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Trenching reduces soil heterotrophic activity in a loblolly pine (*Pinus taeda*) forest exposed to elevated atmospheric [CO_2] and N fertilization

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ABSTRACT

Forests return large quantities of C to the atmosphere through soil respiration (R_{soil}), which is often conceptually separated into autotrophic C respired by living roots (R_{root}) and heterotrophic decomposition (R_{het}) of soil organic matter (SOM). Live roots provide C sources for microbial metabolism via exudation, allocation to fungal associates, sloughed-off cells, and secretions such as mucilage production, suggesting a coupling between the activity of roots and heterotrophs. We addressed the strength of root effects on the activity of microbes and exo-enzymes by removing live-root-C inputs to areas of soil with a trenching experiment. We examined the extent to which trenching affected metrics of soil heterotrophic activity (proteolytic enzyme activity, microbial respiration, potential net N mineralization and nitrification, and exo-enzyme activities) in a forest exposed to elevated atmospheric [CO_2] and N fertilization, and used automated measurements of R_{soil} in trenched and un-trenched plots to estimate R_{root} and R_{het} components. Trenching decreased many metrics of heterotrophic activity and increased net N mineralization and nitrification, suggesting that the removal of root-C inputs reduced R_{het} by exacerbating microbial C limitation and stimulating waste-N excretion. This trenching effect was muted by N fertilization alone but not when N fertilization was combined with elevated CO_2, consistent with known patterns of below-ground C allocation at this site. Live-root-C inputs to soils and heterotrophic activity are tightly coupled, so root severing techniques like trenching are not likely to achieve robust quantitative estimates of R_{root} or R_{het}.

1. Introduction

Soil respiration (R_{soil}) is the primary path by which ecosystems lose C to the atmosphere, and is a globally important flux of ~75–80 Gt C yr^{-1} (Raich et al., 2002; Schlesinger and Andrews, 2000), roughly ten times the flux attributed to anthropogenic emissions (Raupach et al., 2007). Forests dominate this flux, contributing 50–60% of global R_{soil} (Raich et al., 2002). R_{soil} is often conceptually divided into autotrophic respiration of live roots (R_{root}) and heterotrophic decomposition (R_{het}) of soil organic matter (SOM; Hanson et al., 2000; Subke et al., 2006). Much C-cycling research has focused on physically separating root and microbial activity as components of total ecosystem C loss (i.e. contribution of R_{root} vs. R_{het} to R_{soil}) in an effort to better understand the environmental controls of these processes (Comstedt et al., 2011; Diaz-Pines et al., 2010; Hanson et al., 2000; Lavigne et al., 2004; Rey et al., 2002; Scott-Denton et al., 2006; Subke et al., 2006, 2011).

However, there is increasing evidence that a substantial portion of decomposition in forests is driven by inputs from rhizodeposition, suggesting that a sharp conceptual separation between root and microbial activity (i.e. R_{root} vs. R_{het}) may be unrealistic. Radiocarbon analyses demonstrate that the vast majority of R_{soil} is derived from relatively short-lived C compounds that represent a small portion of total soil C stocks (Trumbore, 2000) and that R_{het} in the mineral soil is dominated by recently fixed C attributable to root inputs (Cisneros-Dozal et al., 2006; Högberg et al., 2008). Girdling studies and time-lag analyses show a strong coupling between photosynthesis and R_{soil} that is at least partially driven by the use of recently produced photosynthate by soil microorganisms (e.g., Drake et al., 2008; Högberg et al., 2001; Johnsen et al., 2007; Stoy et al., 2007). Additionally, the presence of roots can strongly stimulate the loss of soil organic C in controlled pot studies (Cheng et al., 2003; Kuzyakov, 2010), and priming of decomposition is increasingly recognized as a driver of decomposition in

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intact forests (Drake et al., 2011; Janssens et al., 2010; Langley et al., 2009; Phillips et al., 2011). These relatively recent investigations follow a long history of coupled root–microbe research, particularly regarding symbioses such as mycorrhizal associations (reviews and syntheses by Johnson et al., 1997; Marschner and Dell, 1994; Read, 1991; van der Heijden et al., 1998; Wardle et al., 2004).

The purpose of this study was to evaluate the degree to which live-root-C inputs drive heterotrophic activity in a warm-temperate forest at the Duke free air CO_2 enrichment (FACE) site. We provide an independent evaluation of the hypothesis that live-root-C inputs accelerate N turnover from SOM by stimulating the activity of soil microbes and exo-enzymes, as recently proposed for this site by Drake et al. (2011) and Phillips et al. (2011). We used a trenching technique to remove live-root-C inputs from 1 m × 1 m areas and measured rates of mineral and organic N cycling, microbial respiration, and the activity of soil exo-enzymes in these trenched areas relative to unmanipulated reference areas. We also derived estimates of R_{root} and R_{het} from automated R_{soil} measurements in and outside of the trenched areas using methods common to trenching and girdling studies that assume no dependence of R_{het} on live-root-C inputs (Hanson et al., 2000).

2. Methods

2.1. Site

This experiment was performed at the Duke free air CO_2 enrichment (FACE) site, in Chapel Hill, NC, USA (35.97 N, 79.08 W). Duke FACE consisted of eight experimental plots 30 m in diameter. FACE technology (Hendrey et al., 1999) was used to maintain atmospheric [CO_2] at 200 ppm above ambient concentrations in four plots, while four control plots were exposed to ambient [CO_2] only. Ambient and elevated CO_2 plot pairs were grouped into blocks based on net N mineralization rates (Finzi and Schlesinger, 2002). These blocks captured spatial variation in soil fertility, leaf area index and net primary productivity across the site (Finzi et al., 2002; McCarthy et al., 2007, 2010) and thus enabled more sensitive statistical tests of treatment effects than a purely random design. Fumigation with CO_2 began in 1994 for block 4 (plots 7–8; Oren et al., 2001) and in 1996 for blocks 1–3 (plots 1–6; DeLucia et al., 1999). A CO_2 × N fertilization study was implemented in 2004 by fertilizing half of each plot with ammonium nitrate at a rate of 11.2 g N m^{-2} yr^{-1}. Fertilizer was hand-broadcasted in two applications in 2004 (half in March and half in April), and once annually in March thereafter. An impenetrable polyvinyl tarp buried vertically to 70 cm depth prevented N-fertilizer movement between plot halves.

2.2. Trenching treatment

We trenched one 1 m × 1 m area to 30–45 cm depth in each N treatment of each experimental plot in March 2010 (8 plots × 2 N treatments = 16 trenches total). The trenches were lined with landscaping cloth to enable lateral water movement but inhibit root ingrowth. We expected that this severed nearly all fine roots in the trenched areas, as the vast majority of fine roots at this site were present in shallow soils from 0 to 30-cm depth; soil sampling to 50-cm depth early in the experiment indicated that nearly 0% of fine roots were present in soils deeper than 30 cm (Matamala and Schlesinger, 2000). However, recent sampling to 90-cm depth in 2010 found more deep roots; 20% of the fine roots and 11% of the coarse roots were found in soils from 30 to 90-cm depth (Cook C.W. and Jackson R.B., *personal communication*), suggesting that while the trenches likely severed the majority of roots, some deep roots may have been unaffected.

2.3. Soil sampling and root biomass

Soil samples were collected inside and just outside of each trench on October 20, 2010. This sampling date was chosen because we had a large quantity of R_{soil} data from automatic chambers during this time period (described below, Sections 2.5 and 3.3). Additionally, this sampling date allowed seven months to elapse between trenching and soil sampling, which was chosen as a compromise between giving enough time for the severed fine roots to die, but sampling before the pulse of root litter began to artifactually affect decomposition. Limited space in the experimental plots precluded sampling soils multiple times for this experiment. A single 10 cm × 20 cm monolith of the organic horizon was collected in addition to three 4.5-cm-diameter soil cores of the top 15 cm of the mineral soil. These samples were transported to Boston University the day after sampling and processed the following day. The mineral soil cores from each plot were composited and sieved through 2-mm mesh. The roots remaining in the 2-mm-mesh sieve were separated by hand and categorized as fine (less than 2-mm diameter) or coarse (greater than 2-mm diameter) and live or dead. Live roots were identified as containing an intact cortex that made the root supple (i.e. the root could bend without breaking) with some tensile strength (i.e. the root could be gently pulled along its main axis without snapping). Roots in the organic horizon were separated by hand and similarly categorized.

2.4. Soil process rates

We measured a number of soil processes to evaluate the degree to which trenching influenced heterotrophic activity. We measured the activity of proteolytic enzymes, microbial respiration in lab incubations, potential net N mineralization and nitrification, and the activity of extracellular enzymes related to phosphorus mineralization (acid phosphatase, herein abbreviated as AP) and the decomposition of amino-sugars (β-1,4-N-acetyl-glucosaminidase, abbreviated as NAG), cellulose (β-1,4-glucosidase, abbreviated as BG), and lignin (peroxidase and phenol oxidase, abbreviated as PerOx and PhenOx, respectively).

Proteolysis was measured as the gross rate of increase in extractable free amino acid concentrations (measured using the OPAME method of Jones et al., 2002) over a 4-h incubation using 2 g of sieved soil as described previously (Brzostek and Finzi, 2011; Lipson et al., 1999; Watanabe and Hayano, 1995). Proteolytic rates, or the rate at which complex insoluble organic matter is broken down into soluble amino acid monomers, is increasingly recognized as a rate-limiting step for decomposition and a regulatory point for C and N cycling in soils (Schimel and Bennett, 2004). Three sub-replicates were measured per sample and averaged before statistical analysis.

The rate of CO_2 production by heterotrophs (i.e. microbial respiration) was measured in lab incubations; 20 g of sieved soil was placed in septum-sealed 500 mL glass jars and incubated at lab temperature (~23 °C). Headspace samples (10 mL) were taken three times at hourly intervals and injected into an infrared gas analyzer (Model EGM-4; PP-Systems, Amesbury, MA, USA) to determine the concentration of CO_2. These concentrations increased at a linear rate for all samples (minimum r^2 = 0.95, average r^2 = 0.99). The rate of CO_2 production per g dry soil was calculated from these slopes, the jar volume, soil mass, and soil gravimetric water content. These measurements were performed 1, 8, and 15 days after sieving. Rates of microbial respiration were stable and highly correlated across the first two measurement time points (r^2 = 0.85), but microbial respiration strongly declined for the third measurement, so the 1- and 8-day measurements were averaged and the 15-day measurements were excluded. Two sub-replicates were measured for each sample and averaged prior to statistical analysis.

Potential net N mineralization and nitrification were measured using a standard 28-day incubation at lab temperature (23 °C) by quantifying the change in 2 M KCl extractable pools of NH_4^+ and NO_3^- (Brzostek and Finzi, 2011; Finzi et al., 1998). The day following soil processing, 20 g of sieved soil was placed into 500 mL glass jars. These samples were either extracted immediately (to measure the initial pool sizes) or after 28 days (to measure the final pool sizes) by shaking with 100 mL of 2 M KCl for 1 h. Concentrations of NH_4^+ and NO_3^- were quantified using a flow injection analyzer (Lachat Quickchem 8000; Hach Company, Loveland, CO, USA). Three sub-replicates were measured for each sample and averaged prior to statistical analysis.

Extracellular enzyme activities were measured by mixing 1 g of soil with 100 mL of 50 mM sodium acetate buffer adjusted to pH = 5.0. The samples were continuously stirred and twenty-four 200 μL aliquots of the suspension were transferred to 96-well microplates; enzyme activities were subsequently measured exactly as described previously (Finzi et al., 2006) and expressed as nmol substrate utilized g^{-1} dry soil h^{-1}. Lignolytic enzymes (PerOx and PhenOx) were measured colorimetrically using L-dihydroxyphenylalanine (L-DOPA) substrate with a 4-h incubation; all other enzymes (AP, NAG, and BG) were measured fluorimetrically using substrates linked to a fluorescent tag (4-methylumbelliferone) with a 2-h incubation.

Extractable soil N pools were estimated for each sample. Mineral pools (NH_4^+ and NO_3^-) were estimated from the initial extractions for the potential net N mineralization and nitrification assays. The quantity of extractable amino acids was estimated from the initial concentrations measured for the proteolytic enzyme activity assays. Total extractable N was estimated as the sum of NH_4^+, NO_3^-, and amino acid pools.

2.5. Soil respiration measurements

We measured R_{soil} inside and just outside of each trench location using an automated CO_2 efflux system (ACES) developed by the United States Department of Agriculture Forest Service, Southern Research Station Laboratory (Butnor et al., 2003, 2005; Palmroth et al., 2005). The ACES open-path infrared gas analysis (IRGA) system made automated measurements of R_{soil} from 25-cm-diameter soil chambers. Each chamber was equipped with reflective covers, pressure equilibration ports, and air and soil thermocouples (soil temperature was measured at 5 cm depth). An ACES system was previously established in each of the eight experimental FACE plots (Oishi et al., submitted); we repurposed four chambers of each system for the measurements presented here. We placed a chamber inside and just outside each of the two trenches in each plot. We established two locations per chamber ~0.5-m apart (i.e. A and B locations), and moved the chambers every 3–4 days between locations to minimize precipitation and litterfall exclusion. Thus, continuous measurements of R_{soil} were recorded inside and outside of each trench, but the exact measurement location varied by ~0.5-m bi-weekly. Average monthly measurements of R_{soil} did not differ across the A and B locations (paired t-test, $p > 0.1$). Volumetric water content (θ) was measured using four frequency-domain reflectometry probes (CS615, Campbell Scientific, Logan, UT, USA) in the top 30 cm of mineral soil in each of the 8 plots in untrenched locations only. A number of equipment failures occurred in 2010, reducing the temporal span of R_{soil} measurements, particularly in the spring and early summer. We excluded days for which we did not have reliable data for at least four of the eight plots.

The quantity of R_{soil} attributable to the decomposition of fine roots within the trenched plots (i.e., a pulse of root litter) was modeled as a function of temperature by the compartment flow method (Santantonio and Grace, 1987) using the mean-residence times measured in a root decomposition experiment at this site (Matamala and Schlesinger, 2000) and the measured total fine root biomass. We modified the earlier approach which used a monthly time step (Matamala and Schlesinger, 2000) to a daily time step to estimate the quantity of R_{soil} caused by the extra decomposing roots in the trenched plots for each day, and subtracted this value from the R_{soil} measurements of the trenched plots. This correction was small in all cases and reduced the calculated R_{het} by an average of 5% (regression of corrected vs. not-corrected R_{soil} was: $y = 0.957x$, $p < 0.001$, $r^2 = 0.99$). This minor correction did not alter any of the statistical inferences or conclusions of this study. R_{het} and R_{root} were calculated using the following equations, which implicitly assume that R_{root} was reduced to zero in the trenched plots and that R_{het} was unaffected by trenching other than the slight increase in respiration from the pulse of decomposing fine roots:

$$R_{het} = (R_{soil} \text{ in trenched location}) - (\text{extra } R_{soil} \text{ from fine root decay}) \quad (1)$$

$$R_{root} = (R_{soil} \text{ in intact location}) - R_{het} \quad (2)$$

2.6. Compilation of R_{root}/R_{soil} estimates

We compiled all values of R_{root}/R_{soil} estimated for the ambient and elevated CO_2 treatments at the Duke FACE site, which included the estimates from non-drought conditions derived in this study and five other literature estimates. Unless otherwise noted, R_{soil} data for these literature estimates of R_{root}/R_{soil} were taken from a recently published re-analysis of survey measurements taken at monthly to twice-monthly intervals from 1996 to 2007 (Drake et al., 2011). Five R_{root}/R_{soil} estimates were derived from the literature. (1) Andrews et al. (1999) utilized the depleted $\delta^{13}C$ composition of the gas used for CO_2 fumigation to trace recent photosynthate in R_{soil}; this method did not rely on independent R_{root} and R_{soil} estimates. As there was no isotopic tracer in the ambient CO_2 treatment, Andrews et al. (1999) did not estimate R_{root}/R_{soil} for this treatment. (2) Matamala and Schlesinger (2000) measured oxygen consumption of cut roots obtained from soil cores to estimate R_{root}. (3) Schäfer et al. (2003) applied the R_{root} measurements of Matamala and Schlesinger (2000) to a new estimate of fine root biomass and modeled R_{soil} (no standard deviation was reported because R_{soil} was modeled). (4) George et al. (2003) estimated R_{root} by measuring the CO_2 production of intact roots placed into a standard gas-exchange cuvette in the field (model 6400-05 conifer chamber, Licor Biosciences, Lincoln, NE, USA). (5) Drake et al. (2008) estimated R_{root} by measuring CO_2 evolution in chambers buried around intact roots in the field. We did not compile estimates of R_{root}/R_{soil} in response to N fertilization, as there were no other available data for this site.

2.7. Statistical analyses

This experiment was a split–split plot randomized complete block design with four replicates. The main plot factor (CO_2 treatment) was split by a sub-plot factor (N fertilization), which was then split by a second sub-plot factor (trenching). Each split introduced a restriction on randomization (e.g., the N-fertilization study could only be applied within the confines of the existing CO_2 experiment), and thus each factor required a different error term and denominator degrees of freedom (ddfm) for proper statistical treatment. Otherwise, the ddfm for the CO_2 and N treatments would be inflated to match the higher ddfm of the trenching treatment, which would artificially increase the statistical power associated with these treatments, possibly causing type-I errors (Littell et al., 2006). The split-plot error terms were implemented by adding block × CO_2 and block × CO_2 × N terms to the RANDOM statement of the MIXED

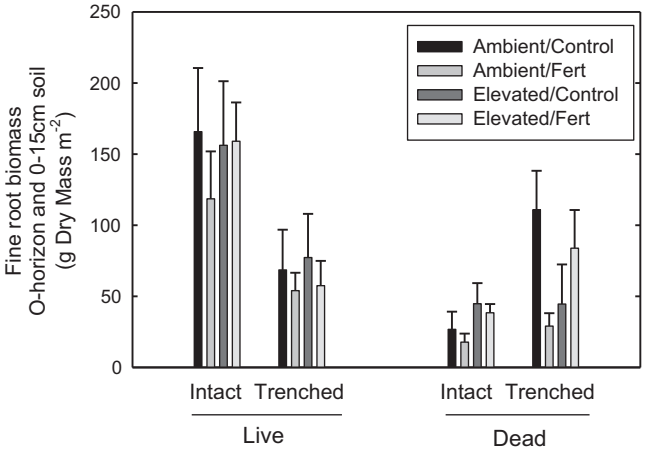

Fig. 1. Live and dead fine-root biomass in response to trenching, CO_2 enrichment (ambient or elevated), and N fertilization (control or fertilized) in a loblolly pine (*Pinus taeda*) forest. Values reflect the sum of the organic horizon and top 15 cm of mineral soil averaged across 4 replicate plots; error bars reflect ±1 SEM. Trenching reduced live and increased dead fine-root biomass ($p < 0.05$).

procedure within the SAS system (SAS 9.1; SAS Institute Inc., Cary, NC, USA). Blocks were treated as random factors, and ddfm were modeled using the Kenward–Roger procedure (Kenward and Roger, 1997). The MIXED procedure was used for all analysis except the linear regression in Fig. 2c, which was fit in Sigmaplot (SigmaPlot 10.0; Systat Software Inc., San Jose, CA, USA), and the log-normal distributions in Fig. 5, which were fit in the UNIVARIATE procedure in SAS. The assumptions that model residuals were normally distributed with mean zero and constant variance were met in all cases; no transformations were necessary.

3. Results

3.1. Root biomass

The trenching treatment was effective in reducing fine root biomass. Trenching reduced live fine root biomass by 57% (Fig. 1, $p < 0.01$). There were no other significant main effects or interactions for live fine root biomass (minimum $p = 0.40$); while previous studies have reported an increase in fine root biomass under elevated CO_2, our relatively limited sampling did not detect this effect (Jackson et al., 2009). Dead fine root biomass increased by 110% with trenching (Fig. 1, $p = 0.02$). There was a significant three-way interaction between CO_2, N fertilization, and trenching for dead fine root biomass; the effect of trenching was significantly stronger in the absence of either CO_2 or N fertilization treatments, or in the combination of elevated CO_2 and N fertilization, relative to the application of either treatment alone (interaction $p = 0.04$). Trenching decreased the total pool of fine roots (live + dead) by 28% ($p = 0.02$).

3.2. Soil process rates

In general, soil processes related to heterotrophic activity and decomposition were affected by N fertilization and trenching, while treatment with CO_2 had little effect. Proteolytic rates decreased by 25% with trenching (Fig. 2a, $p = 0.02$) and 50% with N fertilization ($p < 0.01$). Similarly, trenching and fertilization decreased microbial respiration by 15% (Fig. 2b, $p = 0.02$) and 32% ($p < 0.001$), respectively. There were no significant main or interactive effects of CO_2 treatment ($p > 0.1$) on proteolysis or microbial respiration. Rates of proteolysis and microbial respiration were correlated (Fig. 2c, $y = -0.0023x^2 + 0.42x - 8.8$, $p < 0.0001$, adjusted $r^2 = 0.50$). We used

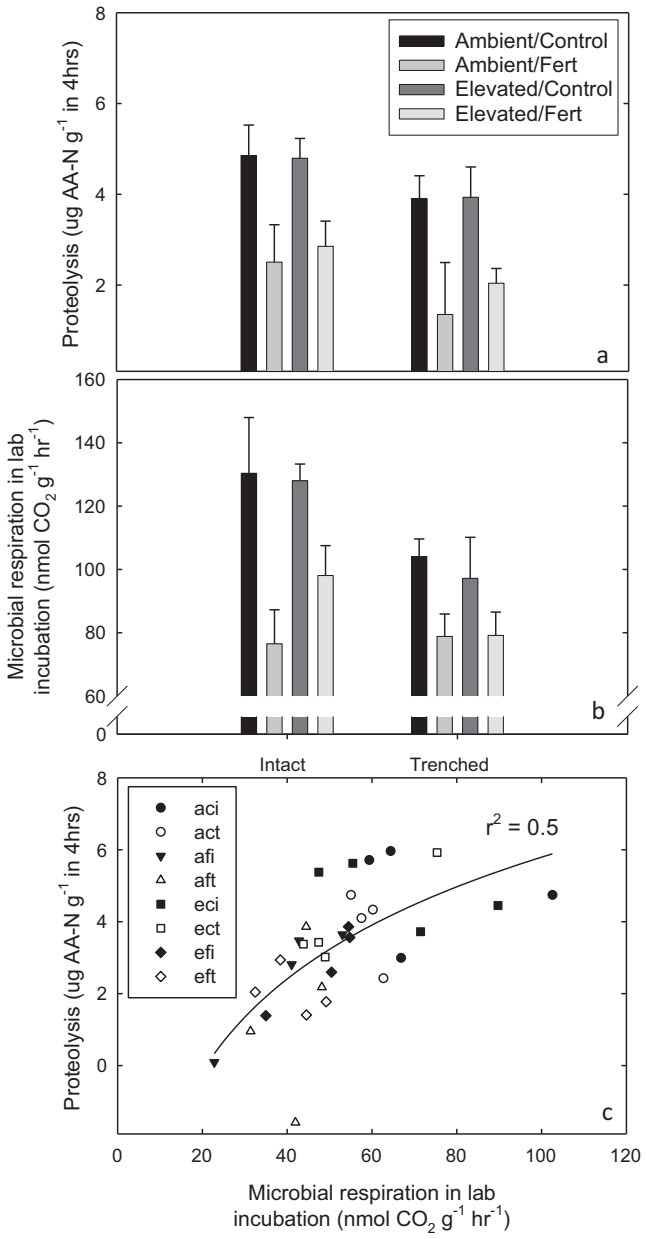

Fig. 2. Metrics of heterotrophic activity in the top 15 cm of mineral soil in response to trenching, CO_2 enrichment, and N fertilization in a loblolly pine (*Pinus taeda*) forest. Proteolysis, or the gross rate of free amino acid production (a), was significantly reduced by trenching and N fertilization ($p < 0.05$). The rate of microbial respiration in lab incubations (b) was similarly reduced by trenching and N fertilization ($p < 0.05$). Bars reflect the mean of 4 replicate plots and error bars reflect ±1 SEM. Proteolysis and microbial respiration were correlated (c); each value reflects a sub-plot of the 4 replicate plots. Treatments are abbreviated as: a, ambient CO_2; e, elevated CO_2; c, control N treatment; f, fertilized with N; i, intact soil; and t, trenched. The line shows the best fit ($y = -0.0023x^2 + 0.42x - 8.8$, $p < 0.0001$, adjusted $r^2 = 0.50$).

a second-order function to describe this relationship because it decreased the Akaike information criteria score (AIC values were 23.6 for the linear fit and 19.9 for the quadratic fit) and significantly increased the adjusted r^2 of the relationship relative to a linear function (linear $r^2 = 0.42$, F-test $p < 0.001$), both of which indicate a better fit with the quadratic equation.

Similar to the proteolytic enzyme observations, extracellular enzyme activities were generally affected by N fertilization and trenching, but not by CO_2 treatment (Table 1). Trenching reduced AP activity, although this effect was only marginally significant ($p < 0.1$). Trenching increased the activity of lignolytic enzymes, as

Table 1
Extracellular enzyme activities at the Duke free air CO_2 enrichment site. Values are the mean of four replicates (±1 SEM). AP releases inorganic P from organic P substrates; NAG releases glucosamine monomers from chitin and amino sugars; BG releases glucose monomers from cellulosic substrates; PerOx and PhenOx oxidize recalcitrant substrates such as lignin. Treatment effects on enzyme activities are shown across the bottom: T indicates a significant effect of trenching, N indicates an effect of N fertilization, and N × C indicates a significant interaction between N fertilization and CO_2 enrichment. Trenching marginally decreased AP and significantly increased PerOx; N fertilization marginally decreased NAG and decreased BG in the elevated CO_2 treatment only.

Treatments			Acid phosphatase (AP) (nmol g^{-1} h^{-1})	β-1,4-N-Acetyl-glucosaminidase (NAG) (nmol g^{-1} h^{-1})	β-1,4-Glucosidase (BG) (nmol g^{-1} h^{-1})	Peroxidase (PerOx) (nmol g^{-1} h^{-1})	Phenol oxidase (PhenOx) (nmol g^{-1} h^{-1})
CO_2	N	Trenching					
Ambient	Control	Intact	127 (18)	39 (5)	47 (8)	340 (48)	430 (76)
Ambient	Fertilized	Intact	122 (18)	27 (5)	44 (3)	290 (50)	480 (70)
Ambient	Control	Trenched	107 (14)	34 (5)	43 (5)	330 (45)	500 (74)
Ambient	Fertilized	Trenched	119 (12)	28 (5)	41 (4)	380 (80)	540 (154)
Elevated	Control	Intact	120 (21)	36 (10)	48 (5)	440 (85)	500 (63)
Elevated	Fertilized	Intact	124 (16)	26 (4)	30 (5)	390 (123)	430 (46)
Elevated	Control	Trenched	96 (18)	37 (5)	48 (11)	450 (79)	580 (138)
Elevated	Fertilized	Trenched	85 (9)	30 (6)	33 (4)	460 (114)	590 (121)
Significant effects			T‡	N‡	N, N × C	–	T

‡Marginally significant effect at $p < 0.1$.

PhenOx activity increased significantly ($p < 0.05$) and there was a non-significant trend of increasing PerOx activity with trenching ($p = 0.15$). N fertilization marginally decreased NAG activity ($p < 0.1$) and significantly decreased BG ($p < 0.05$). The decline in BG activity was larger under elevated CO_2 (CO_2 × trenching interaction, $p < 0.05$).

Potential net N mineralization and nitrification were significantly increased by trenching, but these effects were only present in the absence of N fertilization (Fig. 3). Net N mineralization increased with trenching (~70%, $p < 0.001$). However there was a significant interaction between trenching and N fertilization ($p < 0.01$); trenching stimulated N mineralization by ~180% in the non-fertilized subplots, whereas there was no significant effect in the fertilized subplots. Similarly, trenching significantly increased net nitrification (~130%, $p < 0.001$), with a larger effect in the non-fertilized subplots (~800%, $p < 0.001$) and no significant effect in the fertilized subplots, where nitrification rates were already high.

Variations in the concentration of different N forms in the soil (Table 2) were similar to variations in net N fluxes (Fig. 3) and proteolytic rates (Fig. 2a). Trenching significantly increased extractable NH_4^+ (~45%, $p < 0.01$) but this effect was larger in the non-fertilized subplots (~90%) relative to the non-significant change in N-fertilized subplots (~5%, trenching × fertilization interaction, $p = 0.01$). Trenching and N-fertilization increased the pool of extractable soil NO_3^- by ~25% and 100%, respectively ($p < 0.01$). Trenching decreased the pool of extractable amino acids by ~25% ($p < 0.01$). There were no significant treatment effects on the total of these three pools.

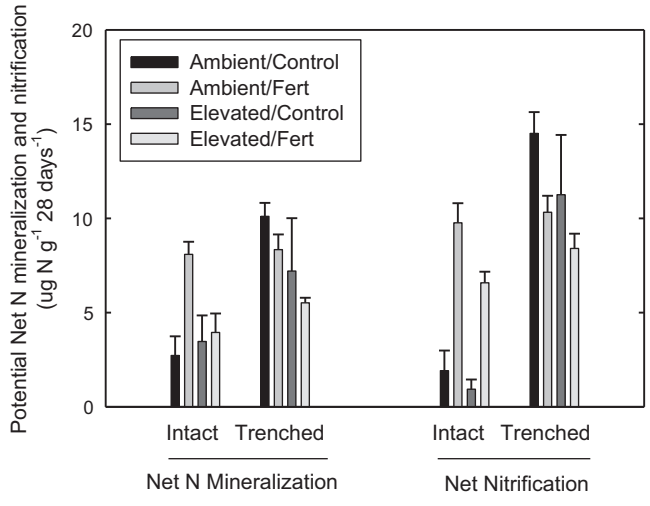

Fig. 3. Potential net N mineralization and nitrification in the top 15 cm of mineral soil from the Duke free air CO_2 enrichment (FACE) site, incubated for 28 days at 23 °C. Values reflect the mean of 4 replicate plots, and error bars reflect ±1 SEM. N fertilization and trenching significantly increased net N mineralization and nitrification ($p < 0.05$), although the effect of trenching was only significant in the absence of N fertilization (interaction, $p < 0.05$).

3.3. Soil respiration

Frequent equipment failures reduced the duration of reliable R_{soil} data, particularly prior to July for all treatments and during August for the elevated CO_2 treatments (Fig. 4a–d). However, we collected ~7000 daily average R_{soil} observations throughout the late summer and fall of 2010, with good coverage from August through December for the ambient CO_2 treatment (Fig. 4a and b) and from September through November for the elevated CO_2 treatment (Fig. 4c and d). This period of R_{soil} data overlaps with the sampling of soils from the field on the 20th of October.

There was a strong drought from July through September of 2010, and soil moisture levels were frequently below a volumetric water content (θ) of 0.2 m^3 m^{-3} (Fig. 4e), which was previously established as the threshold value below which soil water content begins to affect R_{soil} (Palmroth et al., 2005). This drought was followed by a series of storms that wet soils (θ up to 0.4 m^3 m^{-3}) in late-September (Fig. 4e). R_{soil} followed the same pattern as soil moisture, declining throughout the summer drought and increasing following the wet-up in late-September (Fig. 4a–d). It appears that R_{soil} in the intact areas was more strongly reduced by drought than in the trenched areas (Fig. 4a–d), however, it is possible that the lack of root water uptake in the trenched areas maintained a higher level of soil moisture relative to the intact areas. We did not measure soil moisture in the trenched areas. Thus these data do not necessarily indicate that R_{root} was more sensitive to drought than R_{het}.

Averaged across the month of October (when R_{soil} measurements are available for all treatments and soil water content was above drought levels), R_{soil} was significantly reduced by trenching in all treatment combinations, except for the N-fertilized, ambient CO_2 treatment (Fig. 4f; $p < 0.05$). Relative to untrenched reference locations, R_{soil} in the trenched areas was reduced by 46%, 16%, 42%, and 49%, in the treatment combinations of ambient CO_2 unfertilized, ambient CO_2 N-fertilized, elevated CO_2 unfertilized, and elevated CO_2 N-fertilized, respectively (Fig. 4f).

Table 2
Extractable soil N pools at the Duke free air CO_2 enrichment site. Values are the mean of four replicates (±1 SEM). Treatment effects on pool sizes are shown across the bottom: T indicates a significant effect of trenching, N indicates an effect of N fertilization, and T × N indicates a significant interaction. N fertilization increased nitrate, while trenching increased nitrate and reduced amino acids (AA). Trenching increased ammonium, but only in the absence of N fertilization. No treatment significantly affected the sum of these pools (total).

Treatments			NH_4^+ ($\mu g\,N\,g^{-1}$)	NO_3^- ($\mu g\,N\,g^{-1}$)	AA ($\mu g\,N\,g^{-1}$)	Total ($\mu g\,N\,g^{-1}$)
CO_2	N	Trenching				
Ambient	Control	Intact	2.1 (0.4)	3.7 (0.4)	9.8 (1.1)	15.7 (0.7)
Ambient	Fertilized	Intact	1.9 (0.5)	6.8 (1.0)	7.1 (1.0)	16.6 (0.6)
Ambient	Control	Trenched	4.5 (0.6)	4.2 (0.5)	6.9 (1.2)	16.7 (0.8)
Ambient	Fertilized	Trenched	2.1 (0.2)	9.2 (1.3)	6.7 (1.5)	18.6 (0.8)
Elevated	Control	Intact	2.6 (0.9)	3.5 (0.2)	9.0 (1.5)	16.3 (1.9)
Elevated	Fertilized	Intact	3.0 (0.5)	7.2 (1.4)	8.1 (5.4)	18.2 (2.4)
Elevated	Control	Trenched	4.2 (0.7)	4.3 (0.6)	7.5 (1.2)	15.8 (1.1)
Elevated	Fertilized	Trenched	3.1 (1.0)	8.5 (0.4)	5.6 (0.5)	18.8 (1.8)
Significant effects			T, T × N	N, T	T	–

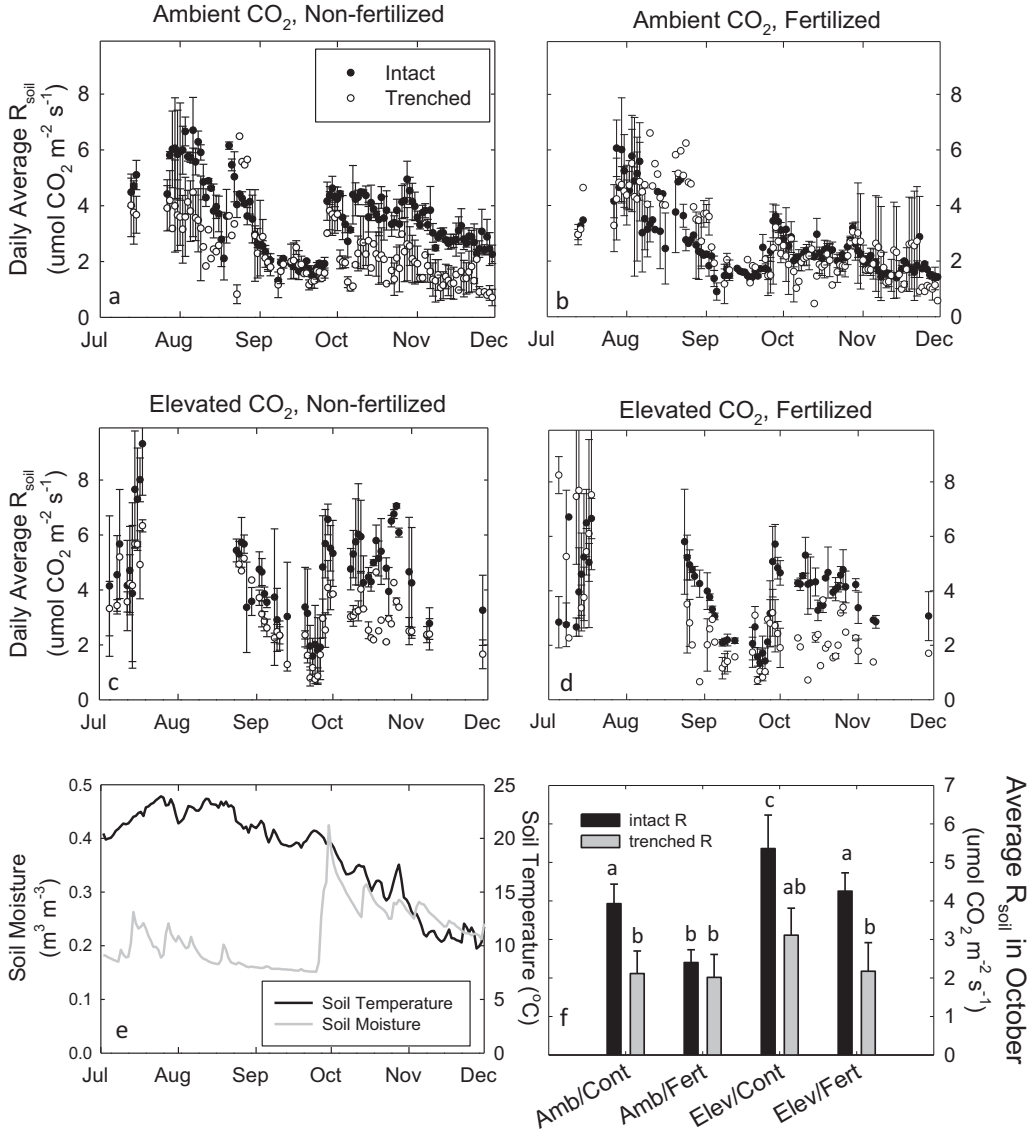

Fig. 4. Soil respiration (R_{soil}) in response to CO_2 enrichment, N fertilization, and trenching measured in the field at the Duke free air CO_2 enrichment (FACE) site (a–d). Daily average R_{soil} vales were averaged across plots (n = 2–4); error bars reflect ±1 SE of this mean. Abiotic conditions (e); soil temperature at 5-cm depth and volumetric soil moisture in the top 30 cm of mineral soil. Values were averaged for each experimental plot for each day, and the average across the 8 plots was presented. R_{soil} averaged across the month of October (f) shows the treatment means for the period of highest data overlap and non-drought conditions.

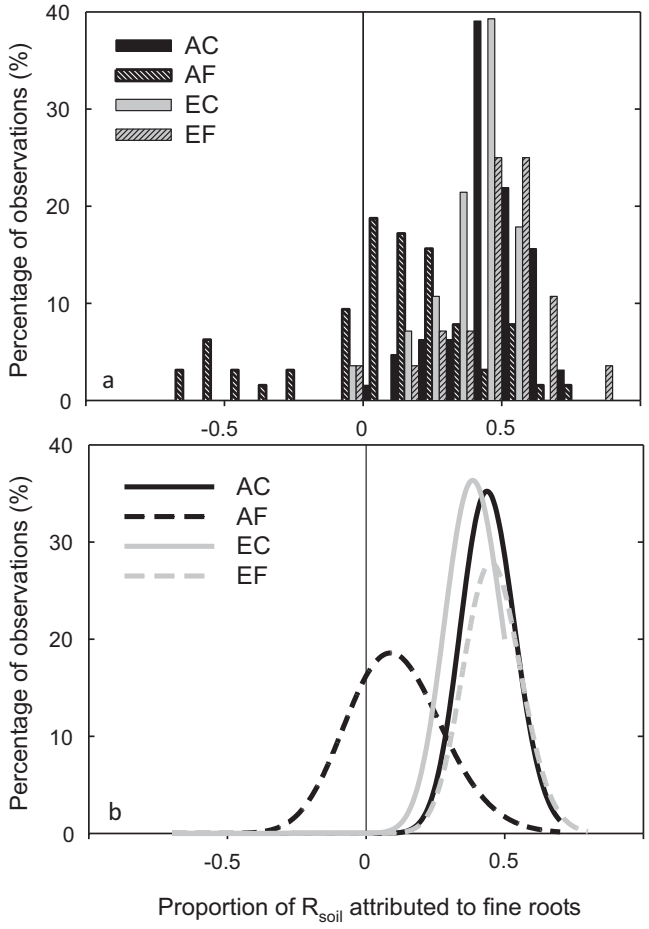

Fig. 5. Distributions of the fraction of soil respiration (R_{soil}) attributable to root respiration (R_{root}) in response to CO_2 enrichment and N fertilization at the Duke free air CO_2 enrichment (FACE) site. Treatments are abbreviated as follows: A, ambient CO_2; E, elevated CO_2; C, control N conditions; and F, fertilized with N. Periods of low soil moisture (less than 0.2 $m^3\ m^{-3}$) were excluded. Histograms of observed R_{root}/R_{soil} (a) were used to fit log-normal distributions (b).

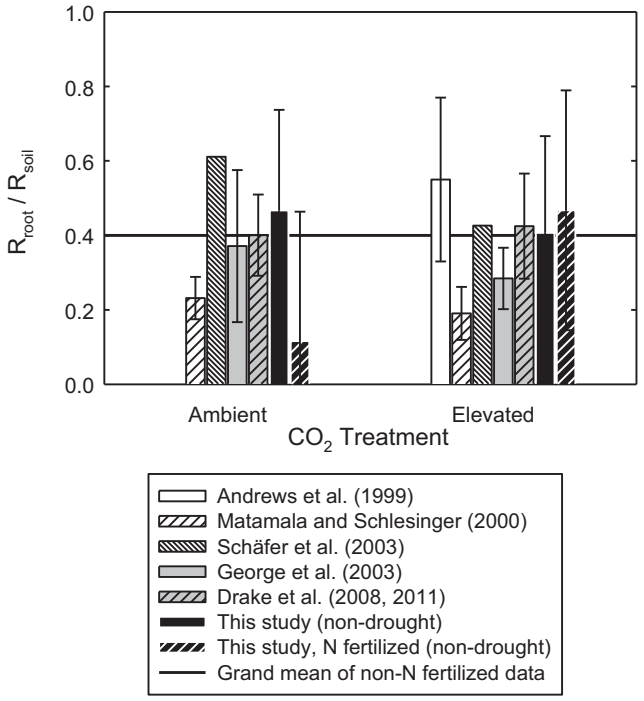

Fig. 6. Compilation of all R_{root}/R_{soil} estimates from the Duke free air CO_2 enrichment (FACE) site. Bars show the mean, error bars reflect the standard deviation, and the horizontal line reflects the grand mean.

The fraction of R_{soil} attributed to R_{root} tended to follow a log-normal distribution (Fig. 5), which is a normal distribution with a heavy tail. The peak of each distribution reflects the most commonly observed estimate of R_{root}/R_{soil} across the entire dataset. This peak (±1 SE) was 0.44 (±0.02) under ambient CO_2 without fertilization, 0.09 (±0.03) under ambient CO_2 with N fertilization, 0.38 (±0.02) under elevated CO_2 without fertilization, and 0.45 (±0.02) under elevated CO_2 with fertilization (Fig. 5b). Some low and negative R_{root}/R_{soil} values occurred at low soil moisture (September drought, Fig. 4) when R_{soil} was low and small changes in fluxes resulted in large changes in the calculated R_{root}/R_{soil} estimates. Thus, dates where θ was less than 0.2 $m^3\ m^{-3}$ were excluded from this analysis of R_{root}/R_{soil} distributions (Fig. 5). Even after filtering the R_{soil} data by θ, some measurements of R_{soil} in the trenched locations exceeded R_{soil} in the untrenched locations in the ambient CO_2 N-fertilized plots (Fig. 4b), leading to some negative calculations of R_{root}/R_{soil} in this treatment (Fig. 5). We do not suggest that root respiration was actually negative during these periods. Rather, we suggest that the distribution of R_{soil} fluxes were similar for the trenched and untrenched locations of the ambient CO_2 N-fertilized plots. Given the temporal and spatial variation in R_{soil}, occasionally a low R_{soil} flux from an intact location was paired with a high R_{soil} flux from a trenched location, resulting in a negative R_{root}/R_{soil} estimate. We did not remove these negative values to avoid positively biasing the mean R_{root}/R_{soil} estimate (Clark and Clark, 1999; Clark et al., 2007).

Across all reported estimates at the Duke FACE site, estimates of R_{root}/R_{soil} varied from 0.19 to 0.61, and the standard deviations for studies that provided error estimates were generally large (Fig. 6). The average estimate (±standard deviation) of R_{root}/R_{soil} for ambient and elevated CO_2 was 0.42 (±0.18) and 0.38 (±0.17), respectively. These estimates were similar across CO_2 treatments – the grand mean was 0.40 (±0.18). Fertilization with N substantially reduced R_{root}/R_{soil} in the ambient CO_2 treatment, but not in the elevated CO_2 treatment (Figs. 5b and 6).

4. Discussion

Trenching reduced fine root biomass and many metrics of heterotrophic activity, indicating that trenching affected R_{root} and R_{het}. Trenching reduced microbial respiration and the activity of proteolytic enzymes while increasing potential net N mineralization and nitrification, suggesting that microbes processed less C and excreted excess mineral N as waste. The repressive effect of trenching on heterotrophic activity was of similar magnitude as N fertilization, which consistently reduces measurements of heterotrophic activity at the Duke FACE site (Butnor et al., 2003; Palmroth et al., 2006; Phillips et al., 2011; Ziegler and Billings, 2011). Thus, trenching had a relatively large effect on heterotrophic activity, which is consistent with other studies illustrating the importance of live-root-C inputs in driving microbial activity (Brzostek and Finzi, 2011; Cheng et al., 2003; Jenkinson et al., 1985; Johnsen et al., 2007; Kuzyakov, 2010; Kuzyakov and Cheng, 2001; Kuzyakov et al., 2000; Langley et al., 2009; Phillips et al., 2011). A strong influence of live-root-C inputs on soil microbial activity suggests that variation in C allocation belowground can influence the availability of soil nutrients released through SOM decomposition, as has been suggested for this site (Drake et al., 2011; Phillips et al., 2011).

Trenching appears to have exacerbated microbial C limitation relative to the untrenched plots. This effect is likely driven by the cessation of rhizodeposition and allocation to the ectomycorrhizal fungi (ECM) that dominate the soil in this ecosystem (Parrent et al., 2006). When aboveground inputs of C to soil microbes were reduced, either by trenching or following reduced C-allocation belowground following N fertilization (Palmroth et al., 2006), the result was a decline in microbial respiration and proteolytic enzyme activities, with an increase in N mineralization. The large decline in proteolytic enzyme activity is particularly notable because ECM fungi produce proteolytic enzymes, take up organic N from the soil, and eventually transfer some N to host plants (Chalot and Brun, 1998; Hobbie and Colpaert, 2003). Several lines of evidence support the idea that microbial C limitation increased in the trenched plots. First, the consistent decrease in CO_2 production (Fig. 2) indicates that soil microbes were metabolizing less C in the trenched plots. Second, the increase in phenol oxidase activity, an enzyme that degrades recalcitrant forms of C in SOM, suggests a decline in the pool of labile C and N in the soil (Sinsabaugh, 2010). Third, the sharp increase in N mineralization and nitrification suggests that the supply of N to microbes was in excess of microbial demand, which typically occurs when C supply limits microbial activity (Anderson et al., 2005; Barraclough and Puri, 1995; Boersma and Elser, 2006; Robertson and Groffman, 2007). It is worth noting that not all enzymes responded similarly; trenching had no effect on NAG and BG. Nevertheless, the preponderance of the microbial and exo-enzymatic responses reflect increases in microbial C limitation with trenching.

A strong effect of live-root-C inputs on microbes is consistent with the literature. Brant et al. (2006) used phospholipid fatty-acid analysis (PLFA) to characterize the soil microbial communities in and outside of trenched plots in three different temperate forest types and found distinct microbial communities in the trenched plots, characterized by more actinomycete and fewer fungal biomarkers relative to un-trenched plots. Similarly, other root removal studies have documented shifts in microbial composition or function, suggesting that strong root effects on microbial activity are relatively common in forest ecosystems (Kaiser et al., 2010, 2011; Lindahl et al., 2010). While we have no data to address possible changes in microbial community composition following trenching, we recognize that altered community composition may explain some of the altered fluxes of C, N, and soil enzyme activities measured in this experiment.

The estimate of R_{root}/R_{soil} for non-drought conditions fell within the wide range of previous estimates from this site (Fig. 6). This suggests that the trenching-based estimate of R_{root}/R_{soil} gave the "right" answer despite artifacts related to a root-removal effect on R_{het}. One explanation may be that trenching introduced two compensating artifacts: trenching likely decreased R_{het} but probably did not totally eliminate R_{root}, as live root biomass was still present in the trenched plots (Fig. 1). Severed roots can remain alive for extended periods, determined by their initial content of stored carbohydrates and soil temperature (Marshall and Waring, 1985). We suggest that trenching reduced R_{het} but did not fully eliminate R_{root}, the combination of which reduced R_{soil} in the trenched plots. Thus, the absolute magnitude of the R_{root} and R_{het} estimates derived from trenching may not reflect in situ rates, even though our estimates ($R_{root}/R_{soil} \approx 0.3$–$0.4$) are similar to those reported at this site (Fig. 6) and in other trenching studies (reviewed by Subke et al., 2006). This uncertainty regarding the absolute magnitude of R_{root}/R_{soil} estimates from root removal techniques has been discussed previously, although most studies addressed uncertainties related to the pulse of root decomposition following trenching, not the loss of heterotrophic activity following the removal of live-root-C inputs (Bond-Lamberty et al., 2011; Comstedt et al., 2011; Diaz-Pines et al., 2010; Lee et al., 2003).

The negative estimates of R_{root}/R_{soil} for the ambient CO_2 plots fertilized with N are not realistic, as they imply a negative R_{root} rate (Fig. 5). The estimates of R_{root}/R_{soil} in this treatment were normally distributed with a mean just above zero (Fig. 5), indicating that R_{soil} was similar in the trenched and un-trenched areas for this treatment. Given the large variability in R_{soil} and allowing for sampling error, we suggest that it is reasonable to occasionally observe higher R_{soil} fluxes in the trenched relative to the un-trenched areas. Clearly, root respiration occurs in all treatments at Duke FACE (Drake et al., 2008). We suggest that these estimates of R_{root}/R_{soil} derived from trenching should not be interpreted as robust quantitative measures, but as indicators of tree C investment belowground. That is, a large reduction in R_{soil} in trenched areas relative to nearby intact areas is indicative of substantial tree C allocation belowground, likely to a number of mechanisms (e.g., root respiration, exudation, and allocation to fungal symbionts). The reduced R_{root}/R_{soil} estimates with N fertilization under ambient CO_2 (Fig. 5) are consistent with other observations of reduced C allocation belowground and R_{soil} following N fertilization at this site (Butnor et al., 2003; Drake et al., 2011; Palmroth et al., 2006) and in forests generally (Janssens et al., 2010).

Despite possible methodological concerns of trenching, the overall results of the Duke FACE experiment are clear. Extensive research at Duke FACE has demonstrated that elevated CO_2 increased R_{soil} by ~20% relative to ambient CO_2 without altering the ratio of R_{root}/R_{soil} (Fig. 6, Bernhardt et al., 2006; Butnor et al., 2003; Drake et al., 2011; Jackson et al., 2009; Palmroth et al., 2006) but see Oishi et al. (submitted). Thus, the increased R_{soil} under elevated CO_2 was likely caused by increases in R_{root} associated with the metabolic costs of maintaining greater root biomass under elevated CO_2 (Drake et al., 2008, 2011; Jackson et al., 2009) and by increases in R_{het} associated with greater rhizodeposition, exudation, and priming of SOM decomposition (Drake et al., 2011; Phillips et al., 2011). This study provides independent support for this body of literature by demonstrating that soil heterotrophic activity is dependent on live-root-C inputs at this site, suggesting that trees can exert a degree of control on decomposition rates and nutrient availability by modifying total belowground C flow in response to N requirements (Drake et al., 2011; Finzi et al., 2007; Palmroth et al., 2006).

This study is broadly consistent with the literature that surface N additions to forests (i.e. N fertilization and atmospheric N deposition) reduces belowground C allocation by trees and thus reduces SOM decomposition by depriving heterotrophs of live-root-C inputs (Allison et al., 2007; Janssens et al., 2010; Palmroth et al., 2006; Treseder, 2008). In this experiment, N fertilization reduced many metrics of heterotrophic activity (Fig. 2, Table 1), increased potential N mineralization (Fig. 3), and reduced R_{root}/R_{soil} to near zero in ambient CO_2 (Figs. 5–6). The combination of N fertilization and elevated CO_2 maintained R_{root}/R_{soil} at ~0.45. These results are consistent with a previous analysis of this site (Palmroth et al., 2006); elevated CO_2 shifts C allocation toward belowground C flow, N fertilization shifts allocation toward aboveground production, while the combination of elevated CO_2 and N fertilization treatments lead to proportional increases C flux above and belowground with no change in relative allocation. That is, the combination of elevated CO_2 and N fertilization provided trees with additional above- and below-ground resources, so the ratio of above vs. belowground C-flow did not change, although both were increased in absolute magnitude. Thus, temperate forest trees appear to adjust the magnitude of C "spent" belowground in response to the N required to support growth and the relative availability of soil N (Drake et al., 2011; Janssens et al., 2010; Palmroth et al., 2006).

5. Conclusions

Trenching reduced many metrics of heterotrophic microbial activity and SOM decomposition, suggesting that microbial activity was heavily subsidized by live-root-C inputs in this forest. The trenching effect was substantially reduced by N fertilization alone (Figs. 3, 4f and 6), suggesting that trees in this forest reduced belowground C allocation in response to N fertilization, but not in the combination of elevated CO_2 and N fertilization. The close coupling between live-root-C inputs and heterotrophic activity indicates that root removal techniques are unlikely to eliminate R_{root} without also affecting R_{het}.

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