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Title: Formation of Water-Soluble Organic Matter Through Fungal Degradation of Lignin

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Abstract: Lignin is a major component of decaying terrestrial vegetation in soils and has been reported to contribute substantially to the formation of soil carbon humus and associated water extracts of soil. To better understand this process of humification, lignin from brown-rot degraded wood is subjected to a white-rot fungus (*Phanerochaete chrysosporium)* whose enzymes are particularly effective in lignin degradation. This enzymatic attack was monitored by ultrahigh resolution mass spectrometry of water soluble extracts of the fungal cultures. The ensuing molecular level characterizations showed that the *P. chrysosporium* fungi induced aromatic ring oxidations followed by ring opening as expected. However, we also observed the production of new molecules, some of which are aliphatic. These results are consistent with recent findings that hydroxyl radical attack of lignin involves ring opening reactions followed by electrocyclic condensations combined with radical scavenging/disproportionation reactions.

Keywords: Humification, Humic substances, Lignin, Fungal degradation, Aromatic ring oxidation

1. Introduction

One of the basic processes influencing the carbon cycle is the transformation of plant and microbial debris into soil humus, commonly referred to as humification (Lara and Thomas 1995; Zech et al., 1997; Gougoulias et al., 2014). The chemical composition of the source organic matter, along with redox conditions, pH and climate, are all factors that affect the progress of humification (Kogel-Knabner et al., 1991). However, uncertainties regarding the pathways for organic matter transformation in soil makes our understanding of humification limited (Schmidt et al., 2011). In spite of recent comprehensive research, chemistry and source of compounds involved in the formation of soil organic matter (SOM), specifically, the role of aromatic compounds originating from plants, mainly lignin and tannins, is under intense debate (Klotzbücher et al., 2016).

Lignin, a phenolic heteropolymer, exists as the second most abundant natural organic substance on Earth and accounts for approximately 20% of the organic matter (OM) input to soil (Field et al., 1998). Annually, 1.1 x 10^{13} kg lignin is produced globally (Thevenot et al., 2010). Many consider this lignin to be substantially more resistant to microbial degradation, compared with many other plant biopolymers such as cellulosic and proteinaceous materials (Zech et al., 1994). These facts have, in the past, nurtured the idea that lignin is a major potential source for the formation of SOM. Surprisingly, the alternate view on SOM formation suggests that SOM in mineral soils is dominated by non-aromatic compounds thought to be microbially derived while lignin-derived aromatics disappear quickly in soil (Zech et al., 1994; Gallet and Pellissier 1997; Kaiser et al., 2004; Thevenot et al., 2010). Recent review indicates that the fate of lignin in

different soils is contradictory. Besides, the process for lignin degradation and it's stabilization in SOM is unresolved (Thevenot et al., 2010).

Typically, support for this microbial origin of SOM involves studies characterizing the chemical structure of SOM applying a variety of analytical methods that include both average spectroscopic methods such as NMR or UV absorbance, fluorescence spectroscopy and molecular methods (Dai et al., 1996; Grünewald et al., 2006; Sanderman et al., 2008; Hansson et al., 2010). The main molecular evidence for the disappearance of lignin in soil comes from CuO oxidation, pyrolysis, or (Tetramethylammonium hydroxide) TMAH thermochemolysis, all of which are conventional molecular methods that rely on a few distinct lignin-derived monomers to evaluate lignin contribution to SOM (Grünewald et al., 2006; Buurman et al., 2007; Nierop and Filley, 2007). In many instances, these molecular "biomarkers" for lignin are quantified analytically to yield an estimate for the amount of intact or slightly modified lignin substructures.

There are some concerns, however, as they are applied for evaluating lignin contributions to SOM. First, lignin does not completely depolymerize during CuO oxidation as this may well be the case also for conventional pyrolysis or TMAH thermochemolysis. Second and most importantly, a minor or severe molecular alteration or reorganization of lignin-derived aromatics might be invisible to the analytical window for CuO, where the presence of specific biomarkers (e.g., lignin phenols) is a requisite for lignin identification (Hernes et al., 2013; Klotzbücher et al., 2016). If the alteration involves ring-opening reactions commonly observed for fungal degradation of lignin (Higuchi, 2004) and the resultant products remain un-assimilated, then this would preclude the identification of lignin phenols and be interpreted as a complete degradation of the lignin. Recent studies (Chen et al., 2014; Waggoner et al., 2015) give us reason to suspect that this is a very probable explanation for why lignin biomarkers are significantly attenuated in

soil perhaps leading to an erroneous evaluation of the role of lignin in soil organic matter accumulation.

Biodegradation of lignin in soil due to the ubiquitous presence of microorganisms is considered one of the most important and primary degradation pathways for wood. Among many microorganisms capable of degrading wood, fungi play an important role (Brown and Chang, 2014). According to extensive previous studies, fungal degradation of wood by brown rot fungi causes a major depletion of cellulose and a minor modification of lignin (Goodell et al., 2003). Lignin is also subjected to severe modification by other different types of fungi called white-rot fungi (Leonowicz., 1999). Numerous studies have suggested that lignin is attacked by fungal enzymes to induce ring opening and production of low molecular weight carboxylated compounds (Kirk and Chang, 1975; Chen et al., 1983). Mechanisms proposed by Higuchi (2004) show oxidation and ring opening of lignin phenolic compounds by oxidative enzymes produced by *P. chrysosporium* -a well-known species of white rot fungi. These enzymes effectively yield some new products like muconic acids (Higuchi, 2004), which are completely invisible to the CuO oxidation or TMAH thermochemolysis methods. The role of these products and their contribution to the formation of soil organic matter has never been investigated, mainly because it was thought that the compounds produced are microbially degraded further and mineralized (Crawford et al., 1981).

Recent studies of humic substances by Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR-MS) has opened a new window for identifying molecular components of SOM (Kim et al., 2003; Grinhut et al., 2011). Analysis by ESI-FTICR-MS has allowed categorization of molecules having common structural features. This is accomplished by first determining the unique elemental compositions of mass spectral peaks and then utilizing the van

Krevelen diagram to plot molar H/C ratios vs. molar O/C ratios. Recently, numerous studies have applied this approach to characterize humic substances (Sleighter and Hatcher, 2007; Nebbioso and Piccolo, 2013).

The current study employs this new molecular approach to examine the formation of new compounds from fungal degradation (*P. chrysosporium*) of a brown-rotted wood to simulate sequential fungal degradation of lignin in soil. Our work combines ESI-FTICR-MS and solid-state ^{13}C NMR to characterize these processes at the molecular level. We chose the brown-rotted wood because these fungi effectively isolate and concentrate lignin in a relatively unaltered state while removing the cellulosic components of wood (Goodell et al., 2003). Moreover, such fungi are likely playing an important role in SOM formation in most soil systems globally.

2. Material and methods

2.1 Organisms.

Spore inoculum cultures of *P. chrysosporium* (strain BKM-F-1767; ATCC 24725) are maintained on supplemented malt agar slants; the medium is described by Tien and Kirk (1988).

2.2 Media and cultures.

The medium used in the experiment contained the following: Basal III medium (per liter): KH_2PO_4, 20 g L^{-1}; $MgSO_4$, 5 g L^{-1}; $CaCl_2$, 1 g L^{-1}; Trace elements solutions (per liter): $MgSO_4$, 3 g L^{-1}; $MnSO_4$, 0.5 g L^{-1}; NaCl, 1.0 g L^{-1}; $FeSO_4 \cdot 7H_2O$, 0.1 g L^{-1}; $COCl_2$, 0.1 g L^{-1}; $ZnSO_4 \cdot 7H_2O$, 0.1 g L^{-1}; $CuSO_4$, 0.1 g L^{-1}; $AlK(SO_4)_2 \cdot 12H_2O$, 0.010 g L^{-1}; H_3BO_3, 0.010 g L^{-1}; $Na_2MoO_4 \cdot 2H_2O$, 0.010 g L^{-1}; Nitrilotriacetate, 1.5 g L^{-1}. The following items were added to shallow stationary cultures: Basal III medium (autoclaved), 100 mL; 10% glucose (autoclaved) 100 mL; thiamin (100 mg/Liter stock, filter sterilized) 10 mL; ammonium tartrate (8 g/Liter

stock, autoclaved) 25 mL; fungal spores (populations measured by absorbance at 650 nm=0.5) 100 mL; trace elements (filter sterilized) 60 mL, make water to 1L.

2.3 Lignin

A lignin sample was prepared from a brown rot fungally degraded wood, white oak (*Quercus alba*), collected from a mixed deciduous/pine forested site in Suffolk, Virginia. The 50-cm diameter tree from which the sample was collected had been killed, probably by lightning, and remained upright for countless years prior to being felled by the simple push of a hand. The remnant tree contained an intact bark layer but the inner xylem tissue had been almost completely transformed by the brown rot fungi of unspecified genus-species characterization. Large portions of the xylem were harvested by hand from the tree and freeze dried and ground to a fine powder. Before using the powdered lignin in microcosm degradation experiments, the sample was placed for 24 h in ethanol to kill indigenous microorganisms. After evaporation of ethanol, samples are used for fungal degradation experiments.

2.4 Growth and Harvest

Shallow stationary cultures (10 mL + 4mg powdered lignin) were grown in ten rubber-stoppered 125-mL Erlenmeyer flasks at 39° under 100% oxygen. They were flushed with oxygen at the time of inoculation and again on day three (Tien and Kirk, 1988). After seven days of incubation, the contents of all ten rubber-stoppered 125-mL Erlenmeyer flasks were added to a centrifuge tube to separate mycelia from the medium by centrifugation followed by filtration through a 0.45-micron filter. It is important to mention that, when the experiment was initiated, we intended to analyze each replicate, but we quickly realized that insufficient material would be available for the planned NMR analyses. At this point, we decided to combine all the replicates into a single sample that provided sufficient material for NMR analysis. Thus, our results

average out the biological variability which is usually the dominant contributor to variability. The solid residue (lignin and mycelia) was then frozen and later freeze dried for solids NMR analysis. The filtrate was collected and prepared for mass spectrometry as described below. Lignin added to a control experiment was left un-inoculated. In addition, a fungal control of only *P. chrysosporium* was prepared by growing the fungus in media without lignin but growing on glucose as a carbon source. The culture was harvested, filtered as described above, and freeze-dried for analysis.

2.5 Extraction of DOM

To prepare the above-mentioned filtrate for mass spectrometry, solid-phase extraction (SPE) of the soluble organic matter was necessary (Dittmar et al., 2008). Briefly, methanol (HPLC grade, Fisher Scientific) is used to rinse solid phase extraction (SPE) cartridges (PPL, 3 mL, Agilent). Cartridges are activated with MilliQ water (MQW, pH 2, HCl ACS grade, Fisher Scientific). Prior to loading the filtrates on the cartridge, the solutions were acidified to pH 2. The cartridges were rinsed multiple times with MQW (pH 2) and dried with N_2 gas prior to elution with methanol. The methanol eluates were then collected for further treatment described below.

2.6 FT-ICR mass spectrometry analysis

The samples were eluted from PPL immediately prior to analysis by ESI-FTICR-MS. The samples were diluted with methanol and water 1:1 ratio and analyzed in the negative ion mode using an Apollo II electrospray ionization source of a Bruker Daltonics 12 T Apex Qe FTICR-MS. ESI-FTICR-MS analyses were operated as described previously (Sleighter and Hatcher., 2008; Sleighter et al., 2010). The molecular formula for each peak was only considered if the corresponding ^{13}C isotope peak existed. To assign molecular formulae from 200 to 800 m/z, an in-house MATLAB script, written at Old

Dominion University according to the following criteria: $^{12}C_{2–50}$, $^{1}H_{5–100}$, $^{14}N_{0–6}$, $^{16}O_{1–30}$, $^{32}S_{0–2}$, and $^{31}P_{0–2}$ within an error of 1 ppm, was employed (Grinhut et al., 2011; DiDonato et al., 2016).

2.7 Solid-state 13C NMR spectroscopy

Solid-state ^{13}C NMR analyses of samples were performed using a 400 MHz Bruker AVANCE II spectrometer equipped with a solid-state MAS probe. Samples were packed into a 2.5 mm Zirconia rotor and sealed with a Kel-F@ cap. Samples were spun at 15 kHz at the magic angle (54.7º). All spectra were acquired with 2048 scans, a contact time of 2.5 ms, and a 1 s recycle delay. Cross polarization ^{13}C NMR spectra were obtained with the multi-CP cross polarization pulse program with a two-pulse phase modulated decoupling as described previously (Johnson and Schmidt-Rohr, 2014).

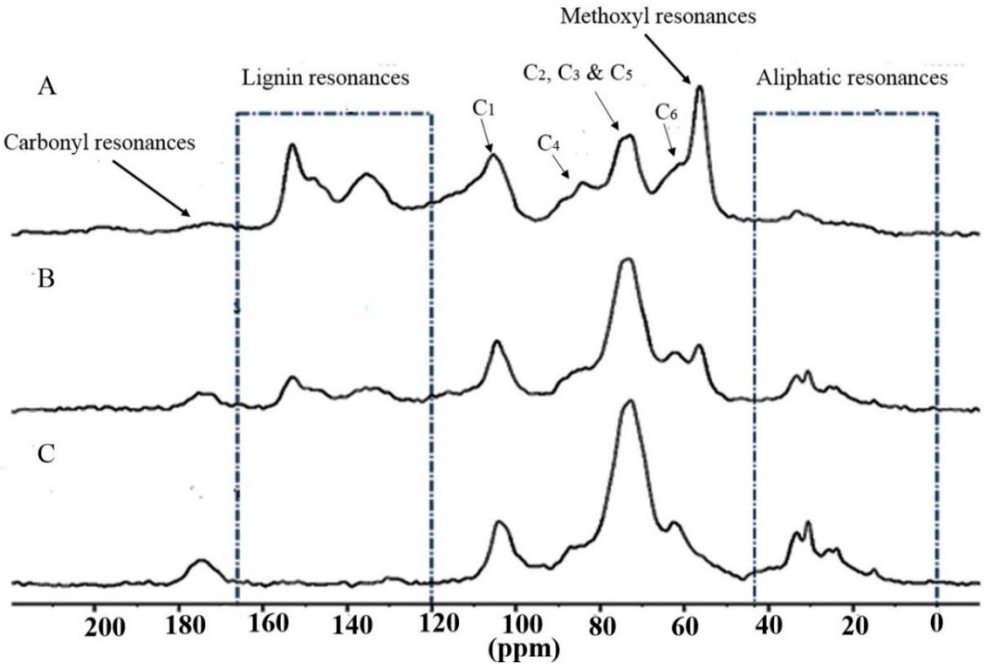
3. Results and Discussion

3.1 Solid-State ^{13}C NMR

Fig. 1 shows the ^{13}C NMR spectra of the lignin control, *P. chrysosporium* control and fungal-treated brown rotted wood after seven days. The three spectra demonstrate the presence of peaks corresponding to cellulosic substances (indicated in the figure by C_i where *i* is the hexose carbon number for both cellulose and hemicellulose), and lignin (indicated by resonances for methoxyl and aromatic units). Peak assignments are based on previous literature (Hatcher, 1987). Additionally, identical multi-CP acquisition parameters were used to obtain these spectra, and the same amount of sample was loaded into sample rotors, allowing comparison of the resultant changes in the spectra. As shown in Fig. 1, the 60−115 ppm region displays peaks typical of polysaccharides, while the side-chain groups (oxygenated C_α, C_β, and C_γ carbons) of the phenyl-propane lignin structural units also provide a minor contribution to this region of the spectra. Cellulose resonance signals at different carbon positions were located at 105 ppm(C_1), 84 ppm (C_4), 72 ppm (C_2, C_3, and C_5), and 65 ppm (C_6). Signals in the 110−165 ppm region are attributed to lignin aromatic units with peaks at 152 ppm representing O-substituted aromatic

carbons, 135 ppm representing C-substituted aromatic carbons, and 112 ppm representing

protonated aromatic carbons (the peak overlaps with that of the C_1 carbons of carbohydrates). Additional signals are observed in the aliphatic region (0-45 ppm) and these are attributed to fungal biomass for the most part. White-rot degradation of lignin from the brown-rotted white oak sample is hypothesized to impart some discernible changes in solid-state ^{13}C NMR spectra of the solids and the ESI-FTICR-MS spectra of water extracts of the lignin. It is apparent from the ^{13}C NMR spectra that initially, the sample is primarily enriched in lignin-derived structures as would be expected from wood subjected to degradation by brown rot fungi and that growth of the fungi *P. chrysosporium* alters the sample and its spectrum by mainly enhancing signals attributable to cellulosic materials. These are most likely from the fungi as deduced from the spectrum for the fungal control. Visually, the sample was completely permeated by fungal hyphae. Concomitant with this enrichment in carbohydrates, a diminution of the signal from



lignin is observed. Because the fungal biomass dominates the spectrum of the fungal-treated lignin, it is difficult to determine from the solid-state ^{13}C NMR that the lignin has been modified structurally.

Fig. 1. ^{13}C NMR spectra of (A) brown rotted wood (lignin control); (B) fungal-treated brown rotted wood after seven days; (C) *P. chrysosporium* control

We attempted to determine changes by calculating a ratio of peaks that are predominantly attributable to lignin. This ratio of peaks, from integration of the spectra, shows that there is relatively little change that can be detected in the overall structural composition of the lignin. Basically, the solid-state NMR is not sensitive enough to discern the changes in lignin brought about by the attack of *P. chrysosporium*. We suggest that changes in the solids portions of the lignin sample may not be detectable over the short time span of exposure (seven days) to *P. chrysosporium* because they are subtle, and the remnant lignin and fungal biomass overwhelms the overall sample make-up.

3.2 FTICR-MS

Fig. 2 shows the negative ion FT-ICR mass spectrum of water extracted brown-rotted wood (lignin control), fungal-treated brown rotted wood and *P. chrysosporium* control after seven days. The general mass distribution of compounds that were ionized in all samples was in the range of 200 -800 m/z, which is consistent with previous studies of dissolved organic matter in natural waters using ESI-FTICR-MS (Sleighter and Hatcher, 2008).

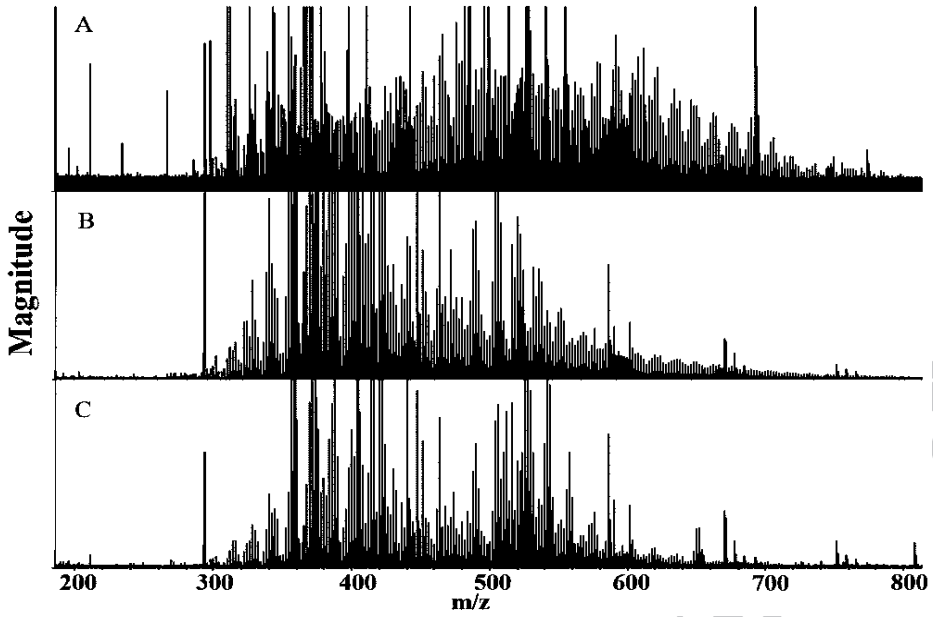


Fig. 2 Distribution of ions in (A) brown rotted wood (lignin control); (B) fungal-treated brown rotted wood after seven days; (C) *P. chrysosporium* control.

At first glance, the distribution of ions in the fungal-treated sample appears more similar to the fungal control which shows strong evidence of a sample dominated by fungal derived compounds. However, closer examination of the individual peaks for each of the samples provides a more valuable comparison to discern how fungal degradation of brown rotted wood modifies lignin and related substructures. As an example, Fig. 3 depicts an expanded (single nominal mass) region for peaks originating from lignin control (A), peaks that are generated after fungal degradation of brown rotted wood (B) and peaks originating fungal control (C). Water extraction of lignin (Fig. 3A) resulted in the assignment of 865 molecular formulas containing only C, H, and O atoms while 276 formulas were disappeared after 7 days of degradation. Besides, 303 new molecules (CHO) are produced.

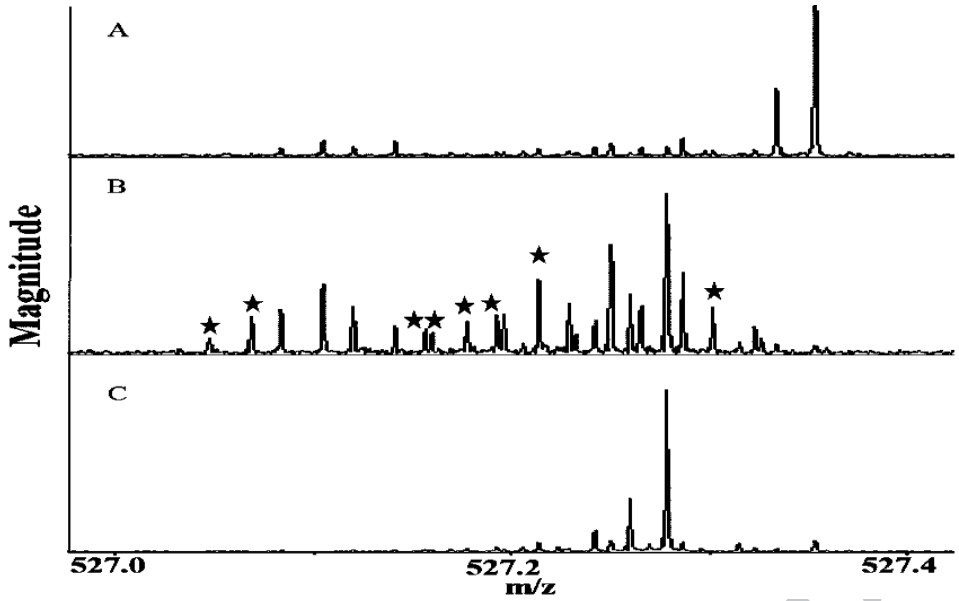


Fig. 3. Evaluation of individual peaks (A) brown rotted wood (lignin control); (B) fungal-treated brown rotted wood after seven days; (C) *P. chrysosporium* control (Peaks marked with star are new peaks).

From the comparisons in Fig. 3, one can readily observe that, in this nominal mass region (also observed in other nominal mass regions), the lignin subjected to fungal degradation contains peaks that are original to the sample, new peaks that are introduced from the fungal biomass, and new peaks that are not due to fungal biomass but represent degradation products of the original lignin sample. Moreover, some peaks present in the original sample (lignin control) completely disappear from this nominal mass region when subjected to the fungal degradation. Interestingly, a major portion of produced compounds that are not part of fungal biomass have mass defects less than 0.1 (their m/z is close to the value of the nominal mass). Compounds with a low mass defect (<0.1) typically have low amounts of hydrogen and/or high amounts of oxygen. These include compounds such as benzenecarboxylic acids or carboxylated condensed aromatics. Likewise, a higher mass defect (0.2 to 0.4) indicates the presence of compounds having higher amounts of hydrogen, and this includes lipids as well as alicyclic structures

(Sleighter and Hatcher, 2008). It is important to mention that these observations are apparent at most of the other nominal mass regions. Our results provide evidence for consumption of lignin-derived compounds and the production of new compounds that are not fungal in origin, some of which are aliphatic or alicyclic molecules and others are aromatic. However, evaluation of each individual peak to generalize a proposed mechanism is tedious.

Previous studies applied van Krevelen diagrams to observe the general trend of changes of all molecules during reaction (Kim et al., 2003). In these studies, peaks were reduced to elemental formulas whose ratios of H/C and O/C were plotted on a diagram to characterize molecular formulas for each sample being compared. Fig. 4 shows the van Krevelen plots for the water extracts from the original brown-rotted wood, the brown rotted wood subjected to seven days of fungal degradation, and fungi growth without the brown-rotted lignin. Based on the formula assignments we identified formulas in each sample that were unique to that particular sample and not observed in other samples. Fig. 4A shows all molecular formulas of water extracted brown-rotted wood that belong to the class of formulas containing only CHO atoms and there is no ambiguity in the assignment of that formula because we consider the ^{13}C isotope distributions to verify the assignment. Although the major portion of these molecular formulas are lignin-like molecules because they plot in a region of the diagram previously noted to be characteristic of lignin (Kim et al., 2003; DiDonato et al., 2016), the presence of some other formulas in regions ascribed to aliphatic molecules and oxidized lignin/tannins are observed. The presence of these molecules in the original sample is most likely related to the ability of brown rot fungi to partially degrade lignin and consequently modify lignin, consistent with previous suggestions (Hyde and Wood, 1997; Goodell et al., 2003). It is most interesting that the van Krevelen plot for the original brown-rotted wood shows two separate clusters, one for molecules

that have a lignin-like structural character, including that of oxygenated lignin, and another cluster for molecules that appear to be aliphatic in nature. They appear to plot in a region of the van Krevelen diagram that has previously been assigned to carboxyl-rich alicyclic molecules (CRAM) (Hertkorn et al., 2006; Stubbins et al., 2010). Interestingly, these aliphatic molecules plot in a region that is similar to that obtained from a fungal control (Fig. 4C) where *P. chrysosporium* were grown with a glucose substrate and no lignin.

Fig. 4B shows all formulas for fungal degradation of brown rotted wood after seven days by *P. chrysosporium*. In addition to the original formulas present in the brown rotted wood, some new formulas are observed. Many of these can be related to compounds produced by the fungi during degradation. Others represent molecules from the original lignin and new molecules resulting from modification of lignin subunits. In order to differentiate the original formulas from those of fungi and those new formulas produced by fungal degradation of the wood, a separate fungal control experiment was examined (Fig. 4C). Fig. 4D depicts newly produced CHO molecules that are not from the fungi and not from the original lignin after lignin degradation of the brown rotted wood.

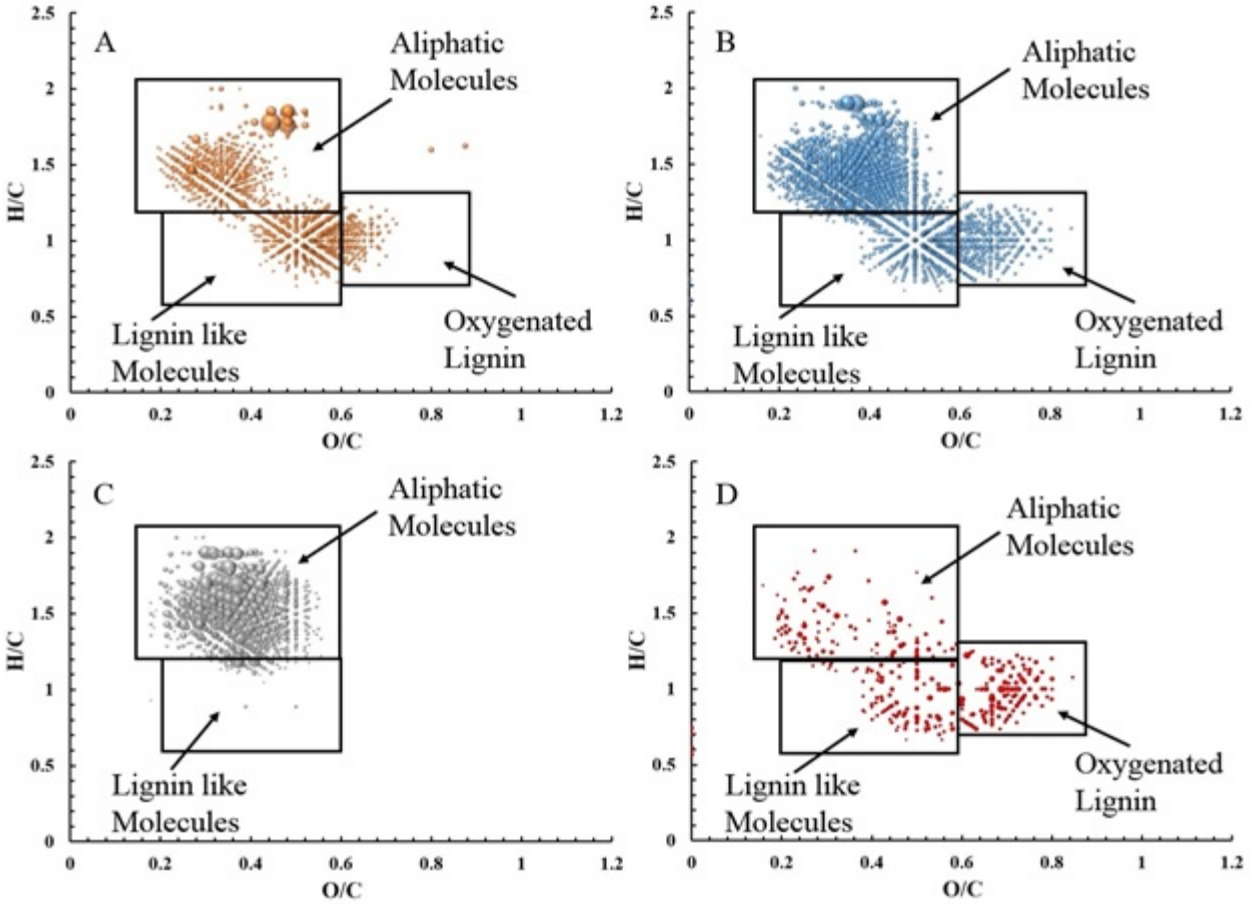


Fig. 4. Van Krevelen plots of CHO formulas from FTICR mass spectra of (A) water extracted brown-rotted wood lignin; (B) fungal degradation of brown-rotted wood lignin after seven days; (C) fungi growth without the brown-rotted lignin after seven days; (D) newly produced molecules by fungal degradation after seven days. The boxed-in regions are based on previous studies (Kim et al., 2003; Sleighter and Hatcher, 2007; DiDonato et al., 2016). Point's sizes (bubbles) are employed to indicate relative magnitudes of the peaks

Unlike the solid-state ^{13}C NMR data, the ESI-FTICR-MS analysis shows a major change after seven days of fungal degradation indicating that this technique is more sensitive to alterations by fungal attack than NMR. We conclude that this is the case because changes affect greatly the nature of molecules that might be rendered soluble in aqueous extracts by the actions of *P. chrysosporium*. After seven days of fungal degradation newly-molecules produced are

partly in a lower O/C and higher H/C region of the diagram, and mostly in the aromatic region of higher O/C.

We note also that Fig. 4D shows that few peaks remain in the aliphatic region of the van Krevelen plot. This can be an artifact of the subtraction process. It is also likely that the brown-rot fungi altered the original wood similarly (Hyde and Wood, 1997; Goodell et al., 2003) and such a peak would have already been present in the original brown-rotted wood. Because the subtraction process was limited to removal of peaks based on presence or absence, a modification of the kind mentioned above would not have been recorded and the modification would have led to the removal of the peak. Thus, the plot in Fig. 4D shows only compounds not original to brown-rotted wood and fungi. It is important to mention that the few formulas for newly produced molecules lying in the aliphatic region of the van krevelen diagram could arise from other components of brown-rotted wood not analyzed by the FTICR-MS. The white-rot fungi could have utilized these other components to produce new compounds that had an aliphatic composition.

KMD analysis of the elemental data was performed as reported previously by Grinhut et al. (2011) to identify trends relating to possible reactions that occurred during the fungal transformation of the brown-rotted wood. We chose to display multiple reaction trends (Fig. 5) which included 1) ring opening reactions (KMD O_2 series), 2) carboxylation/decarboxylation (KMD COO series), 3) methylation/demethylation (KMD CH_2 series), and 4) hydrogenation/dehydrogenation (KMD H_2 series).

Molecules whose elemental formulas lie on a horizontal line (same KMD) constitute part of a series separated in m/z by O_2, COO, CH_2, or H_2. In the original brown-rotted wood, KMD plots appear to show bimodal clustering of the KMD plotted against m/z. The upper cluster of

molecular formulas (higher KMD) are for aliphatic molecules while the lower cluster relate to aromatic molecules. This clustering is tied to the fact that molecules from the original brown-rotted wood exist as separate cluster entities that are either aromatic and structurally similar to lignin or are aliphatic and related to either fungally-derived structures or are produced by oxidative alteration of lignin by hydroxyl radicals typically produced by brown-rot fungi as suggested previously (Waggoner et al., 2015; Waggoner and Hatcher, 2017).

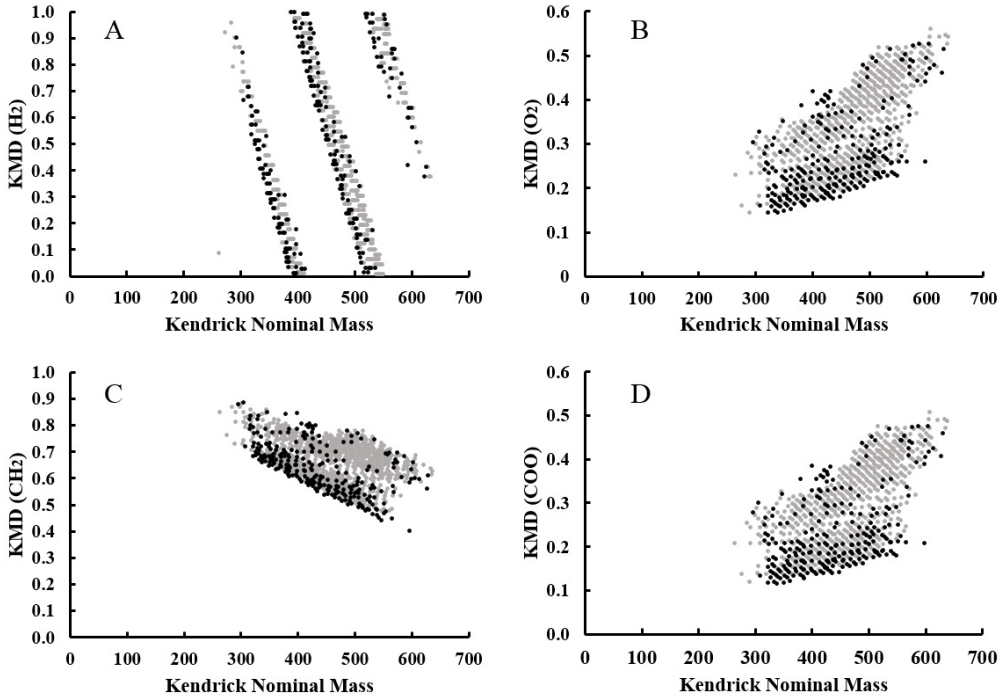


Fig. 5. Kendrick mass defect of (A) Hydrogen series, (B) Oxygen series, (C) CH_2 series and (D) COO series. (New formulas are black and initial formulas are gray)

KMD analysis of the new molecules produced upon fungal degradation of the brown-rotted wood (Fig. 5) appears to preserve this dual clustering observation. Admittedly, fewer formulas exist compared with the original wood extract but the dual clustering is faintly observable. For the KMD CH_2 and KMD H_2 series, there is a definite shift towards lower m/z values which shows side chain oxidation and aromatic oxidation of lignin like molecules,

respectively. However, for the KMD O_2 and KMD COO series, there is a definite shift towards higher m/z values for the series which refer to ring opening of aromatic rings and carboxylation of new molecules, respectively. This trend is consistent with a lignin transformation involving demethylation and carboxylation induced by ring-opening and polymerization as suggested previously. (Waggoner and Hatcher, 2017).

In soils, the major alteration pathway for lignin is microbial degradation by fungi. These organisms excrete extracellular enzymes that chemically attack lignin by generating radicals, in some cases through hydroxyl radicals (Hyde and Wood, 1997) or by direct electron transfer to lignin subunits (Goodell et al., 2003; Higuchi, 2004). On the other hand, Fenton chemistry and photo degradation are considered the other major modifications of lignin in nature. Previous studies have shown that lignin oxidized abiotically by hydroxyl radical oxidation (Fenton chemistry) displays new molecules produced in generally the same regions as is observed here (Waggoner et al., 2015). Likewise, abiotic photodegradation that generates hydroxyl radicals achieves similar transformations (Chen et al., 2014). It is clear from these previous studies and the current study that oxidative alteration of lignin produces a variety of new molecules that imply a modification that produces 1) oxygen addition to the lignin ring structures (increased O/C), and 2) ring opening followed by electrocyclic condensation to seed the production of aliphatic structures (increased H/C and decreased O/C). Condensation to produce condensed aromatic molecules (decreased H/C), observed in previous abiological studies (Waggoner et al., 2015: Waggoner and Hatcher, 2017), was not observed in this current study, perhaps because the lignin modifications occurred over a relatively short time period of seven days. Considering the similarity of some of the products when comparing hydroxyl radical-driven abiotic

transformations of lignin with enzyme-mediated transformations described here, it perhaps suggests that *P. chrysosporium* may generate radicals, or ROS, much like brown rot fungi.

4. Conclusion

The results presented here strongly suggest that lignin modified by fungi contribute to structures observed in SOM. The more highly oxygenated structures possessing higher O/C ratios are usually not observed in SOM but are apparent in fungal-degraded lignin. Recent studies suggested that such molecules are intermediates in the conversion of lignin to aliphatic and condensed aromatic structures in soil (Waggoner and Hatcher, 2017). Besides, it is proposed that oxygenation of the aromatic rings of lignin facilitates ring-opening reactions as suggested previously by Higuchi (2004). Once the ring is opened to produce conjugated olefins (Higuchi, 2004), these can undergo electrocyclic condensation to produce aliphatic molecules, most of which may be alicyclic as observed previously (DiDonato and Hatcher, 2017).

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Highlight

- ESI-FTICR-MS analysis opens a new window for examining the fate of lignin in nature.
- Fungal degradation of lignin plays an important role in the soil humification process by transformation of lignin-derived aromatic molecules to aliphatic molecules
- A similarity in biotic and abiotic oxidation of lignin to create new molecules is observed