Title

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Lignin decomposition is sustained under fluctuating redox conditions in humid tropical forest soils

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Abstract

Lignin mineralization represents a critical flux in the terrestrial carbon (C) cycle, yet little is known about mechanisms and environmental factors controlling lignin breakdown in mineral soils. Hypoxia is thought to suppress lignin decomposition, yet potential effects of oxygen (O_2) variability in surface soils have not been explored. Here, we tested the impact of redox fluctuations on lignin breakdown in humid tropical forest soils during ten-week laboratory incubations. We used synthetic lignins labeled with ^{13}C in either of two positions (aromatic methoxyl or propyl side chain C_β) to provide highly sensitive and specific measures of lignin mineralization seldom employed in soils. Four-day redox fluctuations increased the percent contribution of methoxyl C to soil respiration relative to static aerobic conditions, and cumulative methoxyl-C mineralization was statistically equivalent under static aerobic and fluctuating redox conditions despite lower soil respiration in the latter treatment. Contributions of the less labile lignin C_β to soil respiration were equivalent in the static aerobic and fluctuating redox treatments during periods of O_2 exposure, and tended to decline during periods of O_2 limitation, resulting in lower cumulative $C_\beta$mineralization in the fluctuating treatment relative to the static aerobic treatment. However, cumulative mineralization of both the C_β- and methoxyl-labeled lignins nearly doubled in the fluctuating treatment relative to the static aerobic treatment when total lignin mineralization was normalized to total O_2 exposure. Oxygen fluctuations are thought to be suboptimal for canonical lignin-degrading microorganisms. However, O_2 fluctuations drove substantial Fe reduction and oxidation, and reactive oxygen species generated during abiotic Fe oxidation might explain the elevated contribution of lignin to C mineralization. Iron redox cycling provides a potential mechanism for lignin depletion in soil organic matter. Couplings between soil moisture, redox fluctuations, and lignin breakdown provide a potential link between climate variability and the biochemical composition of soil organic matter.

Introduction

One of the primary functions of lignin in plant tissues is to shield cellulose and hemicelluloses from microbial attack, making lignin disruption prerequisite for the microbial decomposition and assimilation of most plant biomass (Kirk & Farrell, 1987; Haider, 1992; Hammel, 1997). Lignin is second only to these structural polysaccharides, cellulose, and hemicelluloses, as the most abundant organic substance in terrestrial ecosystems (ibid.). Thus, the decomposition of lignin strongly influences the terrestrial carbon (C) cycle both directly and indirectly. Numerous studies have examined the temporal dynamics of lignin turnover in forest floor litter (Melillo *et al*., 1989; Berg, 2000; Talbot & Treseder, 2011; Duboc *et al*., 2014), as well as the ultimate fate of lignin in soil organic matter (Baldock *et al*., 1997; Kiem & Kögel-Knabner, 2003; Grandy & Neff, 2008; Kögel-Knabner *et al*., 2008; Carrington *et al*., 2012; Kramer *et al*., 2012; Dümig *et al*., 2013). Less is known, however, about the biotic and abiotic factors that control rates of lignin degradation in mineral soil environments (Thevenot *et al*., 2010). Litter decomposition processes generate fine particulate matter that is typically enriched in lignin relative to initial litter substrates (Melillo *et al*., 1989; Baldock *et al*., 1997; Grandy & Neff, 2008), and much of this debris enters mineral soil horizons as a consequence of root senescence, and due to the bioturbation and burial of aboveground litter. Lignin and its derivatives were long thought to comprise a large fraction of soil organic matter (SOM) as a consequence of their relative biochemical recalcitrance (e.g., Bollag *et al*., 1997). Recent evidence has challenged this paradigm, suggesting that turnover rates of lignin are often similar or faster than those of bulk SOM, and implying that lignin may represent a minor component of stable soil organic matter (Baldock *et al*., 1997; Kiem & Kögel-Knabner, 2003; Grandy & Neff, 2008; Schmidt *et al*., 2011; Carrington *et al*., 2012). Other work has further emphasized the uncertainty of soil lignin dynamics by demonstrating that methodological artifacts may account for the apparent paucity of lignin in SOM reported in these studies, which typically examined lignin residues using cupric oxide oxidation (Hernes *et al*., 2013). These findings highlight the need for further examination of the mechanisms and environmental factors that control degradation of lignin-rich litter in mineral soils.

Studies of litter decomposition in terrestrial ecosystems routinely show that lignin decomposes slowly in comparison with other organic constituents, and that the presence of lignin ultimately limits rates of litter mass loss (Melillo *et al*., 1989; Berg, 2000; Talbot & Treseder, 2011). The initially slow turnover of lignin is linked to its molecular structure. Lignin is characterized by a heterogeneous, stereoirregular assemblage of phenylpropanoid subunits connected via stable ether and carbon–carbon bonds, which together comprise a hydrophobic, high molecular weight structure that cannot be cleaved by hydrolytic enzymes analogous to those that degrade other plant polymers (Kirk & Farrell, 1987; Hammel, 1997). Instead, lignin biodegradation is an

oxidative process of relatively low specificity and is thought to be restricted to a relatively narrow suite of microorganisms. These microbes primarily include a subset of filamentous fungi, but also some bacteria. The most efficient delignifiers are wood-degrading white-rot fungi that apparently attack lignin using secreted oxidative enzymes requiring molecular oxygen (O_2) or hydrogen peroxide (H_2O_2) as co-oxidants, while also employing various organic redox mediators and reactive oxygen species (Kirk & Farrell, 1987; Hammel *et al*., 2002; Baldrian, 2006; Hammel & Cullen, 2008; Bugg *et al*., 2011). The requirement for enzymatic and nonenzymatic oxidants to degrade lignin is consistent with observations that high molecular weight lignin decomposes very slowly, if at all, in constitutively anaerobic environments in wetlands and deep sediments (Zeikus *et al*., 1982; Benner *et al*., 1984; Kirk & Farrell, 1987).

In terrestrial ecosystems, O_2 is often abundantly available to drive decomposition of surface litter and coarse woody debris, yet O_2 depletion can potentially limit oxidative enzyme activities in surface horizons of mineral soils. A broad spectrum of soils exhibits O_2 limitation (hypoxia or anaerobiosis) over varying spatial scales, ranging from microsites in soil aggregates (mm–cm) to entire soil profiles (tens of cm) (Sexstone *et al*., 1985; Silver *et al*., 1999; Fimmen *et al*., 2008; Liptzin *et al*., 2011). Variation in O_2 availability can affect a wide range of biogeochemical process rates (Ponnamperuma, 1972; Silver *et al*., 1999; Davidson *et al*., 2012; Hall *et al*., 2014), and importantly, hypoxia is widely thought to retard lignin decomposition in soils and limit the growth of lignin-degrading fungi (Kirk & Farrell, 1987; Haider, 1992; Ekschmitt *et al*., 2008). Humid tropical forests provide an important example where a combination of warm temperatures, high rainfall, clay-rich soils, bioturbation, high biological O_2 demand, and large inputs of organic matter generates large spatial and temporal variation in surface soil O_2 availability. In these ecosystems, bulk gas-phase O_2 concentrations can vary between 0% and 21% over timescales of hours to weeks at shallow depths (e.g., 10 cm) (Silver *et al*., 1999; Liptzin *et al*., 2011). In spite of the potential impacts of O_2 limitation, however, lignocellulose typically decomposes rapidly in surface horizons of per-humid tropical forests, where roots are often completely depleted from buried litterbags in <12 months (Cusack *et al*., 2009). Furthermore, fine particulate matter accounts for a very small component of SOM in these ecosystems (Marin-Spiotta *et al*., 2008; Cusack *et al*., 2011a). These findings imply rapid degradation of lignocellulose (a dominant constituent of plant litter) in the mineral soil environment.

Wood-decaying fungi are prevalent in surface litter and organic horizons of humid tropical forests, yet few macrofungi grow exclusively in the mineral horizons where a substantial portion of lignocellulose is ultimately decomposed (Lodge & Cantrell, 1995; Meier *et al*., 2010). Rather,

bacteria dominate mineral horizons of humid tropical soils in terms of biomass and species richness, and few canonical lignin-degrading organisms are known to occur (Pett-Ridge & Firestone, 2005; Cusack *et al*., 2011b; DeAngelis *et al*., 2011, 2013). Previous studies suggested that bacteria dominate the decomposition of buried wood, resulting in slow breakdown of lignin relative to carbohydrates (Baldock *et al*., 1997). While a direct contribution of bacterial enzymes to lignin decomposition appears possible, additional mechanisms linked to the environmental dynamics and O_2 fluctuations characteristic of these soils may also provide important contributions to lignin breakdown.

Recent research has focused on identifying the mechanistic role of oxidative enzymes in lignin decomposition (Baldrian, 2006; Hammel & Cullen, 2008), but the potential for lignin degradation via other mechanisms has received less attention. It is known that some fungi can decompose lignocellulose indirectly, without the use of peroxidase or laccase enzymes, by generating reactive oxygen species such as hydroxyl radical ($^{\cdot}OH$) via Fe redox cycling (Cohen *et al*., 2002; Hammel *et al*., 2002; Yelle *et al*., 2011). Fungal enzymes and/or organic electron shuttles reduce Fe(III) to Fe(II), while separate enzymes produce hydrogen peroxide (H_2O_2); these species react to produce $^{\cdot}OH$, a strong oxidant capable of lignin decomposition, via the Fenton reaction ($H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + {}^{\cdot}OH$). Fenton chemistry has been studied and applied in wastewater treatment and bioremediation (Pignatello *et al*., 2006). It also has a recognized role in lignocellulose breakdown by fungi that cause brown rot of wood (Cohen *et al*., 2002; Hammel *et al*., 2002; Yelle *et al*., 2011). However, possible contributions of Fenton chemistry to organic matter oxidation in natural soils have only recently been noted (Hall & Silver, 2013).

Humid tropical forest soils are characterized by high rates of Fe redox cycling driven by a combination of rapid O_2 fluctuations, high rates of microbial metabolism, and abiotic reactions that could stimulate lignin decomposition indirectly by producing reactive oxygen species. In the absence of O_2, microbes in Fe-rich humid tropical soils can sustain high rates of anaerobic respiration via dissimilatory Fe reduction, producing Fe(II) at rates of tens to hundreds of μg Fe g soil^{-1} day^{-1} (Peretyazhko & Sposito, 2005; Chacon *et al*., 2006; Dubinsky *et al*., 2010). A broad spectrum of heterotrophic bacteria can couple anaerobic C oxidation directly or indirectly to Fe oxides via organic electron shuttles, generating Fe(II). Across a gradient of representative humid tropical soils (Oxisols, Ultisols, and Inceptisols), *in situ* Fe(II) concentrations remained at high and ecologically relevant concentrations (hundreds–thousands of μg Fe(II) g soil^{-1}) despite high rainfall, likely due to the high cation exchange capacity of these soils and the predominance of macropore flow, which typically preclude complete moisture saturation (Hall *et al*., 2013, 2014).

Reactive Fe remains abundant and ecologically relevant even in extremely wet, highly weathered, and leached tropical forest soils due to rapid Fe redox cycling and precipitation of short range-order mineral phases during Fe(II) oxidation (Thompson *et al*., 2011), thus decreasing the removal of Fe^{2+} by O_2-rich water. When O_2 encounters an anaerobic microsite via diffusion or advection, Fe(II) is vulnerable to abiotic and biotic oxidation (Ahmad & Nye, 1990; Dubinsky *et al*., 2010), although abiotic oxidation typically dominates in cases of high O_2fluxes (Druschel *et al*., 2008). Abiotic reactions between Fe(II) and O_2 alone are sufficient to produce reactive oxygen species: Fe(II) reacts with O_2 to produce superoxide ($O_2^{\cdot-}$), which protonates to form hydroperoxyl radical ($HO_2^{\cdot}$). These oxyradicals then dismutate or oxidize additional Fe(II) to produce H_2O_2 (McKinzi & DiChristina, 1999), which then drives Fenton chemistry via further reactions with Fe(II). Additional Fe(II) to drive these reactions is then regenerated via microbial reduction of Fe(III) in a subsequent anaerobic phase, if the soil is experiencing periodic O_2 fluctuations. This cyclic production of reactive oxygen species could contribute to lignin decomposition with or without additional contributions from canonical lignin-degrading organisms. Reactions between H_2O_2 and reduced humic substances, which can serve as electron donors analogous to Fe(II), provide a similar mechanism for $^{\cdot}OH$ production (Page *et al*., 2012).

Several previous studies used ^{14}C-labeled synthetic or plant-derived lignins to examine rates of lignin breakdown under laboratory conditions and the relative mineralization of different lignin functional groups over time (Kirk *et al*., 1975; Martin & Haider, 1979; Haider & Martin, 1981; Martin *et al*., 1982; Stott *et al*., 1983). Kirk *et al*. (1975) found that methoxyl ($-OCH_3$) functional groups were usually mineralized most rapidly, aliphatic side-chain carbons less rapidly, and aromatic carbons yet more slowly (see Fig. 1 for an illustration of a representative lignin macromolecule). In the remainder of the above studies, side-chain and aromatic ring C from lignin macromolecules were mineralized at similar rates. Importantly, the depolymerization of lignin macromolecules appeared to control rates of lignin degradation, as lignin-related monomers added to soils were mineralized at much greater rates than high molecular weight lignins (Haider & Martin, 1981). However, these studies were all conducted under static aerobic conditions, and the influence of environmental factors on lignin mineralization received little attention, aside from soil type and pH.

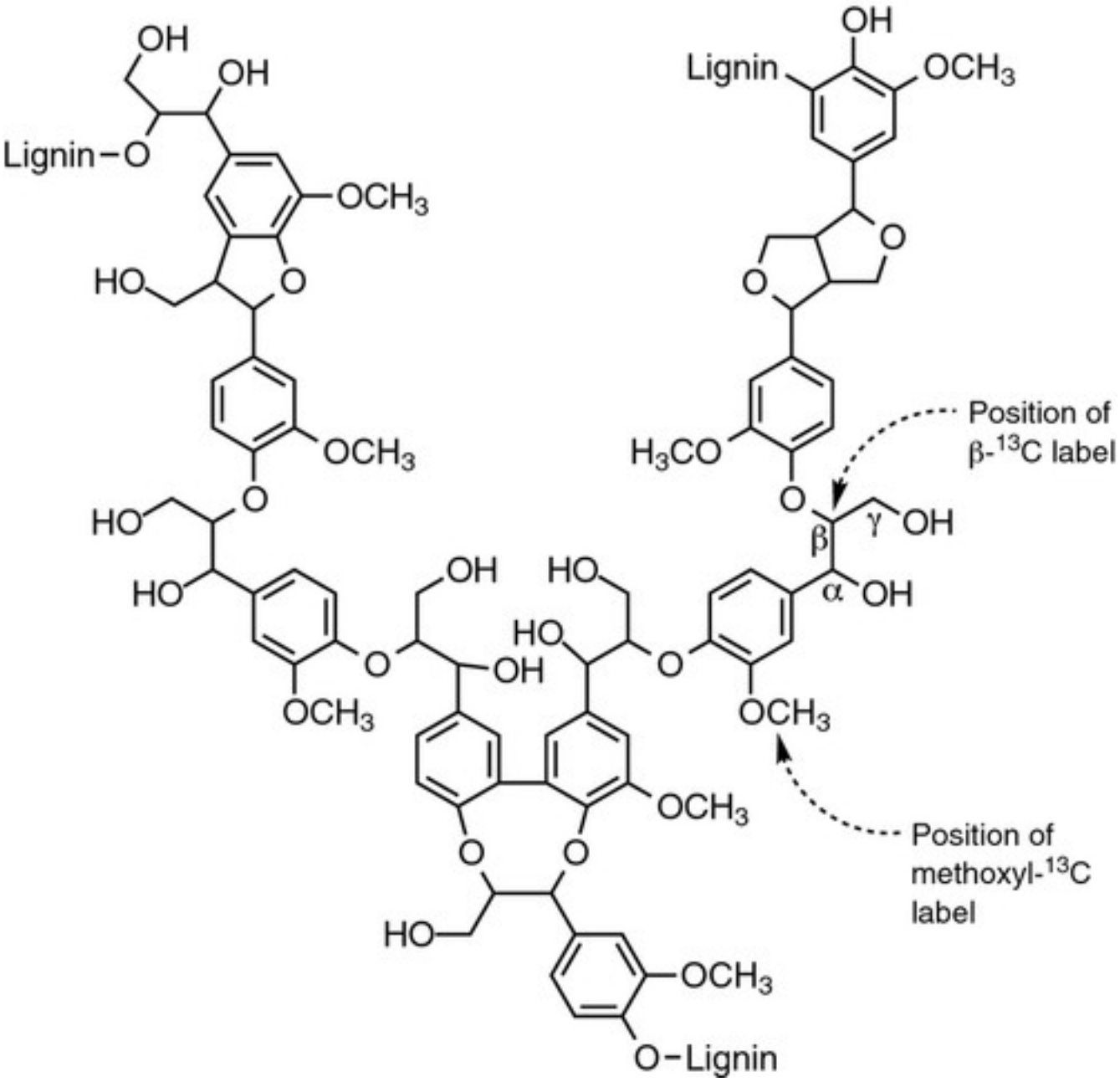

Figure 1

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Typical guaiacyl lignin structures. The positions of ^{13}C enrichment in the two synthetic lignins used are indicated on one of the polymer's phenylpropane structures. Release of a labeled methoxyl (OCH_3) group as $^{13}CO_2$ can occur without scission of the lignin, whereas release of labeled C_β as $^{13}CO_2$ requires ligninolysis.

Caption

Here, we examined whether humid tropical soils characterized by rapid O_2 fluctuations and associated Fe redox cycling could sustain high rates of lignin decomposition. We used high molecular weight ^{13}C-labeled synthetic lignins as tracers, because they lack the labeled

polysaccharide impurities that are inevitably present in natural lignins prepared via isotope labeling of growing plant material (Crawford, 1981). As polysaccharides are much more biodegradable than lignin, their presence even at low levels in a lignin preparation can lead to invalid results when relatively low rates of ligninolysis are being monitored. We compared the percent of soil respiration derived from lignin (P_{lignin}) as well as the total mass of mineralized lignin across laboratory treatments that imposed fluctuations and constitutive differences in O_2 availability. We hypothesized that fluctuations in O_2 availability that stimulated Fe redox cycling would increase P_{lignin} relative to other organic compounds, given that ·OH is a nonspecific yet ligninolytic oxidant whose production should increase with overall rates of Fe reduction and oxidation.

As lignin is a heterogeneous polymer, we predicted that the strength of these relationships would vary according to the position of the lignin ^{13}C label, which we supplied either at aromatic methoxyl positions or at C_β of the polymer's propyl side chains (Fig. 1). Removal of an aromatic methoxyl via electrophilic attack is a characteristic reaction of ·OH, and a typical lignin modification caused by fungi that employ Fenton chemistry to attack lignocellulose (Hammel *et al*., 2002). Demethoxylation does not result in depolymerization of the lignin, but does increase its polarity and reactivity by introducing hydroxyl groups into its aromatic rings. Release of C_β from the lignin also occurs following electrophilic aromatic attack by ·OH, although this reaction is not so prevalent as demethoxylation (Hammel *et al*., 2002; Yelle *et al*., 2011). Importantly, production of CO_2 from C_β is diagnostic for lignin depolymerization, because C_β cannot be released without breaking the aliphatic side chains that link lignin aromatic rings. Depolymerization is a critical step in lignin breakdown because small lignin fragments can be degraded by a much broader suite of microbes, even in the absence of O_2(Kirk & Farrell, 1987; Haider, 1992). Thus, methoxyl release is expected to be most diagnostic for Fenton chemistry, yet C_β release shows that lignin has been degraded to lower molecular weight material, and once this has occurred, the low molecular weight aromatic fragments that result can be readily metabolized by nonligninolytic organisms.

The above hypotheses are consistent with the importance of Fenton reactions as a nonspecific oxidative mechanism contributing to lignin decomposition in soils, a phenomenon that could contribute to the relative paucity of lignin in stabilized soil organic matter despite its biochemical recalcitrance. Improving our mechanistic understanding of lignin degradation represents a critical step toward predicting the direction and magnitude of soil C fluxes in a changing climate. The precise chemical mechanisms of lignin degradation are difficult to evaluate in natural soil environments, which exhibit heterogeneous interactions between biotic communities and the

physical/chemical environment over scales of nm. Imposing realistic variation in O_2 dynamics in conjunction with the sensitive measurements afforded by position-specific ^{13}C enrichment allowed us to assess whether Fe redox cycling has the potential to contribute to lignin breakdown under typical field conditions.

Materials and methods

Site description

Surface soil (0–10 cm) was collected from an upland valley site in the Bisley Research Watersheds of the Luquillo Experimental Forest, Puerto Rico, a NSF Long-term Ecological Research and Critical Zone Observatory site. These soils experience temporal fluctuations in bulk soil O_2 concentrations, which vary from 0% to 21% over scales of hours to weeks (Silver *et al*., 1999; Liptzin *et al*., 2011). Oxygen fluctuations, in turn, stimulate Fe redox cycling: Anaerobic periods stimulate dissimilatory Fe reduction, while aerobic periods drive oxidation of Fe(II) (Peretyazhko & Sposito, 2005; Chacon *et al*., 2006; Dubinsky *et al*., 2010; Hall & Silver, 2013). Annual precipitation at this site varied between 2600 and 5800 (mean 3800) mm $yr^{-1}$from 1988 to 2011, with relatively little seasonality, and mean annual temperature is 22 °C (F. Scatena, unpublished data). Soils are Oxisols (Aquic Hapludox) formed from basaltic to andesitic volcaniclastic sediments. The diverse evergreen forest is dominated by tabonuco (*Dacryodes excelsa*) and sierra palm (*Prestoea montana*). Several kg of A horizon soil (0–10 cm) was collected in February 2013 and express-shipped at ambient temperature to UC Berkeley.

Synthetic lignins

We synthesized isotopically labeled lignins in the laboratory to provide a conclusive measure of lignin breakdown. Workers in some previous studies have injected lignin precursors into live plants to generate labeled lignins via natural biochemical processes and reported marginally higher rates of mineralization of these plant-synthesized lignins as compared with synthetic laboratory lignins (Haider & Martin, 1981). However, plant biochemical processes inevitably transfer the added isotope label to other compounds, including structural polysaccharides that remain as contaminants in isolated natural lignins (Crawford, 1981). As these labeled polysaccharides are biochemically less recalcitrant than the labeled lignin, their biodegradation artificially inflates apparent rates of lignin breakdown.

Synthetic guaiacyl lignins were produced by polymerizing natural abundance coniferyl alcohol, [methoxyl-^{13}C]coniferyl alcohol (99 atom % ^{13}C), or [2-^{13}C]coniferyl alcohol (99 atom

% ^{13}C) *in vitro* with horseradish peroxidase (Sigma-Aldrich, type VI, St. Louis, MO, USA) as described in Kirk & Brunow (1988). Natural abundance coniferyl alcohol and the other reagents listed below, including ^{13}C-labeled precursors, were purchased from Sigma-Aldrich. A complete description of the syntheses of ^{13}C-labeled coniferyl alcohols, subsequent production of high molecular weight lignins (>1000 u), and validation with ^{13}C NMR analysis is provided in the Appendix S1.

The lyophilized synthetic lignins were dissolved in acetone/water, 4 : 1, and added to leaf litter collected from the field site. Litter (45% C, 1.4% N by mass) was oven-dried at 65 °C, ground, and sieved through a 53-μm mesh. Litter was immersed in the lignin solution, using a 5 : 1 mass ratio of litter to lignin, and the acetone/water solution was evaporated in a fume hood, allowing precipitation of the lignins on the surface of litter particles.

Coarse roots, organic debris, and fauna were removed from the soil monolith, which was gently homogenized while leaving coarse (1–2 cm) aggregates intact. Aliquots of the lignin/litter mixture (120 mg) were gently homogenized with replicate soil subsamples (1 g dry mass equivalent) at field moisture, for a total addition of approximately 20 mg lignin/1000 mg soil. Assuming that each lignin subunit had a molecular mass of ~200 g mol^{-1}, each experimental unit received approximately 100 μmol of ^{13}C label. Soils were incubated in glass jars (237 ml) sealed with gas-tight aluminum lids equipped with a Swagelok fitting and a butyl rubber septum. Incubation experiments commenced within 7 days of soil sampling for the aerobic and the fluctuating treatments that received methoxyl-^{13}C-labeled lignin; the rest of the treatments were initiated one to two weeks later.

Incubation treatments

Samples amended with methoxyl- and β-^{13}C-labeled lignins (Fig. 1) and unlabeled lignin (control) each received three headspace treatments: (i) CO_2-free air (static aerobic treatment); (ii) nitrogen (N_2; static hypoxic treatment); (iii) three days of CO_2-free air followed by four days of N_2 (fluctuating treatment). We use the adjective ‘hypoxic’ as opposed to ‘anaerobic’ given that we could not guarantee the complete absence of O_2 from our samples. However, periodic tests with an electrochemical O_2 sensor showed O_2 concentrations below detection limit (tens of ppm), and the incubation vessels have previously been shown to be leak-tight using a sulfur hexafluoride tracer. Each treatment was replicated five times for both the methoxyl- and β-^{13}C-labeled lignins and three times for unlabeled control lignins. All samples received 0.5 ml of de-oxygenated (N_2-flushed) ultrapure water immediately prior to the experiment, and additional water was added as necessary during the experiment to replace moisture lost during headspace

flushing. Experimental units were flushed with the appropriate gas to purge the jar headspace at three- and four-day intervals, according to the above treatments, for the duration of the experiment (66 days). Due to a technical error, the fluctuating headspace treatment for the β-^{13}C-labeled samples erroneously received N_2 instead of CO_2-free air during one three-day period. Samples were incubated in the dark under ambient laboratory temperatures (19–24 °C), which are consistent with variation in soil temperatures at the field site.

Chemical analysis

We measured headspace concentrations of CO_2 and its ^{13}C content over three- and four-day intervals immediately prior to flushing the headspace. Thus, we measured the entire production of $^{13}CO_2$ over the course of the experiment. At each sampling event, two replicate 30 ml gas samples were collected from each jar via a gas-tight syringe and stored in helium-purged and evacuated 20-ml glass serum flasks capped with Teflon septa sealed with aluminum crimps. Concentrations of CO_2 were measured on one replicate sample with a gas chromatograph and thermal conductivity detector (Shimadzu, Columbia, MD). The $^{13}C/^{12}C$ isotope ratio of CO_2 was determined from the other replicate sample on an IsoPrime 100 isotope ratio mass spectrometer (Elementar, Hanau, Germany). The percent contribution of ^{13}C-labeled lignin mineralization to total CO_2 production was determined using a two-source mixing model:

$$P_{\text{lignin}} = 100\frac{x(^{13}\text{C})_{\text{mixture}} - x(^{13}\text{C})_{\text{control}}}{x(^{13}\text{C})_{\text{lignin}} - x(^{13}\text{C})_{\text{control}}}.$$

Here, $x(^{13}C)_{mixture}$ and $x(^{13}C)_{control}$ represent the atom fraction of ^{13}C in respiration from samples with ^{13}C-labeled lignin and control samples, respectively, and $x(^{13}C)_{lignin}$ is 0.99 of either the methoxyl C or C_β. Total mineralization of ^{13}C-labeled lignin over each three or four-day timestep was calculated by multiplying P_{lignin} by total CO_2 production over that period. Note that this measure greatly underestimates total mineralization of added lignin because the labeled C atoms constitute a minor fraction of total C in the synthetic lignins (Fig. 1).

To illustrate patterns of net Fe reduction and oxidation accompanying headspace treatments, we incubated additional replicate samples under static aerobic and fluctuating headspace conditions, as described above, for the first five weeks of the experiment. We extracted all the soil in each jar in a 1 : 30 slurry with 0.5 M HCl, which was shaken for two hours and centrifuged for 10 min at 1500 rcf. Concentrations of soil Fe(II) were determined colorimetrically using a modified ferrozine method (Viollier *et al*., 2000). Soils from the static hypoxic treatment were not analyzed for Fe(II) content given that Fe reduction predominates in the absence of O_2 and Fe(II) oxidizes very slowly, if at all under hypoxic conditions (Ahmad & Nye, 1990).

Results

Decomposition of methoxyl-^{13}C-labeled lignin

The percent contribution of lignin mineralization to soil respiration (P_{lignin}) differed significantly among incubation treatments over time, and also according to the molecular position of the ^{13}C label (methoxyl vs. C_{β}). We define a 'cycle' of headspace fluctuations in the fluctuating treatment as the sequence of hypoxic and aerobic conditions applied during a given 7-day period; there were nine cycles of headspace fluctuations during the experiment. During the first two cycles, P_{lignin} was similar between the static aerobic and the fluctuating treatments during the aerobic phase and was lower in the hypoxic phase of the fluctuating treatment (Fig. 2a). By the third cycle, P_{lignin} was similar in the static aerobic and fluctuating treatments during both the aerobic and hypoxic phases, and by the fourth cycle, P_{lignin} in the fluctuating treatment significantly exceeded P_{lignin} in the static aerobic treatment during the aerobic phase ($P < 0.05$). A pattern of significantly greater P_{lignin} in the aerobic phase of the fluctuating treatment relative to the static aerobic treatment, and equivalent P_{lignin} in the hypoxic phase, persisted until the end of the experiment (Fig. 2a). Between cycles 4 and 9, P_{lignin} in the aerobic phase of the fluctuating treatment measured 140% of the value in the aerobic treatment, and ^{13}C-labeled lignin accounted for 0.75% and 0.53% of total respiration, respectively. The static hypoxic treatment had significantly lower P_{lignin} than the static aerobic and fluctuating treatments throughout most of the experiment, although the hypoxic phase of the fluctuating treatment and the static hypoxic treatment had similar P_{lignin} values during cycles eight and nine (Fig. 2a).

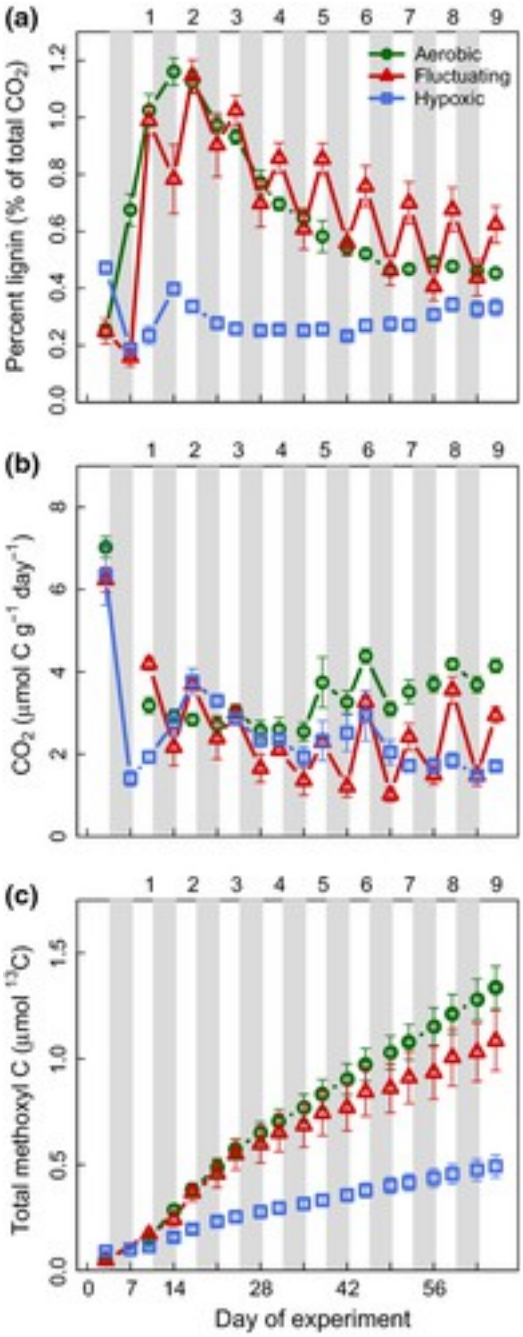

Figure 2

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Mineralization of methoxyl ^{13}C-labeled lignin compared by treatment. Panel (a) shows P_{lignin}, the percent contribution of labeled lignin to total CO_2 production, (b) shows total CO_2 production, and (c) shows cumulative methoxyl ^{13}C mineralization on a mass basis. Vertical gray bars denote hypoxic phases of the fluctuating treatment, and numbers above the graph denote headspace fluctuation cycles as described in the Results. Data represent means ± SE, which are additive for panel (c).

Caption

Despite the differences in P_{lignin} between the aerobic and fluctuating treatments early in the experiment, total methoxyl-^{13}C lignin mineralization (i.e., $^{13}CO_2$ production from labeled lignin) was essentially identical between these treatments in cycles 1–3 due to an opposite pattern in total CO_2 production (Fig. 2b). Total CO_2 production in the fluctuating treatment was significantly greater than in the static aerobic treatment during the aerobic phase of cycles one and two. This pattern was reversed later in the experiment, where increased P_{lignin} during the aerobic phase of the fluctuating treatment (Fig. 2a) was offset by decreased total CO_2production (Fig. 2b), resulting in similar total methoxyl-C lignin mineralization (Fig. 2c). Total methoxyl-^{13}C lignin mineralization was statistically equivalent between the static aerobic and fluctuating treatments over the entire experiment (Fig. 2c), although the difference between means slowly increased over time. This difference was primarily driven by lower respiration rates in the fluctuating treatment relative to the static aerobic treatment during both phases of the headspace cycle as the experiment progressed. Overall, total methoxyl-^{13}C lignin mineralization in the fluctuating treatment measured 79 ± 12% of that in the static aerobic treatment. Mineralization of methoxyl-^{13}C under static hypoxic conditions was similar to the other treatments over the first week but quickly and significantly declined thereafter (Fig. 2c); total mineralization through cycle nine measured only 35 ± 10% of that in the static aerobic treatment. Assuming that the presence of O_2 was a rate-limiting step for initial lignin oxidation, cumulative methoxyl-^{13}C lignin mineralization can also be compared on the basis of total O_2exposure after subtracting background methoxyl-C mineralization under static hypoxic conditions. The fluctuating treatment mineralized 23 nmol methoxyl-^{13}C g^{-1}soil per day of O_2exposure, as compared with 14 nmol C g^{-1} day^{-1} for the static aerobic treatment.

Decomposition of β-^{13}C-labeled lignin

For the β-^{13}C-labeled lignin, P_{lignin} was generally similar between the static aerobic and fluctuating treatments during the aerobic phase, but was significantly lower in the fluctuating treatment during the hypoxic phase (Fig. 3a). During the first cycle, however, P_{lignin} was significantly greater during the aerobic phase of the fluctuating treatment than the static aerobic treatment. A period of extended hypoxic conditions due to a technical error in cycle four temporarily decreased P_{lignin} by a factor of two in the fluctuating treatment, but P_{lignin}recovered in subsequent aerobic phases and was statistically equivalent to the value from the static aerobic treatment after cycle six, where it displayed a similar pattern as shown in cycles two and three (Fig. 3a). In the static hypoxic treatment, P_{lignin} was consistently lower than in the other treatments, measuring only 21% of the static aerobic treatment between cycles three and nine.

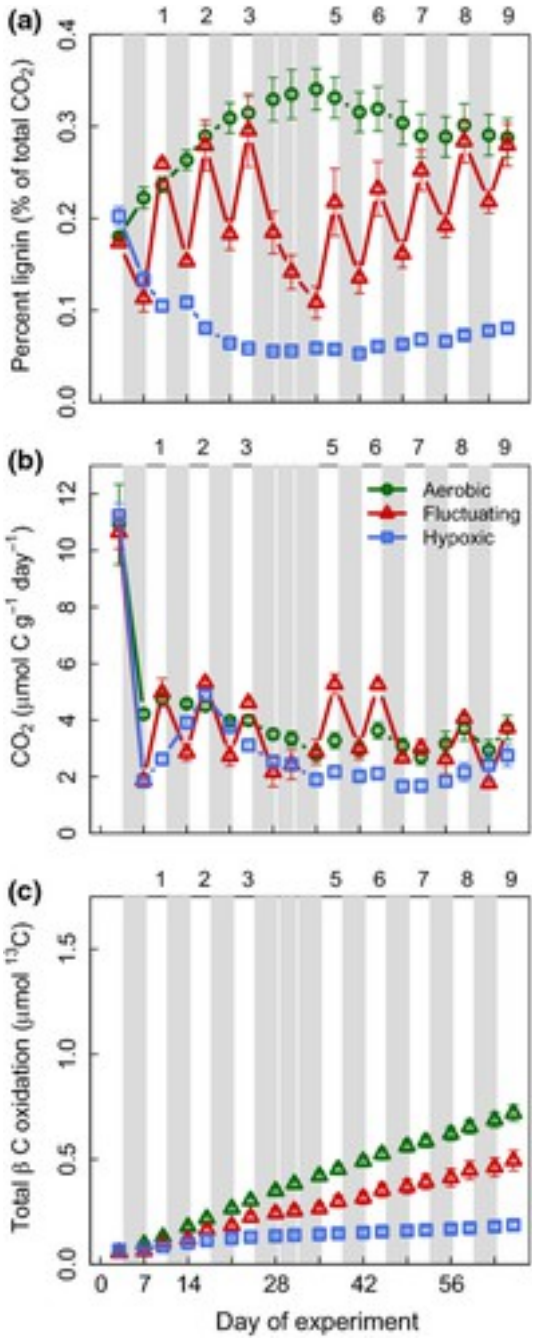

Figure 3

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Mineralization of β ^{13}C-labeled lignin compared by treatment. Panel (a) shows P_{lignin}, the percent contribution of labeled lignin to total CO_2 production, (b) shows total CO_2 production, and (c) shows cumulative β ^{13}C mineralization on a mass basis. Vertical gray bars denote hypoxic phases of the fluctuating treatment, and numbers above the graph denote headspace fluctuation cycles as described in the Results. Data represent means ± SE, which are cumulative for panel (c).

Caption

Total β-^{13}C lignin mineralization in the aerobic and fluctuating treatments diverged during cycle two and remained significantly lower in the fluctuating treatment for the rest of the experiment

(Fig. 3c). Similarly, total β-^{13}C mineralization remained lower in the static hypoxic treatment than the other treatments during and following cycle two. Overall, β-^{13}C lignin mineralization in the fluctuating and hypoxic treatments measured 69 ± 8% and 26 ± 3% of the aerobic treatment, respectively. However, total CO_2 production in the fluctuating treatment exceeded that of the static aerobic treatment during the aerobic phase on multiple occasions (Fig. 3b), as was also observed once for soils incubated with methoxyl-^{13}C-labeled lignin. This phenomenon was especially prominent in the aerobic phase of cycles five and six, where CO_2production in the fluctuating treatment measured 161% and 145% of the aerobic treatment, respectively (Fig. 3b). Cumulative CO_2 production was greatest in the aerobic treatment and lower in the fluctuating and hypoxic treatments during one set of incubations (methoxyl lignin), whereas the fluctuating treatment was ultimately equivalent to the aerobic treatment in the other incubations (β lignin), possibly as a consequence of increased respiration following the extended anaerobic period in the middle of the experiment (Fig. S1).

Expressing β-^{13}C mineralization on the basis of total O_2 exposure indicated a large difference in oxidation efficiency between the static aerobic and fluctuating treatments. After subtracting background β-^{13}C mineralization under static hypoxic conditions, the fluctuating treatment mineralized 33 nmol β-^{13}C g^{-1} soil per day of O_2 exposure, as compared with 18 nmol g^{-1} $day^{-1}$for the static aerobic treatment.

Iron redox cycling over time

Trends in Fe(II) concentrations indicated substantial net Fe reduction and oxidation in the fluctuating treatment, and consistently low net Fe redox cycling in the static aerobic treatment (Fig. 4). Concentrations of Fe(II) measured approximately 80 μg g^{-1} at the beginning of the experiment. In the fluctuating treatment, Fe(II) increased several-fold during the hypoxic phases and decreased during the aerobic phases. In contrast, Fe(II) concentrations were relatively static in the aerobic treatment until cycles four and five, when they declined to nearly zero.

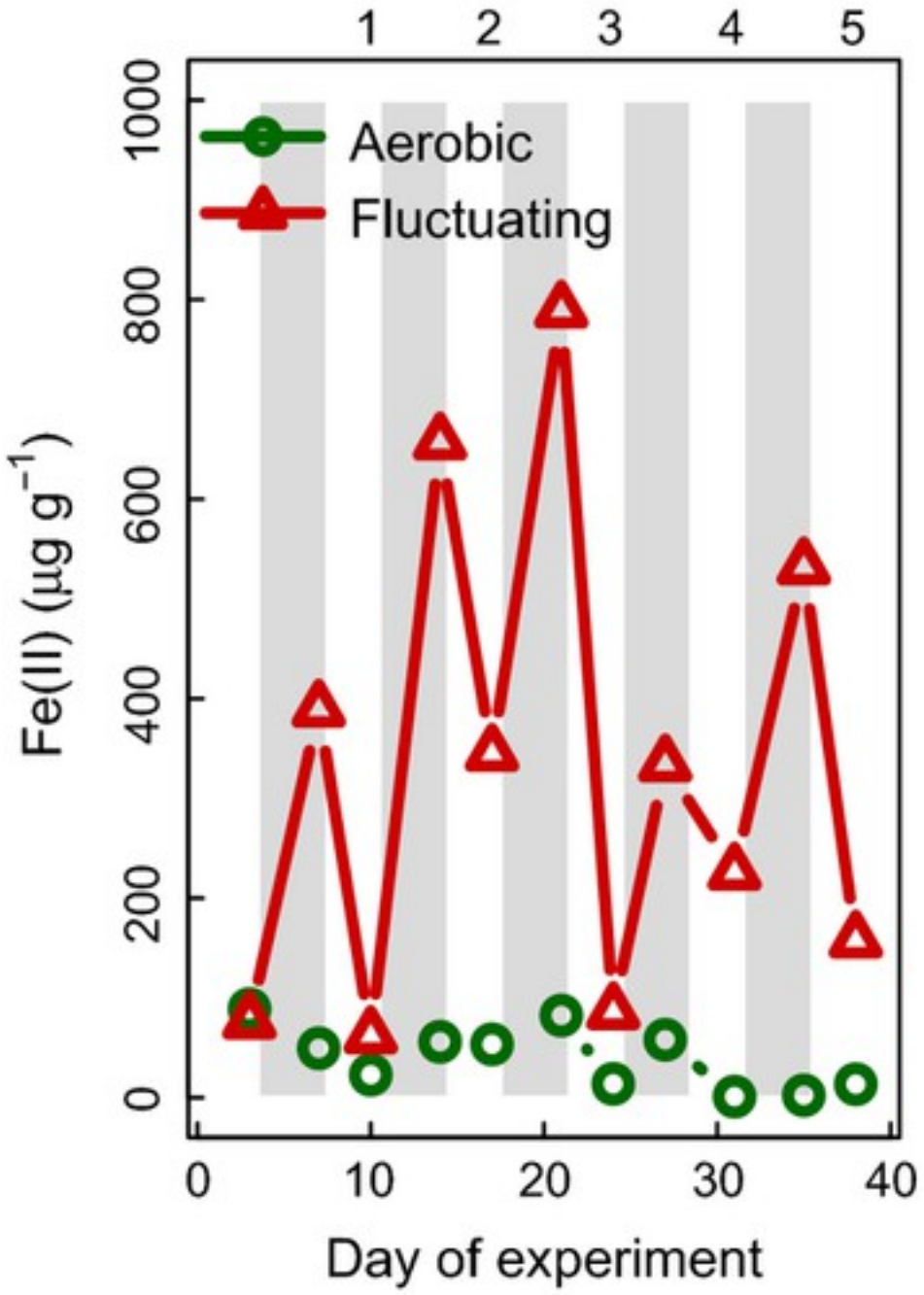

Figure 4

Open in figure viewerPowerPoint

Temporal patterns in Fe(II) concentrations measured in 0.5 M HCl soil extractions by treatment, illustrating the magnitude of net Fe reduction and oxidation in the fluctuating treatment. Replicates were destructively sampled. Symbols represent individual samples for each treatment/date.

Caption

Discussion

Impacts of O_2 variability on lignin breakdown

Lignin decomposition is thought to decrease dramatically with O_2 limitation (Zeikus *et al*., 1982; Benner *et al*., 1984; Ekschmitt *et al*., 2008; Sinsabaugh, 2010), and aromatic lignin derivatives have been widely observed to accumulate in flooded wetland soils and subsurface horizons of humid tropical soils (Fenner & Freeman, 2011; Kramer *et al*., 2012). To our knowledge, however, no previous studies have rigorously tested the impact of *variation* in O_2concentrations, which are characteristic of a wide range of soil types (Sexstone *et al*., 1985; Sierra & Renault, 1998; Silver *et al*., 1999), on lignin breakdown in terrestrial soils. We found that periodic O_2 limitation had relatively little inhibitory effect on most indices of lignin decomposition in a humid tropical soil, in contrast to results from studies employing pure cultures of lignin-degrading fungi (Kirk & Farrell, 1987). In support of our hypothesis, alternation between hypoxic and aerobic incubation conditions substantially *increased* the aerobic contribution (P_{lignin}) of methoxyl-^{13}C lignin mineralization to soil respiration, while the respiratory contribution of β-^{13}C lignin was equivalent to that observed under static aerobic conditions when comparing the aerobic phase alone. Thus, despite decreased CO_2 production under fluctuating relative to static aerobic conditions, methoxyl-^{13}C lignin mineralization was ultimately similar between these two treatments, and β-^{13}C lignin mineralization in the fluctuating treatment was almost 70% of the aerobic treatment. Normalizing lignin mineralization to O_2 exposure indicated a twofold increase in total aerobic mineralization in the fluctuating treatments relative to static aerobic conditions. Static hypoxic conditions resulted in a consistently low contribution of lignin mineralization to respiration and low total lignin mineralization, consistent with previous work in lake and wetland sediments (Zeikus *et al*., 1982; Benner *et al*., 1984).

The high P_{lignin} values measured under the aerobic phase of the fluctuating treatment are conservative estimates given that they reflect the contribution of a single C moiety of lignin, and that multiple oxidative reactions are required to transform lignin C to CO_2. For example, methoxyl groups must first be oxidatively cleaved from an aromatic ring, and methanol subsequently oxidized to CO_2. Rates of CO_2 production from low molecular weight aromatic lignin breakdown products are much less dependent on O_2 than the initial oxidation of lignin (Zeikus *et al*., 1982; Kirk & Farrell, 1987; Haider, 1992). This means that much of the lignin mineralized under hypoxic conditions in the fluctuating treatment was likely to have been initially oxidized in the presence of O_2, given that static hypoxic conditions had consistently low P_{lignin}. We estimated the contribution from lignin oxidized in an aerobic phase but mineralized in a hypoxic phase by subtracting P_{lignin} measured in the static hypoxic treatment from that

measured in the fluctuating treatment during the hypoxic phase. This correction substantially increased our estimates of lignin oxidation during the aerobic phase of the fluctuating treatment, implying that the initial oxidation of β-^{13}C was actually *greater* during its aerobic phase than in the static aerobic treatment. Similarly, we normalized total lignin mineralization in terms of differences in O_2 exposure between the static aerobic and fluctuating treatments. Differences in this metric among treatments implied that fluctuating O_2 conditions were more effective than static aerobic conditions in mineralizing lignin when normalized to the amount of O_2 supplied (33 vs. 18 nmol β-^{13}C g^{-1} soil day^{-1}). Low O_2 availability has been well demonstrated to limit lignin mineralization by white-rot fungi in the laboratory (Kirk & Farrell, 1987), but had a considerably lower impact than expected in our soil microcosms when O_2availability fluctuated. This result indicates the presence of a ligninolytic mechanism that is not attributable to canonical lignin-degrading fungi.

Redox fluctuations and reactive oxygen species

Together, these findings suggest that periodic O_2 limitation does not necessarily lead to the preferential preservation of lignin. Rather, they support the potential importance of redox fluctuations in *increasing* the contribution of lignin decomposition to soil respiration, possibly via nonspecific oxidation mechanisms involving reactive oxygen species. Alternatively, shifts in microbial community composition or abundance under the fluctuating O_2 treatments could also contribute to the observed patterns, although canonical lignin-degrading organisms would not be expected to show increased growth under these conditions (Kirk & Farrell, 1987; Haider, 1992; Ekschmitt *et al*., 2008). Many soils experience episodic fluctuations in O_2availability at scales ranging from aggregates to profiles (Sexstone *et al*., 1985; Silver *et al*., 1999; Fimmen *et al*., 2008; Liptzin *et al*., 2011), which can promote respiratory Fe reduction and subsequent Fe oxidation. In our experiment, the fluctuating headspace treatment showed high rates of Fe redox cycling, in agreement with several studies of terrestrial soils undergoing O_2fluctuations (Thompson *et al*., 2006; DeAngelis *et al*., 2010; Dubinsky *et al*., 2010; Hall *et al*., 2013). Oxidation of Fe(II) by O_2 and H_2O_2 generates reactive oxygen species, including $OH^{\cdot}$(Pignatello *et al*., 2006). This mechanism provides a plausible explanation for the large increase in P_{lignin} of methoxyl C in the fluctuating treatment, as well as for the increased lignin mineralization implied after accounting for time lags and normalizing for O_2 availability. Production of reactive oxygen species could be especially important for maintaining the decomposition of the biochemically recalcitrant aliphatic backbone that links the aromatic rings in lignin. Our experiment with β-^{13}C-labeled lignin showed a fivefold increase in P_{lignin}under fluctuating redox relative to static hypoxic conditions. We note that the oxidation of reduced

humic substances to form H_2O_2 could also have contributed to production of reactive oxygen species in this experiment (Page *et al*., 2012). Finally, we note that Fe redox cycling initially could have contributed to lignin mineralization in the static aerobic treatment as well, given the consistent presence of Fe(II) in static aerobic samples over the first three weeks of the experiment. Gross Fe reduction and oxidation can occur inside soil aggregates as a consequence of spatial O_2 gradients and Fe(II) diffusion (Ahmad & Nye, 1990), even when no changes in net Fe(II) are apparent, and net Fe(II) reduction can occur in unsaturated soil samples exposed to an aerobic atmosphere (Liptzin & Silver, 2009). This mechanism could have contributed to similarities in methoxyl P_{lignin} between the aerobic and fluctuating treatments over the first three cycles, when Fe(II) was greatest in the static aerobic treatment.

Implications for the terrestrial C cycling in a changing climate

Reconciling the accumulation of lignin during late-stage litter decomposition with the apparent depletion of lignin in stabilized soil organic matter (Grandy & Neff, 2008; Schmidt *et al*., 2011) represents an important conceptual challenge for C cycle science. Environmental conditions that increase the contribution of lignin decomposition to soil respiration, such as the redox fluctuations imposed in this experiment, provide one potential explanation for how lignin turnover could be sustained even in soil microsites where canonical lignin-degrading organisms are apparently rare, and where critical cosubstrates for lignin degradation such as O_2 are periodically or constitutively limiting.

Our results also point to the importance of understanding the impact of environmental fluctuations on biogeochemical processes, in addition to changes in mean climatic parameters. For example, climate change is likely to alter rainfall dynamics in tropical regions, leading to changes in the temporal distribution of precipitation in addition to annual mean rainfall (Huang *et al*., 2013). Spatial and temporal variation in precipitation and net radiation is closely coupled to soil O_2 dynamics via soil moisture (Silver *et al*., 1999; Liptzin *et al*., 2011). Based on our present data, variation in the frequency and magnitude of redox fluctuations is likely to impact the contribution of biochemically recalcitrant organic polymers, such as lignin, to soil respiration. The periodicity of fluctuations employed in this experiment (three days of aerobic followed by four days of hypoxic conditions) are well within the range of redox fluctuations observed at our field sites, but *in situ* periods of fluctuation can vary widely according to local environmental conditions (Silver *et al*., 1999; Liptzin *et al*., 2011). Given our present results, the length and periodicity of aerobic and hypoxic phases are likely to impact total as well as relative rates of lignin mineralization among soils. Redox fluctuations thus provide a plausible, and testable, linkage between changes in precipitation and hydrology and the biochemical

composition of SOM. The combination of bacterial metabolism and abiotic Fe(II) oxidation to generate reactive oxygen species could contribute to lignin breakdown across a broad range of soil environments, given the apparent ubiquity of microsites that can sustain anaerobic metabolism and Fe reduction (Fimmen *et al*., 2008).

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