Soil Ecology, second ed. Elsevier, New York.

Coplen, T.B., 1996. New guidelines for reporting stable hydrogen, carbon, and oxygen isotope-ratio data. Geochimica et Cosmochimica Acta 60, 3359–3360.

Edwards, C.A., Bohlen, P.J., 1996. Biology and Ecology of Earthworms, third ed. Chapman & Hall, New York.

Frelich, L.E., Hale, C.M., Scheu, S., Holdsworth, A.R., Heneghan, L., Bohlen, P.J., Reich, P.B., 2006. Earthworm invasion into previously earthworm-free temperate and boreal forests. Biological Invasions 8, 1235–1245.

García, J.A., Fragoso, C., 2003. Influence of different food substrates on growth and reproduction of two tropical earthworm species (*Pontoscolex corethrurus* and *Amynthas corticis*). Pedobiologia 47, 754–763.

Gonzalez-Meler, M.A., Taneva, L., Trueman, R.J., 2004. Plant respiration and elevated atmospheric CO_2 concentration: cellular responses and global significance. Annals of Botany 94, 647–656.

Gonzalez-Meler, M.A., Rucks, J.S., Aubanell, G., 2014. Mechanistic insights on the responses of plant and ecosystem gas exchange to global environmental change: lessons from Biosphere 2. Plant Science 226, 14–21.

Groffman, P.M., Bohlen, P.J., 1999. Soil and sediment biodiversity. Bioscience 49, 139–148.

Hendrey, G.R., Ellsworth, D.S., Lewin, K.F., Nagy, J., 1999. A free-air enrichment system for exposing tall forest vegetation to elevated atmospheric CO_2. Global Change Biology 5, 293–309.

Hopkins, F., Gonzalez-Meler, M.A., Flower, C.E., Lynch, D.J., Czimczik, C., Tang, J., Subke, J.A., 2013. Ecosystem-level controls on root-rhizosphere respiration. New Phytologist 199, 339–351.

Iversen, C.M., Keller, J.K., Garten Jr., C.T., Norby, R.J., 2012. Soil carbon and nitrogen cycling and storage throughout the soil profile in a sweetgum plantation after 11years of CO_2-enrichment. Global Change Biology 18, 1684–1697.

Iversen, C.M., Ledford, J., Norby, R.J., 2008. CO_2 enrichment increases carbon and nitrogen input from fine roots in a deciduous forest. New Phytologist 179, 837–847.

James, S.W., 1999. Oligochaeta: megascolecidae and other earthworms from southern and midwestern North America. In: Dindal, D.L. (Ed.), Soil Biology Guide. John Wiley & Sons, Inc, New York, pp. 379–386.

Jastrow, J.D., Miller, R.M., Matamala, R., Norby, R.J., Boutton, T.W., Rice, C.W., Owensby, C.E., 2005. Elevated atmospheric CO_2 increases soil carbon. Global Change Biology 11, 2057–2064.

Kalisz, P.J., Wood, H.B., 1995. Native and exotic earthworms in wildland ecosystems. In: Hendrix, P.F. (Ed.), Earthworm Ecology and Biogeography in North America. Lewis Publisher, Florida, pp. 117–126.

Kim, D., Oren, R., Clark, J.S., Palmroth, S., Oishi, A.C., McCarthy, H.R., Maier, C.A., Johnsen, K., 2017. Dynamics of soil CO_2 efflux under varying atmospheric CO_2 concentrations reveal dominance of slow processes. Global Change Biology 23, 3501–3512.

Lavelle, P., 2002. Functional domains in soils. Ecological Research 17, 441–450.

Lavelle, P., Decaëns, T., Aubert, M., Barot, S., Blouin, M., Bureau, F., Margerie, P., Mora, P., Rossi, J.P., 2006. Soil invertebrates and ecosystem services. European Journal of Soil Biology 42, S3–S15.

Ledford, J., Norby, R.J., Tharp, M.L., 2008. ORNL FACE Fine Root Data. Carbon Dioxide Information Analysis Center. United States Department of Energy, Oak Ridge National Laboratory, Tennessee, United States. Data available at: https://facedata.ornl.gov/ornl/ornl_data_plantresponse.html. http://cdiac.ornl.gov.

Lee, K.E., 1985. Earthworms: Their Ecology and Relationships with Soils and Land Use. Academic Press, New York.

Lynch, D.J., Matamala, R., Iversen, C.M., Norby, R.J., Gonzalez-Meler, M.A., 2013. Stored carbon partly fuels fine-root respiration but is not used for production of new fine roots. New Phytologist 199, 420–430.

Matamala, R., Gonzalez-Meler, M.A., Jastrow, J.D., Norby, R.J., Schlesinger, W.H., 2003. Impacts of fine root turnover on forest NPP and soil C sequestration potential. Science 302, 1385–1387.

Norby, R.J., Ledford, J., Reilly, C.D., Miller, N.E., O'Neill, E.G., 2004. Fine-root production dominates response of a deciduous forest to atmospheric CO_2 enrichment. Proceedings of the National Academy of Sciences 101, 9689–9693.

Norby, R.J., Wullschleger, S.D., Hanson, P.J., Gunderson, C.A., Tschaplinski, T.J., Jastrow, J.D., 2006. CO_2 Enrichment of a deciduous forest: the Oak Ridge FACE experiment. Ecological Studies 187, 231–252.

Norby, R.J., Warren, J.M., Iversen, C.M., Medlyn, B.E., McMurtrie, R.E., 2010a. CO_2 enhancement of forest productivity constrained by limited nitrogen availability. Proceedings of the National Academy of Sciences 107, 19368–19373.

Norby, R.J., Iversen, C.M., Childs, J., Tharp, M.L., 2010b. ORNL Net Primary Productivity Data. Carbon Dioxide Information Analysis Center. United States Department of Energy, Oak Ridge National Laboratory, Oak Ridge, Tennessee. Data available at: http://facedata.ornl.gov/ornl/npp_98-08.html. http://cdiac.ornl.gov.

Norby, R.J., Zak, D.R., 2011. Ecological lessons from free air CO_2 enrichment (FACE) experiments. Annual review of Ecology, Evolution and Systematics 42, 181–203.

Norby, R.J., Todd, D.E., Fults, J., Johnson, D.W., 2001. Allometric determination of tree growth in a CO_2-enriched sweetgum stand. New Phytologist 150, 477–487.
Pataki, D.E., Ellsworth, D.S., Evans, R.D., Gonzalez-Meler, M., King, J., Leavitt, S.W., Lin, G., Matamala, R., Pendall, E., Siegwolf, R., Van Kessel, C., Ehleringer, J.R., 2003. Tracing changes in ecosystem function under elevated carbon dioxide conditions. BioScience 53, 805–818.
Riggs, J.S., Tharp, M.L., Norby, R.J., 2009. ORNL FACE CO2 and Weather Data. Carbon Dioxide Information Analysis Center. U.S. Department of Energy, Oak Ridge National Laboratory, Oak Ridge, Tennessee. https://facedata.ornl.gov/ornl/ornl_data_co2weather.html.
Sánchez-de León, Y., Lugo-Pérez, J., Wise, D.H., Jastrow, J.D., Gonzalez-Meler, M.A., 2014. Aggregate formation and carbon sequestration by earthworms in soil from a temperate forest exposed to elevated atmospheric CO_2: a microcosm experiment. Soil Biology and Biochemistry 68, 223–230.
Schmidt, O., 1999. Intrapopulation variation in carbon and nitrogen stable isotope ratios in earthworm *Aporrectodea longa*. Ecological Research 14, 317–328.
Scullion, J., Smith, A.R., Gwynn-Jones, D., Jones, D.L., Godbold, D.L., 2014. Deciduous woodland exposed to elevated atmospheric CO_2 has species-specific impacts on anecic earthworms. Applied Soil Ecology 80, 84–92.
Schulze, E.D., 2006. Biological control of the terrestrial carbon sink. Biogeosciences 3, 147–166.
Soil Survey Staff, 2018. Natural Resources Conservation Service. United States Department of Agriculture Web Soil Survey. Available online at: the following link. https://websoilsurvey.sc.egov.usda.gov/ Accessed [Feb/23/2018].
Taneva, L., Pippen, J.S., Schlesinger, W.H., Gonzalez-Meler, M.A., 2006. The turnover of carbon pools contributing to soil CO_2 and soil respiration in a temperate forest exposed to elevated CO_2 concentration. Global Change Biology 12, 983–994.
Valckx, J., Govers, G., Hermy, M., Muys, B., 2011. Optimizing earthworm sampling in ecosystems. In: In: Karaca, A. (Ed.), Biology of Earthworms, Soil Biology 24. Springer-Verlag, Berlin, pp. 19–38.
Walker, A.P., Hanson, P.J., De Kauwe, M.G., Medlyn, B.E., Zaehle, S., Asao, S., Dietze, M.C., Hickler, T., Huntingford, C., Iversen, C.M., Jain, A.K., Lomas, M., Luo, Y., McCarthy, H.R., Parton, W.J., Prentice, I.C., Thornton, P.E., Wang, S., Wang, Y.P., Wårlind, D., Weng, E.S., Warren, J.M., Woodward, F.I., Oren, R., Norby, R.J., 2014. Comprehensive ecosystem model-data synthesis using multiple datasets at two temperate forest free-air CO_2 enrichment experiments: model performance at ambient CO_2 concentration. Journal of Geophysical Research: Biogeosciences 119, 937–964.
Zaborski, E.R., 2003. Allyl isothiocyanate: an alternative chemical expellant for sampling earthworms. Applied Soil Ecology 22, 87–95.