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The effect of long-term CO_2 enrichment on carbon and nitrogen content of roots and soil of natural pastureland

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Abstract

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Increasing levels of atmospheric CO_2 may change C and N dynamics in pasture ecosystems. The present study was conducted to examine the impact of four years of CO_2 enrichment on soil and root composition and soil N transformation in natural pastureland. Plots of open-top growth chambers were continuously injected with ambient CO_2 (350 μL L^{-1}) and elevated CO_2 (625 μL L^{-1}). Soil cores exposed to ambient and elevated CO_2 treatment were incubated and collected each year. Net N-mineralization rates in soil (NH_4^+-N plus NO_3^--N), in addition to total C and N content (%) of soil and root tissues were measured. Results revealed that elevated CO_2 caused a significant reduction in soil NO_3 ($P < 0.05$), however, no significant CO_2 effect was found on total soil C and N content (%). Roots of plants grown under elevated CO_2 treatment had higher C/N ratios. Changes in root C/N ratios were driven by changes in root N concentrations as total root N content (%) was significantly reduced by 30% ($P < 0.05$). Overall, findings suggest that the effects of CO_2 enrichment was more noticeable on N content (%) than C content (%) of soil and roots; elevated CO_2 significantly affected soil N-mineralization and total N content (%) in roots, however, no substantial change was found in C inputs in CO_2-enriched soil.

Keywords

CO_2 enrichment, C sequestration, pastureland, root C/N, soil C, soil N

Introduction

Atmospheric carbon dioxide (CO_2) concentrations have been steadily rising in the past few decades, mainly due to anthropogenic activities. Mean atmospheric CO_2 has increased from approximately 280 ppm, at the beginning of the industrial revolution, to a current concentration of more than 400 ppm and still rising as it is being daily fuelled by the continuing burning of fossil fuels and the destruction of forests (Blunder and Arndt, 2018). This ongoing CO_2 increase has direct and indirect impacts on structure and function of terrestrial ecosystems, particularly, on plant growth as well as plant tissue composition (Cha et al., 2017; Liu et al., 2018).

Several decades of experimental studies on the effect of CO_2 enrichment have provided us with valuable data

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that have improved our understanding of how terrestrial ecosystem would respond to the effect of elevated atmospheric CO_2. These data involve the responses of individual plant species and plant communities (Bazzaz, 1990; 1996; Curtis and Wang, 1998; Zak et al., 2000; Körner, 2003; Mathias and Thomas, 2021). It has been revealed that elevated atmospheric CO_2 has some direct effects on plant growth and development; these effects include quantitative and qualitative changes in above-ground structure (Robinson and Conroy,1999; Kimball et al., 2002; Long et al., 2004; Ainsworth and Long, 2005) and below-ground structure and soil processes (Fitter et al., 1997; Paterson et al., 1997; Zak et al., 2000; Reich et al., 2006; Terrer et al., 2016), involving changes in the plant chemical composition and litter decomposition (Cotrufo and Ineson, 1995; King et al., 1997; 2003).

Soil microorganisms regulate the dynamics of organic matter decomposition and hence, the availability of plant nutrients. Elevated atmospheric CO_2 may affect soil microorganisms indirectly through affecting root growth, rhizodeposition rate, and litter composition (Rogers et al., 1994; Paterson et al., 1997). Several studies have found that CO_2 induced changes in plant tissue composition (Cha et al., 2017; Volder et al., 2015; Liu et al., 2018) causing changes in the composition of rhizosphere and soil microbial activity; and hence, impacting C turnover and storage in soil (Barnard et al., 2005; Muller et al., 2009; Runion et al., 2009; Cha et al., 2017). Increasing CO_2 may increase the availability of labile C through exudation (Cheng, 1999) enhancing C flux from plants to soil (Diaz et al., 1993; Curtis at al., 1994), which may, in turn, stimulate soil microbial processes such as mineralization (Baggs et al., 2003). While several research studies have reported an enhancement in N mineralization under elevated CO_2 due to the stimulation of microbial SOM decomposition caused by enhanced C inputs via rhizodeposition (Rütting et al., 2010), some studies have found that elevated CO_2 caused a decrease in N mineralization (Gill et al., 2006; Reich et al., 2006), yet, neither nitrification nor N mineralization were changed by elevated CO_2 in some other experiments (Zak et al., 2003; Barnard et al., 2005). However, Rütting and Andresen (2015) explained that gross mineralization is unaffected in P-limited ecosystems and it can be stimulated in N-limited ecosystems.

CO_2 enrichment may cause changes in the composition of above as well as below-ground plant tissues, particularly, C and N concentrations (Cotrufo and Ineson, 1995; Wang et al., 2021). Plant tissue N was found to decrease in response to elevated atmospheric CO_2 even when soil N availability is high (Cotrufo et al., 1998), which may be attributed to the reduced ability of plants to assimilate nitrate under elevated CO_2 condition (Bloom et al., 2012; 2014). Altered composition of plant tissues may produce poor litter quality which was found to slow down or retard decomposition by soil microorganisms (Arnone and Hirschel, 1997; Runion et al., 2009; Cha et al., 2017) resulting in a noticeable decrease in microbial biomass and activity.

Intergovernmental panel on climate change suggests that terrestrial ecosystem could sequester more than 20% of anthropogenic CO_2 emissions due to the CO_2 fertilization effect and the enhancement of forest tree growth with CO_2 enrichment (Ipcc 2001, 2007). Soil C is the net effect of carbon inputs and decomposition and it is affected by several factors such as soil chemical and physical factors, litter quality and fertilization. Soils contain the largest amount of C in the terrestrial ecosystem, about twice the amount of C found in the atmosphere (Powlson et al., 2011). Carbon allocated belowground in temperate forests is considered the largest, ranging from 20% to 60% (Waring et al., 1998). Soil organic C is also considered the dominant storage sink in grassland and cropland as it holds about half of the C stored in the forests. Effects of rising atmospheric CO_2 on belowground processes; particularly, soil N and C has been receiving increased attention in the past two decades and substantial changes in C dynamics and the global C cycle have been noticed and reported as studies have shown that elevated CO_2 concentration stimulate photosynthesis and hence, increase net primary productivity of the plant and fine roots (King et al., 2001; Norby et al., 2004; Finzi et al., 2007; Dijkstra et al., 2008; Liu et al., 2018; Martens et al., 2020) causing an increase in the amount of C stored in trees and soil (Follett, 1993; Franzluebbers et al., 2010).

Grassland occupies much of the land area worldwide as it covers about one quarter of the Earth's land area; it includes native grasslands, meadows and pastures. In natural N-limited terrestrial ecosystems such as pasturelands, available soil N for plant uptake is mainly derived from soil N cycle, particularly, N mineralization. Studies suggest that soil C sequestration and plant N availability can be sustained if soil N cycle processes are stimulated by elevated CO_2 (Barnard et al., 2006; Müller et al., 2009; Rütting et al., 2010). However, the impact of elevated atmospheric CO_2 on soil N cycle and its feedback on plant N availability and C sequestration in pastureland is still not thoroughly comprehended. Understanding how belowground processes will be affected by elevated atmospheric CO_2 is becoming necessary to examine future responses of pasture ecosystem to the ongoing increase of atmospheric CO_2 and to predict how much carbon can be allocated and stored belowground in pastureland. Therefore, soil microbial processes, root C and N, as well as soil C and N should be carefully investigated.

The present experiment was conducted to examine the effects of CO_2 enrichment on soil N-mineralization as well as soil and root tissue composition in natural pastureland. The use of well-developed *in situ* technologies (Free Air Carbon Dioxide Enrichment (FACE) and Open Top Chambers (OTC)) have facilitated the investigation of the responses of natural vegetation such as pastureland to long-term CO_2 enrichment. This study was a part of an extensive network of OTC studies conducted to manipulate CO_2 levels and examine their effects on several terrestrial ecosystems around the world, particularly, herbaceous communities such as grasslands and pasturelands, for further details, see (Potvin and Vasseur, 1997; Vasseur and Potvin, 1998; Al-Traboulsi, 1999; Nowak et al. 2003; Kuzyakov et al., 2019). The objective of present study was to follow and investigate *in situ* changes in soil net N-

mineralization rates (NH_4^+-N plus NO_3^--N production) in addition to soil and root C and N content (%) in N-limited pastureland, in response to four years of CO_2 enrichment using OTC technology. We suggest that long exposure to high CO_2 concentration may affect soil and root composition, particularly, C and N content (%) and plant available N in soil. We hypothesize an increase in C/N ratios of CO_2 enriched roots due to the expected increase in C allocation to root tissues. We also hypothesize an enhancement in soil C accumulation and storage due to the possible increase in allocation of photosynthates belowground.

Materials and methods

The study site

The field site was an abandoned pasture located in Farnham, southern Québec, Canada (45°17' N, 72°59' W). The pasture vegetation was a mixture of 11 C_3 species including grasses such as *Poa pratensis* and *Phleum pratense* and dicots such as *Taraxacum officinale* and *Plantago major*. Before the start of this four-year experiment, the pasture site had been grazed by cows for 10 years, fertilizers were added yearly. *In situ* OTC technology involving four open-top growth chambers made of clear lexan (3 m in diameter and 2.4 m in height) were used. The air of the chambers was injected with pure carbon dioxide gas for four years. Two chambers were injected with ambient CO_2 (350 ± 20 μL L^{-1} CO_2), while the other two chambers were injected with elevated CO_2 (625 ± 100 μL L^{-1} CO_2). Two unenclosed circles of the same diameter were used to serve as field control plots to test for chamber effect. Monitoring of CO_2 injection was done by an infrared gas analyzer in conjunction with solenoid valves. CO_2 was sampled every 15 s from each chamber. Pure CO_2 was injected in the air stream of the chamber if CO_2 concentration was below 625. Each year, the experimental period lasted for one growing season, starting in mid-May and closing in the first week of October to avoid frost. Average minimum and maximum temperatures were recorded in and outside the chambers using air and soil probes. Throughout each growing season, water was not a limiting factor for pasture vegetation as average rainfall ranged between 550 and 700 mm during the season.

Soil sampling

To estimate root biomass, total C and N content (%) of roots and soil, and soil N mineralization (NH_4^+-N plus NO_3^--N production) in the field, *in situ* covered core method developed by Adams and Attiwill (1986) was used during the growing season of three years; the 1st, 2nd, and 4th year of the four-year experimental time. This technique was proved to give reliable estimates of N mineralization in the field (Knoepp and Swank, 1995). A total of 36 PVC tubes of 4 cm in diameter and 15 cm deep were used for each year examined to collect soil cores. A total of 6 tubes were inserted into the soil at six random locations in each plot at the beginning of the incubation period (May). Three tubes were immediately removed and kept frozen until analyzed, while the other three tubes were removed and placed back into the soil in the PVC tubes, and left in the field to incubate for three months. They were covered with thin plastic film to prevent rainfall from leaching any nitrogen mineralized. At the end of the incubation period (July), the tubes were collected, soil was removed and kept frozen until analyzed.

Root and soil measurements

Soil cores were taken from the cooler, thawed, and weighed to determine soil mass per volume (bulk density). Soil pH was also measured on a 1:1 (soil:water) slurry using a pH meter (pH 209, HANNA Instruments, Woonsocket, USA). Soil cores were hand-sorted to remove root material. Roots found in each core were gently washed, dried at 75 °C, and weighed to determine root biomass, then ground into a fine powder with a wiley mill. Samples of 5–10 mg of root powder were used to measure total C and N content of roots. Root samples were placed into tin cups. Total C content (%) and total N content (%) were determined by dry combustion using CHN elemental analyzer (ERBA, model EA 1108) at the laboratories of Biology Department at McGill University. Total C content (%) and total N content (%) of soil was also determined using the same method; soils were ground into a fine powder and 10–15 mg of soil powder from each soil core was used for this analysis. NO_3 and NH_4 were measured in each soil core with KCl extractions to estimate plant available N. A solution of potassium chloride (2.0 N KCl) was prepared by dissolving 149 g KCl in approximately 800 ml NH_4-free double-distilled water and diluted to 1 L. All flasks used were acid washed before use to remove any NH_4, NO_3 or any soluble C that was initially present. Five-grams of soil from each core sample was extracted to determine initial N in 50 ml of 2.0 M solution of KCl. All KCl soil extracts were shaken on a mechanical shaker for 30 minutes, then filtered through Whatman No. 42 filter paper and frozen. Samples were later analyzed for NH_4 and NO_3 with the Tecator automated analyzer at the Biodôme laboratory, Montréal, Québec. As net mineralization of nitrogen in field soil can be calculated by measuring changes in the mineral N content of undisturbed soil isolated inside the tubes. Mineralization was calculated by subtracting initial N (cores taken in May) from final N (incubated cores taken in July).

Statistical analysis

Data on soil cores (root biomass, soil pH, total soil C content (%), total soil N content (%), soil NO_3, soil NH_4, total root C (%), total root N (%) and C/N content) were analyzed with split-plot analysis of variance. Each variable and year was analyzed separately. CO_2 was the main plot factor and chamber (CO_2) was the main plot error; month (May and July for each of the three examined years) and its interactions with CO_2 were considered as sub-plot factors. All statistical analyses were carried out using the statistical package SPSS 20. $P = 0.05$ was accepted as the appropriate significance level in this analysis. Data were log

transformed where appropriate to meet normality. Type III sums of squares were used to account for missing data. To test for differences among ambient and elevated CO_2, and control plots, *a priori* contrasts were used to contrast ambient and elevated CO_2 for the CO_2 enrichment effect, and ambient and control plots for the chamber effect.

Results

Root biomass

CO_2 enrichment had no significant effect on roots growing within soil cores. There was no significant main effect of CO_2 nor of any interactions on root biomass of soil cores collected at the beginning and the end of the incubation period of each growing season throughout the time of the study.

Soil carbon and nitrogen dynamics

Neither CO_2 main effect nor CO_2 * time (year) interaction effect was found to be significant for total soil C content (%) examined in May and July for three years (Table 1). Although the effect was insignificant, however, across years, CO_2 enriched soils tended to have the lowest C content (%) (Fig. 1a). It was higher in field control plots, and the highest for soils exposed to ambient CO_2. CO_2 enriched soil had the lowest N content (%) in the last year of the experiment (0.14%) (Fig. 1b), however, neither CO_2 main effect nor CO_2 * time interaction effect was significant in N content of core soils (Table 1). When soil C/N was used as a dependent variable, no significant main effect of CO_2 nor of any interactions tested was significant (Table 1).

CO_2 main effect was significant in soil NO_3 ($P = 0.05$) with less NO_3 under elevated CO_2 than in the other two environments (Table 2). The contrast test that was performed as an a priori test (contrasts) showed significant differences between ambient and CO_2 enriched soils ($F = 40$, $P < 0.05$). The overall mean was 2.0 µg g^{-1} and 1.3 µg g^{-1} for ambient and elevated CO_2, respectively (Table 1). Thus, elevated CO_2 caused a statistically significant reduction of 35% in soil NO_3. An increase in soil NO_3 over time was also observed. NO_3 of enriched soil was 0.53 mg g^{-1} in the first year of the study, then increased to 2.2 mg g^{-1} in two-year time (Fig. 1c), however, it was the lowest compared with ambient and control soil over each of the three years examined. CO_2 enrichment had no effect on ammonification in any of the three years examined. Neither CO_2 main effect nor CO_2 * time interaction was significant in NH_4 content of soil cores examined (Table 2). There were also no significant effects of CO_2 on total nitrogen content of soil ($NO_3 + NH_4$) of all cores examined (Table 2).

A significant main effect of CO_2 was detected in soil pH (Table 2). Soil cores taken from ambient CO_2 plots had pH values with overall mean of 7.33 over the three years of the experiment. The pH of soils taken from elevated CO_2 plots was lower in the second year of the experiment (7.01); and was declined further to 6.81 the next year (Fig. 1d).

Total carbon and nitrogen content (%) of roots

No significant effects were found in total C content (%) of roots growing within cores collected at the beginning and the end of the incubation period in all the years examined (Table 1), however, roots grown under ambient and control condition tended to have higher C content (%) over all the years of the study, compared with those grown in CO_2 enriched soil. In the last year of the experiment, total C content percentage of roots examined for control, ambient and CO_2 enriched soil was 17, 12.2, and 8%, respectively (Fig. 2a). CO_2 main effect was significant in total root N content (%) ($F = 2.67$, $P < 0.05$). The contrast test showed significant differences between N content percentage of ambient CO_2 roots and CO_2 enriched roots ($F = 5.3$, $P < 0.05$). The overall mean was 0.63% DW and 0.89% DW for elevated and ambient CO_2 (Table 1). Across years, total root N content (%) was reduced under elevated CO_2 treatment by 30%. Total root N content (%) was the lowest in CO_2 enriched roots over the three years examined compared with control and ambient CO_2 (Fig. 2b).

A significant main CO_2 effect was detected in C/N ratio of core roots ($F = 6.4$, $P < 0.05$). Significant differences were observed between C/N ratios of ambient and CO_2 enriched roots collected at the end of the incubation period (July) ($F = 17.9$, $P < 0.05$). Roots grown in CO_2 enrichment

Table 1. Total Nitrogen content (%) and total Carbon content (%) of soil and plant roots, C/N ratio and soil NH_4 and NO_3 of control, ambient and elevated CO2 treatment. Each value represents the mean of 36 samples collected in July over a period of 4 years (first, second and fourth year). Data in bold represent statistically significant values. Standard deviations are given within parentheses.

	C (% DW)	N (% DW)	C/N	NH_4 (µg g^{-1})	NO_3 (µg g^{-1})	Total N (µg g^{-1})
I. Soil						
- Ambient CO_2	2.07 (0.5)	0.70 (0.06)	11.9 (1.7)	0.68 (1.3)	2.00 (1.1)	1.28 (1.4)
- Elevated CO_2	1.87 (0.5)	0.15 (0.05)	11.7 (0.5)	0.43 (2.2)	**1.30** (1.0)	0.72 (2.6)
- Control	2.05 (0.3)	0.16 (0.02)	12.3 (0.9)	0.39 (1.0)	1.77 (1.1)	1.34 (1.6)
II. Roots						
- Ambient CO_2	14.0 (6.6)	0.89 (0.7)	17.8 (3.1)			
- Elevated CO_2	13.4 (7.9)	**0.63** (0.3)	**21.0** (4.1)			
- Control	15.3 (8.2)	0.79 (0.3)	19.3 (5.1)			

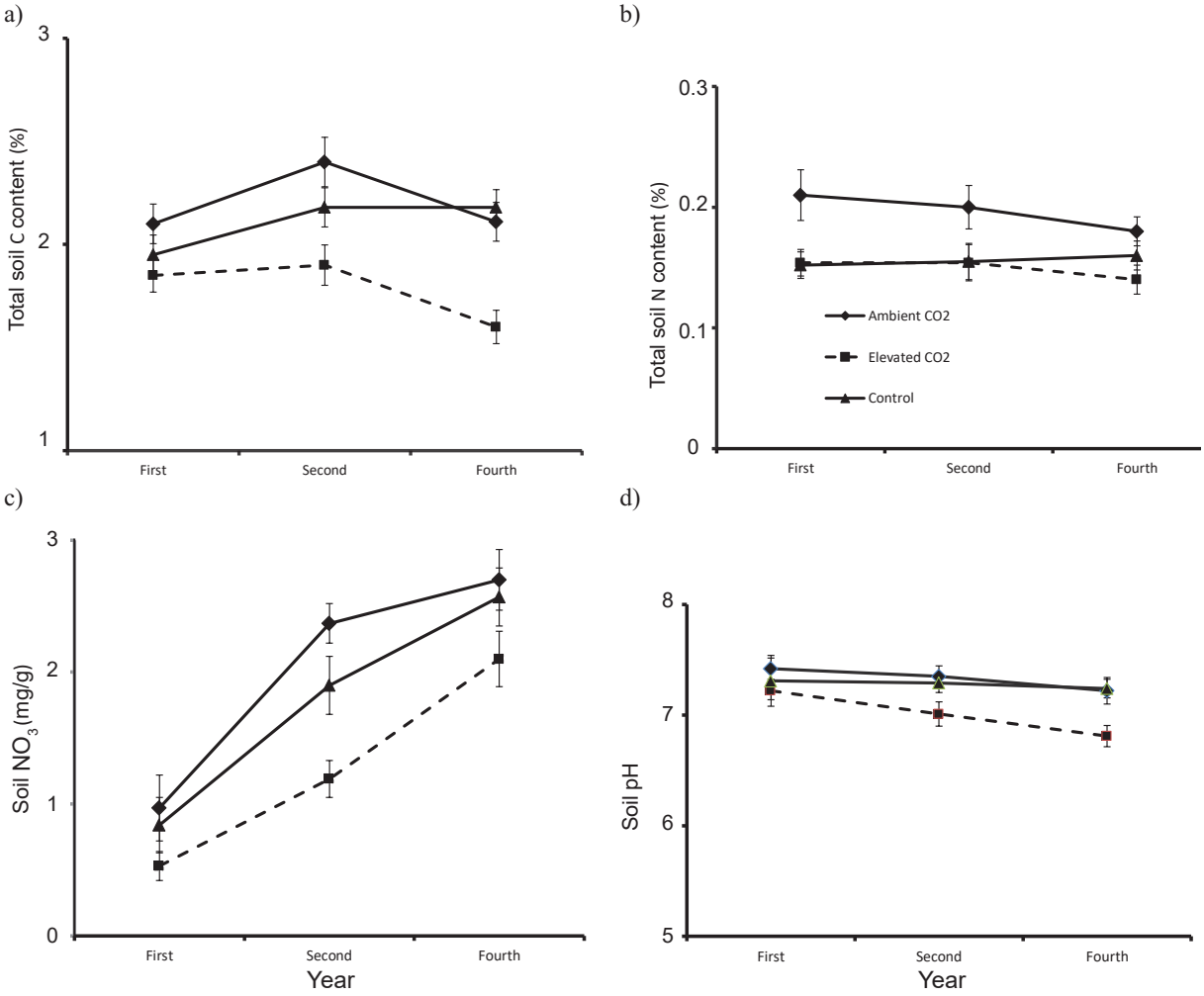

Fig. 1. Total C content (%) (a), total N content (%) (b), NO_3 (µg g^{-1}) (c) and pH (d) of soil samples collected from ambient CO_2, elevated CO_2 and control plots. In (a), (b) and (d), data are means of 6 samples per plot taken at the end of the incubation period (July) for each year. In (c), data are means of 12 samples per plot taken for each year. Net nitrification rate were calculated by subtracting initial NO_3, measured at the beginning of the incubation period (May), from final NO_3, measured at the end of the incubation period. Double standard errors of the means are shown where larger than the symbols.

had higher C/N ratio, an increase of 15% was found when compared with C/N ratio of ambient CO_2 roots. The overall mean was 19.3, 21 and 17.8 for control, elevated and ambient CO_2, respectively (Table 1).

Discussion

The ratio of total C/N is a reflection of the availability of carbon and nitrogen, yet, it is constrained by physiological limits on resource uptake (Koch, 1988). In the present experiment, roots of plants grown under high CO_2 concentrations had significantly higher C/N ratios than those grown under low CO_2 concentrations. This effect is most likely to have resulted from the statistically significant reductions in N concentrations in root tissues grown under elevated CO_2 as the total C content (%) of roots was unaffected by high CO_2 concentrations (Fig. 2b), this clearly indicates that the response of root tissue N to elevated CO_2 was faster than that of root C; it appears that four years of CO_2 enrichment had no significant effect on C allocation to root tissues as it was hypothesized. These findings are consistent with several studies that reported a change in the biochemical composition of plant tissues exposed to high levels of CO_2 (Cotrufo et al., 1998; Easlon and Bloom, 2013; Volder et al., 2015; Cha et al., 2017; Liu et al., 2018), with a noticeable reduction in the tissue N concentration (Cotrufo et al., 1998). An increase in C/N ratios under elevated CO_2 due to a reduction in plant tissue N was also recorded in several previous studies (Owensby et al., 1993; Curtis et al., 1989; Easlon and Bloom, 2013; Volder et al., 2015; Yuan and Chen, 2015; Cha et al., 2017).

The nature of the present study site is natural N-limited pasture grassland, and similar to several elevated CO_2 studies in N-limited grassland plants (Cotrufo et al., 1998; Gill et al., 2006; Volder et al., 2015), N content of pasture plant root was substantially decreased. This nutrient-limited condition of the site may have doubled the impact of

Table 2. Results of analysis of variance to determine the effect of elevated atmospheric CO_2 on soil NH_4, NO_3, ($NH_4 + NO_3$), and pH (n = 2). Values in bold represent significant differences and (n.s) represents non-significant effects.

Variable	Source	F	P
NH_4	CO_2	0.22	n.s
	Time	4.89	**0.013**
	CO_2 * Time	2.07	n.s
	Chamber (CO_2)	1.44	n.s
	Chamber *Time (CO_2)	1.35	n.s
NO_3	CO_2	2.77	**0.05**
	Time	15.76	**0.0001**
	CO_2 * Time	0.32	n.s
	Chamber (CO_2)	0.13	n.s
	Chamber *Time (CO_2)	0.40	n.s
NH_4+NO_3	CO_2	2.20	n.s
	Time	6.36	**0.0043**
	CO_2 * Time	1.68	n.s
	Chamber (CO_2)	2.99	**0.043**
	Chamber * Time (CO_2)	0.85	n.s
pH	CO_2	9.98	**0.0002**
	Time	3.81	**0.0043**
	CO_2 * Time	1.11	n.s
	Chamber (CO_2)	16.07	**0.0001**
	Chamber * Time (CO_2)	1.44	n.s

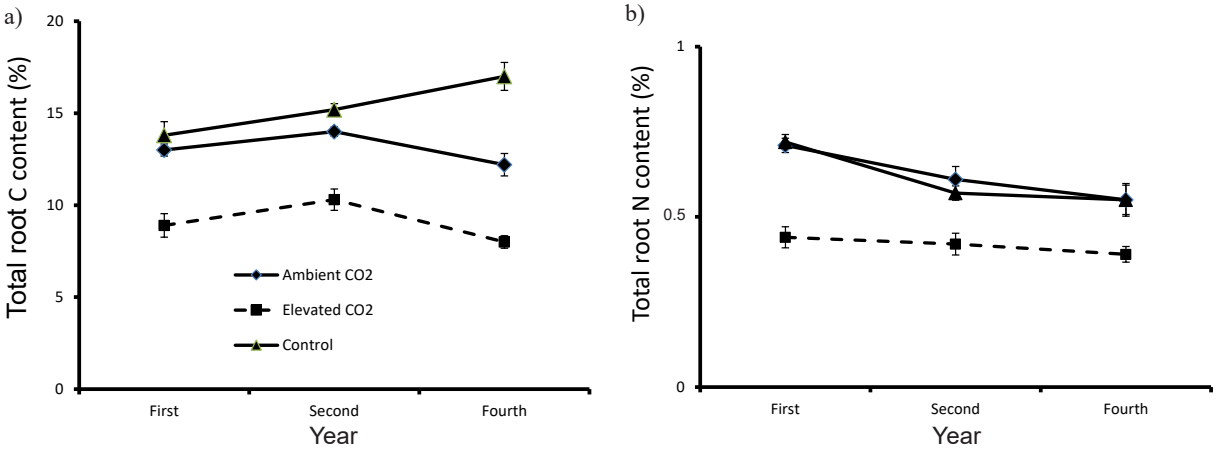

Fig. 2. Total C content (%) (a) and total N content (%) (b) of root samples collected from ambient CO2, elevated CO2 and control plots. Data are means of 6 samples per plot taken at the end of the incubation period (July) for each year. Double standard errors of the means are shown where larger than the symbols.

elevated CO_2 on plant tissue composition suggesting that root N concentration can be further reduced by elevated CO_2 under nutrient deficient condition. Similarly, Baxter et al. (1997) explained that grass plants showed a reduction in root N content when grown under elevated CO_2 and low nutrient supply as the availability of N in soils of grasslands can limit plant response to elevated atmospheric CO_2 (Schneider et al., 2004; Fargione et al., 2007). Our results are also consistent with the response of grass plants grown in nutrient-limited pastureland to elevated atmospheric CO_2 as Runion et al. (2009) noted that tissue N concentration of the plant tissue was reduced by 6% under elevated CO_2, while tissue C concentration was unaffected, which resulted in an increase in C/N ratio under high CO_2 treatment.

The decomposition of senesced plant litter represents

an important intermediate step in the cycling of nutrients between above and belowground systems. The rate of decomposition of plant litter is sensitive to fluctuations in environmental conditions, particularly to changes in the quality of litter (Ball, 1997). C/N ratios of plant material have been shown to be important in determining decomposition rates (Berg, 1984). The significant changes in root tissue composition, particularly N concentration, recorded in the present study, may have impacted the process of litter decomposition of this root material as C/N ratios of plant is crucial in determining the speed with which plant material will be decomposed (Runion et al., 2009). Our findings proposed that higher C/N ratio and lower N concentrations of plant tissues grown under elevated CO_2 treatment may have slowed or suppressed the decomposition of plant litter as the decline in N content of CO_2 enriched roots may have resulted in the production of litter of inferior quality and soil microorganisms usually show a clear preference for good quality litter, e.g., with high N content as it can be decomposed rapidly (Schlesinger and Bernhardt, 2013). Similar to our results, several studies have indicated that elevated CO_2 treatment could alter litter quality (Couteaux et al., 1991) and rhizosphere (Runion et al., 1994), adversely affecting microbial activity and slowing the decomposition rate (Overdieck and Reining, 1986; Bazzaz, 1990; Couteaux et al., 1991; Cotrufo and Ineson, 1995; Cha et al., 2017) particularly, under N-limited conditions (Ginkel and Gorissen, 1998). It is, therefore, possible to suspect that change in litter quality is likely to influence the activity of soil microorganisms with possible negative consequences for mineralization rate and hence, a decline in soil N. That may explain the statistically significant decline in NO_3 concentrations found in the CO_2 enriched soil (Fig. 1c). Soil acidification can be influenced by several environmental factors (Janík et al., 2014). In the present study, CO_2 enrichment caused a significant reduction in soil pH as CO_2 enriched soil had lower pH values than control soil (Fig. 1d). This reported decline in soil pH under elevated CO_2 may have also contributed to a further reduction in N mineralization rate, as it was found that N mineralization is stimulated when soil pH raises (Fu et al., 1987; Curtin et al., 1998; Zhao et al., 2017).

The possible reduction in N mineralization may have induced soil microorganisms to use recalcitrant N-rich C fractions of the soil to meet their N demands, causing C mineralization to increase while decreasing N mineralization. Soil microbial activity and microbial N sink were not investigated in the present study, however, the significant reduction in soil NO_3 (35%) and the observed gradual decline in soil NH_4 support the possibility that the increased microbial N-uptake may have acted to further reduce soil N. These findings substantiate those of Liu et al. (2018), who found that under CO_2 enrichment, the use of inorganic N by soil microorganisms was intensively increased causing a reduction in N content of soil and plant tissues. The significant pH reduction found in the CO_2 enriched soil may have stimulated the denitrification rate (Slmek and Cooper, 2002; Moser et al., 2018), which may, in turn, have acted to further enhance the decline in soil NO_3 concentration recorded in our experiment. Increased denitrification rate in CO_2 enriched soil (Arnone and Bohlen, 1998; Dijkstra et al., 2008) may be the result of the activation of denitrifiers by the enhanced root exudation (Rogers et al., 1998).

In pastureland, roots are considered the primary source of plant-derived organic C input to soils, both from turnover of dead material and release of exudation from growing roots (Farrar et al., 2003). These C inputs can be easily and quickly decomposed by soil microorganisms and their concentrations are likely to increase under elevated CO_2 levels (Paterson et al., 1996) as fine root production increases in some species at elevated CO_2 (Norby et al., 1986). In our study, roots were the primary means for carbon to enter the soil as the aboveground litter was removed and cleared every year during the experimental study time. It has been suggested that to enhance soil C sequestration, plants can be used to extract C from atmosphere and store it in their tissues then deposit it in the soil via their roots, increasing soil C input, which may help mitigate climate change effect (Follett, 1993) as soil can be a major C sink. An enhancement in C storage and sequestration in the CO_2 enriched soil was predicted and hypothesized in this study, however, our results revealed that elevated CO_2 concentrations had no effect in increasing the amount of stored C in the CO_2 enriched soils as no significant effect was found on total soil C content (%). This indicates that four years of CO_2 enrichment was not sufficient to induce significant changes in C inputs in soils of N-limited pasturelands. The amount of carbon stored in pasture soils was found to be largely varied as it can be influenced by a number of factors which may enhance or slow soil C sequestration. These factors include climate, soil type, soil depth, forage type, forage utilization, litter quality, land management and N fertilization (Franzluebbers et al., 2000; 2010) and the interactions between these factors and soil C storage is still largely not understood as they involve several complex multilevel microbial interactions (Fatichi et al., 2016).

Based on the results of the present experiment, it can be concluded that soil N-mineralization in N-limited pastureland is likely to change under elevated levels of atmospheric CO_2 as four years of CO_2 enrichment significantly decreased soil NO_3 concentrations. For roots, elevated CO_2 tended to decrease total root N content (%) resulting in increasing root C/N ratio which may have affected soil microbial activities. In contrast to what we hypothesized, CO_2 enriched soil appeared to have no potential to sequester C at the present time scale suggesting that soil C sequestration is a process that certainly require decades to be accurately assessed in natural highly disturbed pastureland. Additional long-term *in situ* experiments are required to examine how elevated atmospheric CO_2 can influence such processes in N-limited pastureland over a longer timescale.

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