Investigation of growth responses in saprophytic fungi to charred biomass

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Abstract

We present results of a study testing the response of two saprophytic white-rot fungi species, *Pleurotus pulmonarius* and *Coriolus versicolor*, to charred biomass (charcoal) as a growth substrate. We used a combination of optical microscopy, Scanning Electron Microscopy (SEM), elemental abundance measurements, and Isotopic Ratio Mass Spectrometry (IRMS) to investigate fungal colonization of control and incubated samples of Scots Pine (*Pinus sylvestris*) wood, and charcoal from the same species produced at 300°C and 400°C. Both species of fungi colonize the surface and interior of wood and charcoals over time periods of less than 70 days, however distinctly different growth forms are evident between the exterior and interior of the charcoal substrate, with hyphal penetration concentrated along lines of structural weakness. Although the fungi were able to degrade and metabolize the pine wood, charcoal does not form a readily available source of fungal nutrients at least for these species under the conditions used in this study.

Keywords

Charcoal, Fungi, Degradation, Biochar, Stable Isotope

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Introduction

Fire plays a central role in the dynamics of many ecosystems, with over 8.6 x 10^{12} kg of biomass burnt globally each year [1]. Consequently, fire strongly influences vegetation development and soil microbial function, driving patterns of succession and species composition [2]. A major product of biomass burning is charcoal, formed as a result of biomass pyrolysis when material is exposed to elevated temperatures (over c.300°C), where flaming combustion is inhibited by low oxygen availability. The importance of charcoal to global biogeochemical cycles is highlighted by the fact that at present, total global production of pyrogenically altered carbon (including charcoal) from biomass burning has been estimated at 50-270 Gt yr^{-1} [3]. Therefore, the products of biomass pyrolysis, including charcoal, make up an important proportion of many soils, comprising up to 35% of total organic content [4].

The molecular changes during production of charcoal, particularly reorganization of the carbon structure into condensed aromatic ring configurations [5] both raise the carbon content and the resistance to environmental degradation of charcoal relative to un-pyrolysed biomass. As a result, the mineralization rates of some forms of charcoal are extremely slow, apparently resisting degradation over geological timescales [1]. On the other hand, it is also apparent that some charcoal components undergo environmental degradation on decadal timescales (e.g. [6]). The potential environmental resistance to mineralization has resulted in a recent interest in the potential for net sequestration of C over extended time periods, via the large scale amendment of soils with charcoal, also termed 'biochar' [7]. A central question for both specific initiatives such as biochar, and for understanding of wider general ecosystem dynamics, is the interaction between charcoal and soil microbial communities.

Fungi are an important part of dynamic soil systems, with the majority of the over 80,000 presently known species spending at least part of their life-cycle in the soil [8], and fungal hyphae contents estimated in some environments as high as 67 km per 1g of dry soil [9]. Fungi therefore play an essential role in soil ecosystems. For example, the ramification of hyphae through soil has the beneficial effect of stabilizing soil structure, both through the adhesive properties of exo-polysacharides and the physical entrapment of soil particles [10]. These actions have significant influence on soil water infiltration

and water holding capacity. The fungi of interest in this study are the large group of fungi that function as primary degraders of organic material to forms more bioavailable for plants and other soil biota (saprophytic fungi).

There is a growing body of evidence that some fungal communities respond positively to the presence of charcoal in soils, for example, increased activity of arbuscular mycorrhizal fungi due to changes in soil chemistry has been demonstrated following charcoal additions [11]. However, direct empirical evidence for the mechanisms by which charcoal presence may enhance fungal growth is often lacking [12, 13]. Different hypotheses include the availability of C and nutrients from the charcoal surface [14], or the provision of micro-habitats for fungal communities within the char structure itself [15]. In the case of saprophytic fungi, many species are able to utilize aromatic macromolecules, such as lignin, as an energy source, by catabolic degradation with a variety of exoenzymes [16]. In addition to compounds in uncharred biomass, saprophytic species have also been observed to co-metabolise recalcitrant materials such as black shales and charcoal over relatively short timescales of <4 months [17]. However, questions remain surrounding the role of pyrolysed biomass as a growth substrate, and specifically whether fungi are able to utilize carbon from charred material as an energy source.

In this study, we investigate the response of two saprophytic fungal species to charcoal, produced under different pyrolysis conditions. These results feed directly into enhanced understanding and quantification of the role charcoal plays in global biogeochemical cycles via soil microorganism- charcoal interactions. In order to address these research goals, we have used a combination of stable isotopic analysis via Isotope Ratio Mass Spectrometry (IRMS), fungal growth analysis, and observation by Scanning Electron Microscopy. The combination of IRMS with visual techniques represents a new approach to studies of this type, and appears a promising methodological approach to deriving direct empirical evidence for fungi- charcoal interaction mechanisms.

1. Materials and methods

The approach used in this study involved quantitative examination of (i.) the effect on fungal growth of charcoal additions to a minimal nutrient medium, and (ii.) the

utilization of charcoal particles as a growth substrate by soil fungi. The charcoal was prepared from samples of Scots Pine (*Pinus sylvestris*), obtained from Tentsmuir Forest, Fife, in 2005. The bark was removed, and a sub-section of the first 15 rings was taken, and processed to 1.5cm cubes. Charcoal was prepared from the cubes using a Carbolite™ rotary furnace in an inert (N_2) atmosphere for a period of 60 minutes at either 300°C or 400°C, as described in [18]. Molecular structural differences between charcoals produced at these temperatures results from differential thermal degradation of wood structural components. Cellulosic material degrades very rapidly over 250-400°C, while lignin whereas slower degradation of lignin proceeds over the interval 200°C to 720°C [19, 20].

Two species of white-rot, lignin-degrading fungi were used in the study; *Pleurotus pulmonarius* and *Coriolus versicolor* which have a demonstrated ability to break down complex macromolecular lignin structures that contain a relatively large number of aromatic rings. Furthermore, the ability of these fungi to co-metabolise a range of macromolecules from polycyclic aromatic hydrocarbons (PAHs) and pentachlorophenols (PCPs) to tars and oils has also been reported [21, 22, 23, 24]. Therefore, it is hypothesised that the exocelluar enzymes produced by these fungi may also be capable of the biodegradation charcoal.

To determine fungal growth rate response, a study of the two fungal species was conducted on minimal essential media (MEM) [25] with differing carbon sources. The carbon sources were 0 and 0.5 % D-glucose, 0.5% 300°C powdered charcoal and 0.5% 400°C powdered charcoal. The base media components were selected in an attempt reflect the low levels of available nutrient for growth found in the charcoals. Fungi were incubated at 20 °C on the respective carbon sources for a period of 8 days, during which hyphal extension and morphology of filaments were assessed daily.

In the second experiment, charcoal blocks and uncharred blocks of pine wood were incubated with both fungal species for 70 days. At least 12 replicates were used for fungal incubations on charcoals, and 5 replicates for incubations on wood. The blocks were placed upon MEM agar, which contained a starting carbon (0.5 % D-glucose) and nitrogen (0.02% L-aspargenine) source to initiate fungal colonization. Prior to,

and following, the incubation period, the cubes were lyophilized and weighed to estimate the rate of decay of the blocks. In the case of *P. pulmonarius* this was accompanied by visual qualitative analysis of the degree of surface colonization following 56 days incubation. The extent and nature of fungal colonization of the blocks was investigated using scanning electron microscopy (SEM). Lyophilized blocks were split open, and the freshly split surfaces sputter-coated with Au. The outer and inner surfaces of the blocks were then examined at the St. Andrews University Facility for Earth and Environmental Analysis (FEEA), using a JEOL JSM-5410 scanning electron microscope at 10 kV accelerating voltage, with a variable working distance of between 15 and 28 mm.

Isotopic and elemental analyses were performed on samples of untreated wood and charcoal and of the material following fungal incubation. To obtain samples for analysis, dried blocks of both control and incubated material were split open across the centre and material for analysis was extracted to a depth of 2mm at a minimum of four points across the interior cross-section of each block, extending to 2mm from the outer edges of the block surface. Additionally, samples were obtained from the surface 0.5 mm of blocks that had been subject to *P. pulmonarius* fungal incubation (see Figure 2C). This ensured extraction of material with a homogeneous isotopic signal prior to incubation, excluding the effect of inter-ring isotopic variation within the original plant sample. Isotopic and elemental analyses were also made of both the D-glucose and L-Asparagine carbon and nitrogen sources and the two species of fungi separately grown on MEM without the addition of any charcoal. This allowed an assessment of the degree of fractionation between these substrates and the fungi, and of the isotopic signature of the fungi when these nutrient sources were accessed.

Stable isotopic ratios of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) were measured using continuous flow via a Costech elemental analyser (EA) fitted with a zero-blank autosampler, interfaced by a ConFlo II to a ThermoFinnegan Delta^{plus} XL at the University of St. Andrews FEEA. The carbon (%C) and nitrogen (%N) content of the samples was determined following combustion in the EA. Each sample was measured in duplicate, and individual machine runs included a mix of sample material, blanks and laboratory standards. Three internal standards were measured, where the primary standard was acetanilide (IAEA/Sigma Aldrich, %C: 71.09%, $\delta^{13}C$: -30.11‰; %N:

10.36, $\delta^{15}N$: -0.03), and secondary standards were a commercially available protein (B2155, %C: 47.02, $\delta^{13}C$: -26.98‰, %N: 13.63, $\delta^{15}N$: 5.94) and a C_4 cane sugar/uric acid mix (Tesco, %C: 40.20, $\delta^{13}C$: -12.02‰, %N: 9.1, $\delta^{15}N$:15.07). Results are expressed in standard notation ($\delta^{13}C$ and $\delta^{15}N$) as parts per thousand (‰) relative to Vienna PeeDee Belemnite (VPDB) for $\delta^{13}C$ and atmospheric N_2 for $\delta^{15}N$. Measurement precisions were better than 0.1‰ for $\delta^{13}C$, 0.3‰ for $\delta^{15}N$, 1% for %C and 0.05% for %N.

As it was not possible to both analyse a single block for elemental/isotopic composition, and also incubate the same block with the fungal species, it is likely that slight differences existed in the composition of the control and incubated blocks prior to fungal incubation. SEM analysis indicates that most colonization on the exterior of the blocks with the number of hyphae decreasing progressively into the interior of the blocks. Therefore, individual measurements in a single block are normalized to the composition obtained from the centre of each block to make the results comparable between blocks. This allows an assessment of the extent of variability within incubated and control blocks, and whether isotopic variations were randomly distributed throughout the blocks, or followed a spatial pattern.

2. Results

Figure 1 shows a comparison of the mean hyphal extension rates for each fungal species on each growth medium. Hyphal extension rates for both species were significantly higher for media with 0 and 0.5 % glucose than was the case for any media that included charcoal. In media without charcoal, *C. versicolor* appeared to show faster growth rates than *P. pulmonarius* and hyphal extension was greatest with 0% glucose for both fungi. However it is important to note that the growth forms on 0 and 0.5% glucose were very different. On 0% glucose, growth was very sparse, with few branched hyphae, typical of an exploratory growth form [26]. In contrast, much denser growth, typical of a more exploitative growth form, occurred on 0.5 % glucose.

Growth of fungal hyphae on the charcoal-containing media was generally very sparse, with a lag period of 3 days, and it was difficult to visualise the hyphae against the black coloured agar. The slow hyphal extension rate meant it was difficult to measure hyphal branching angles, however small fan-like flushes of hyphal growth were observed, suggestive of an exploratory growth form. There were differences between the two fungal species in growth response to the two different charcoal-containing media. In the case of *P. pulmonarius* there was a clearly faster growth rates on the media containing 300°C powdered charcoal compared to the 400°C powdered charcoal (Figure 1). In contrast, *C. versicolor* showed no significant difference in hyphal extension rate between the two charcoal treatments.

The weight gain recorded for some charcoal blocks following fungal incubation (Table 1) demonstrates colonization of the surface of the charcoal by a significant mass of fungi. However there is no evidence for removal of any significant proportion of the charcoal structure itself via fungal degradation. Weight losses in the wood blocks on the other hand, indicate that >9.50% by weight of the lignocellulosic structure has been metabolized over the fungal incubation period. Differences in colonization were also evident in visual analysis. After 8 weeks incubation, fungal growth of *P. pulmonarius* was visible on the outer surfaces of both the 300°C and

400°C charcoals, however, a greater degree of colonization had occurred on the 300°C charcoal blocks (Figure 2A and 2B).

Both species of fungi appear to be able to colonize the outer surface of both the wood and charcoal blocks (Figure 3, A-D). A relatively dense mass of branched, anastomosed fungal hyphae was observed on the outer surface of all samples, with some morphological features suggestive of an exploitative growth form. In the pine wood, there is evidence for substantial fungal degradation of interior structures within the blocks (Figure 4 A-B), with numerous cracks, small cavities, and holes in the cell walls that are not present in the non-fungal controls. In the charcoal blocks, similar evidence for structural degradation is absent, and penetration of hyphae in significant quantities into the interior of the blocks appears to occur mainly along pre-existing physical cracks and fissures that extend from the surface to varying depths. Fungal colonization of these involves a sparse, exploratory growth form (Figure 4 C-D). SEM comparison of the fungal colonization of the interior of the charcoal blocks again shows differences in colonization degree between 300°C and 400°C charcoal for *C. versicolor*, but a similar degree of colonization for the two charcoals by *P. pulmonarius*.

Internal %C variability through the control blocks is low, with a range of <0.45% in the wood and 300°C charcoal, and <0.85% in the 400°C charcoal (Table 2). Isotopic variation across the control blocks is also relatively small, with a variation of 0.2‰ in the 300°C charcoal, and 0.3‰ in the wood and 400°C charcoal. The nitrogen content of all three sample types is low; in wood and 400°C charcoal, this was lower than the measurement precision, however in the 300°C charcoal %N content averaged 0.3% (giving an average C:N ratio of 197), with a range of 0.04%. The $\delta^{15}N$ values of these samples had a range of 0.66‰ with an average value of -4.27‰ (Table 2). The internal isotopic and elemental variability in the control blocks is slightly greater than the instrument precision, and therefore the maximum range in values across control blocks is taken as the significance level for further interpretation. These values are ±0.85% for %C, ±0.31‰ for $\delta^{13}C$, 0.04% for %N and 0.66‰ for $\delta^{15}N$.

The $\delta^{13}C$ of the D-glucose is -11.25‰, and of the L-Asparagine is -24.23‰, while the $\delta^{13}C$ of *C. versicolor* and *P. pulmonarius* grown on MEM is -8.61‰ and -10.44‰

respectively, showing no significant incorporation of L-Asparagine carbon by the fungi (Table 2). The elemental composition of the two fungi species for C was 37.05% in *C. versicolor* and 41.54% in *P. pulmonarius*, and for N was 9.02 % in *C. versicolor* and 3.22% in *P. pulmonarius*. The D-glucose is obtained from C_4 sugars, that do not contain nitrogen, so the L-Asparagine ($\delta^{15}N$ = +4.23‰) comprises the entirety of the fungal ON source, as reflected in the $\delta^{15}N$ of fungi grown on MEM; +2.93‰ for *C. versicolor* and +3.08‰ for *P. pulmonarius*. This gives fungi $\delta^{15}N$ ~1‰ lower than that of the L-Asparagine, indicating that both species discriminate against uptake of ^{15}N when grown on this substrate, as previously observed [27].

Variation in %C increased following incubation with *P. pulmonarius* and *C. versicolor* in the wood and 300°C charcoal, for example variations of up to 2.57% were observed in the 300°C charcoal incubated with *C. versicolor*. However, in the 400°C charcoal %C variation was only slightly larger than the control samples (Table 2). Fungal incubation raised the %N of all samples to measurable levels, with the highest %N concentrations in the majority of samples were recorded on the outside of the blocks, where fungal hyphae concentration was greatest. Overall though, %N values across the tested samples remained relatively low.

After incubation with both *P. pulmonarius* and *C. versicolor* the range in $\delta^{13}C$ from the exterior to interior of the blocks showed an overall increase relative to the control samples, up to a maximum of 1.40‰ (Figure 5). Variability of $\delta^{13}C$ in charcoal samples incubated with *P. pulmonarius* increased relative to the control in both the 300°C and 400°C charcoal, whereas following incubation with *C. versicolor*, increased $\delta^{13}C$ variability was only observed in the 300°C charcoal. Increases in $\delta^{13}C$ variability appear to relate to a pattern of isotopic offset between samples from the exterior and interior of the blocks, however these are inconsistent in direction between sample types. For $\delta^{15}N$, there was considerable variation in values across the incubated blocks, with differences of up to 3.6‰ in $\delta^{15}N$ (Figure 6). Again, these variations appear to follow a pattern, with separation in values between the exterior and interior of the blocks, where values on the exterior tend to be higher. Average values of $\delta^{15}N$ in all samples decrease in the order wood > 300°C charcoal > 400°C charcoal (Table 2); for example, following incubation with *P. pulmonarius* the average sample $\delta^{15}N$ values are -3.04‰, -4.7‰ and -6.6‰, respectively. In all

samples following incubation, the highest $\delta^{15}N$ values occur on the outer surface of the blocks.

3. Discussion

Fungal colonization occurred after 70 days on the surface of all samples, with a particular concentration of fungal hyphae on the outer surface of the blocks, and it is only in this zone that an exploititative growth form is observed, indicating either a concentration of available nutrients on these surfaces, or that the substrate surface forms a favourable habitat for the fungi to exploit the D-glucose and L-asparagine C and N sources. Penetration of the fungi to the interior of the charcoal blocks appeared limited to physical cracks in the structure. The preferential exploitation of structures such as cracks in the charcoal blocks indicates heterogeneity in the presence of fungal hyphae within the blocks, rather than a progressive radiation of fungi from the exterior to the interior of the blocks. This suggests that colonization of charred organic material by saprophytic fungi may therefore occur more rapidly when the material contains greater amounts of cracks that the fungi can easily penetrate. The resulting spatial heterogeneity in fungal hyphae within the blocks observed by SEM imaging appears to correspond to the heterogeneity observed in the elemental and isotopic values within incubated samples. Overall, both image based, elemental and isotopic analyses suggest that colonization of the samples by the fungi exterior and interior colonization is distinctly different. During the three month period of this study, the majority of fungal growth occurred within the top few mm of the charcoal block surfaces.

Increased variability in measured isotopic and elemental values between the control and incubated samples suggests that for both species, fungal growth on different samples produced sufficient mycelium mass to be detectable by these techniques. In the majority of incubated samples, decreased %C at the block exterior is consistent with a greater contribution of fungal mass to samples taken from these locations. Increases in %N following incubation are also suggestive of the presence of fungal hyphae through the samples, however there is no consistent trend in values with location in the blocks. This may be due to the fact that the C: N ratios of the unincubated substrates are so high. The introduction of even a small proportion of

fungal mass, with a far lower C: N ratio would therefore produce a measurable increase in %N. However, as the %N values of the samples remain uniformly low, it is evident that hyphae comprise a relatively small proportion of the overall sample mass. This is consistent with observations by SEM of only limited penetration by the fungi to the block interiors.

Increased $\delta^{13}C$ variation across the blocks following incubation appears due to significant differences between the outer block, where fungal colonization is greatest, and the interior (Figure 5). In wood and 400°C charcoal following *P. pulmonarius* incubation, $\delta^{13}C$ values are higher on the block exterior, relative to the interior, consistent with a contribution from the isotopically heavier fungi. However, in 300°C charcoal incubated with either species, block exterior values tend to be lower than in the interior. In the case of $\delta^{15}N$, higher values on the block exteriors (Figure 6) are consistent with a contribution from fungi $\delta^{15}N$, which is significantly enriched in ^{15}N relative to the charcoal substrate. The greater observed variability in $\delta^{15}N$ relative to $\delta^{13}C$ in the incubated blocks (figures 5 and 6) may again result from the high C:N ratios of the unincubated control samples. C:N values for the fungi are 4.1 for *C. versicolor* and 12.9 for *P. pulmonarius*, compared to values of 181 to 215 in the control 300°C charcoal. As the %C of the control substrate is so high, a higher proportion of fungal mass would be required to significantly influence the $\delta^{13}C$ of the samples than would be required to influence $\delta^{15}N$.

Comparison of $\delta^{13}C$ and $\delta^{15}N$ values indicates apparent differences between the effect of fungal incubation on wood, 300°C and 400°C charcoal samples, as highlighted in figure 7, where several different sample groups are evident. This includes the control 300°C charcoal, and comparison of the scatter in values between this and the incubated sample groups again emphasises the higher isotopic variability of incubated versus control samples. In wood and 400°C charcoal following *P. pulmonarius* incubation, increases in $\delta^{13}C$ generally correspond to higher $\delta^{15}N$ values. In contrast, for 300°C charcoal incubated with both fungal species the trend appears to be in the opposite direction. The negative correlation between $\delta^{13}C$ and $\delta^{15}N$ in 300°C incubated charcoal samples, in contrast to the positive association in 400°C charcoal (Figure 7) may suggest variation in the isotopic composition of the fungi within samples, possibly as a function of resource partitioning within the hyphae.

Translocation of resources, including nutrients and water, within a continuous hyphal network enables fungi to colonize spatially heterogeneous substrates [28, 29]. This strategy may therefore be useful in colonization of substrates that are poor in certain resources, such as charcoal with a high carbon to nitrogen ratio.

The quality of a fungal substrate is determined by the concentration and physical availability of nutrients, as well their chemical form [29]. The results presented here suggest that among the tested materials, charcoal produced at 300-400°C is a low-quality growth substrate for both *P. pulmonarius* and *C. versicolor* fungal species. The results of the growth rate study, specifically differences in hyphal growth forms, demonstrate that that with 0% glucose the fungi are actively searching for energy sources by devoting resources to rapid development of few long hyphae, whereas with 0.5% glucose the fungi are devoting resources to development of a more branched hyphal network in order maximise resource capture from the nutrient agar plates. In addition, the observed growth forms within the charcoal blocks suggest that an exploratory form developed, and the hyphal penetration within the block is more actively engaged in seeking readily available nutrient sources, rather than in any utilization of the charcoal structure itself. This is supported by the absence of significant weight loss following fungal incubation, with overall increases in mass observed in some charcoal blocks. In contrast, some degradation of the wood structure appears to have occurred, with significant mass loss in the measured samples, and a clear difference in physical structural degradation revealed by SEM imaging of the incubated samples. The destruction of anatomical structures in the wood sample by fungal action contrasts with the apparent lack of evidence of structural degradation of the charcoal samples.

Pyrolysis results in alterations to the wood macro- and micro-structure, with progressive homogenization of the wood cell walls and disappearance of the middle lamella [30]. This occurs in conjunction with increases in overall average carbon abundances with increasing pyrolysis temperature, in this study from 46.0% in wood to 78.8% for 400°C charcoal. The result is decreasing structural heterogeneity as pyrolysis temperature increases, and it appears that this feature plays a major role in inhibiting growth of some fungal species when charcoal is used as a substrate. These results support the interpretation that changes in molecular structure induced by

pyrolysis of wood at temperatures of 300°C or more greatly inhibit the ability of these species of saprophytic fungi to enzymatically degrade charcoal, at least over the timeframe of this study. This is likely to be the result of lower bioavailability of the carbon structure of the charcoal itself, however it is also possible that pyrolysis produces certain substances that actively inhibit the activity of the fungi. Both theories have previously been suggested to explain reduction of fungal attack in wood following heat treatment [31].

4. Conclusions

It is clear from visual and SEM analysis that both species of fungi colonize the surface, and to a lesser extent, the interior, of both 300°C and 400°C charcoals over time periods of less than three months. Overall, it appears that a combined visual and elemental/isotopic approach is a promising methodology for examining fungal response to charcoal as a growth substrate. It is important to note that these results represent a relatively short term experiment, and future work should concentrate on determining whether over more extended time periods a more extensive degradation of charcoal by fungi occurs. However, the results presented here suggest that for these two common species of fungi, breakdown of the structure of charcoal itself does not supply a readily available source of nutrients. This is in contrast to previous work, which suggests that some saprophytic fungi species do degrade highly recalcitrant condensed aromatic structures (e.g. [17]), and highlights the fact that the diversity of microbial species and strategies in soils will likely result in a range of responses to charcoal. Rather than using the charcoal structure itself as a source of nutrients, it is also possible that colonization of the charcoal samples by the fungi could be the result of different nutrients (e.g. P, K, Ca) that are present on the charcoal surface as a result of pyrolytic degradation of the original biomass. Positive effects for soil saprophytic fungi communities consisting of species similar to the ones tested here are therefore more likely to result from pyrolysed material as a favourable habitat or source of nutrients, than direct utilization of the charcoal structure itself, even in material produced at relatively low temperatures (i.e. <300°C).

Further research within this field is clearly required in order to enhance understanding of interactions between fungi and charcoal in soils, not least because of the truly global importance of these within such systems. Future directions include a focus upon variation in the range of conditions for fungal incubation, such as temperature-dependent initiation of more rapid phases of fungal growth.

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Tables

Table 1: Weight changes within fungal decay study, showing changes in weight of samples of wood and two charcoals following fungal incubation with *Pleurotus pulmonarius* and *Coriolus versicolor*.

Species	***P. pulmonarius***			***C. versicolor***		
Media	**Before Incubation (g)**	**After Incubation (g)**	**% wt loss**	**Before Incubation (g)**	**After Incubation (g)**	**% wt loss**
Wood						
	2.622	2.220	15.332	2.392	2.264	5.351
	1.724	1.584	8.121	1.553	1.431	7.856
	1.756	1.608	8.428	1.688	1.572	6.872
	1.961	1.804	8.006	2.423	2.343	3.302
	2.336	2.157	7.663	2.654	2.577	2.901
300°C	1.219	1.218	0.082	1.235	1.235	0.000
charcoal	1.740	1.739	0.057	1.470	1.469	0.068
	1.904	1.909	-0.263	0.968	0.969	-0.103
	1.481	1.478	0.203	1.612	1.613	-0.062
	0.958	0.965	-0.731	1.227	1.229	-0.163
	1.496	1.503	-0.468	1.502	1.501	0.067
	1.190	1.194	-0.336	1.714	1.717	-0.175
	3.101	3.106	-0.161	1.339	1.344	-0.373
	0.865	0.870	-0.578	1.428	1.432	-0.280
	0.999	1.353	-35.435	1.361	1.363	-0.147
	1.590	1.597	-0.440	0.896	0.897	-0.112
	3.678	3.696	-0.489	0.763	0.763	0.000
400°C	0.326	0.333	-2.147	0.563	0.562	0.178
charcoal	0.522	0.527	-0.958	0.579	0.581	-0.345
	0.426	0.439	-3.052	0.483	0.484	-0.207
	0.381	0.383	-0.525	0.513	0.511	0.390
	0.476	0.481	-1.050	0.364	0.361	0.824
	0.391	0.399	-2.046	0.374	0.376	-0.535
	0.367	0.378	-2.997	0.601	0.602	-0.166
	0.430	0.439	-2.093	0.569	0.568	0.176
	0.340	0.350	-2.941	0.396	0.397	-0.253
	0.346	0.345	0.289	0.328	0.327	0.305
	0.484	0.500	-3.306	0.469	0.470	-0.213
	0.488	0.493	-1.025	0.547	0.548	-0.183

Table 2: Results of elemental and isotopic analysis of control samples and material following fungal incubation with *Pleurotus pulmonarius* and *Coriolus versicolor*. PW: pine wood, PC-300: 300°C charcoal, PC-400: 400°C charcoal. 1: Exterior block surface, 2: 1-2mm depth from top, 3: 7-8 mm depth from top, 4: 9-10 mm depth from top, 5: 7-8 mm depth from base, 6: 1-2mm depth from base.

Sample	**%C**	**$\delta^{13}C$**	**%N**	**$\delta^{15}N$**	**CN**
D-glucose	39.40	-10.98	-	-	-
L-aspargenine	35.89	-23.95	16.00	4.23	2.24
C. versicolor	37.05	-8.61	9.02	2.93	4.11
P. pulmonarius	41.54	-10.44	3.22	3.08	12.90
PW_2	45.75	-25.07	-	-	-
PW_3	46.09	-25.05	-	-	-
PW_5	46.19	-24.76	-	-	-
PW_6	46.06	-24.82	-	-	-
PW_ *P. pulmonarius* _1	44.26	-23.93	0.31	-1.42	143.01
PW_ *P. pulmonarius* _2	45.16	-24.71	0.10	-4.73	465.35
PW_ *P. pulmonarius* _3	46.44	-25.19	0.04	-2.98	319.62
PW_ *P. pulmonarius* _5	46.29	-25.28	-	-	-
PW_ *P. pulmonarius* _6	46.83	-25.33	-	-	-
PC-300_2	58.17	-25.37	0.28	-3.93	215.33
PC-300_3	58.34	-25.57	0.31	-4.56	189.59
PC-300_5	58.62	-25.52	0.29	-4.00	201.72
PC-300_6	58.54	-25.39	0.32	-4.59	181.64
PC-300- *C. versicolor* _2	56.75	-26.65	0.26	-0.36	217.19
PC-300- *C. versicolor* _3	56.05	-26.15	0.24	-1.43	238.54
PC-300- *C. versicolor* _4	58.25	-25.85	0.31	-3.81	187.10
PC-300- *C. versicolor* _5	57.77	-25.98	0.34	-1.92	174.46
PC-300- *C. versicolor* _6	56.51	-26.14	0.24	-3.28	241.77
PC-300- *P. pulmonarius* _1	60.84	-26.41	0.27	-2.27	223.12
PC-300- *P. pulmonarius* _2	60.49	-26.60	0.19	-4.98	324.66
PC-300- *P. pulmonarius* _3	61.48	-26.01	0.21	-4.56	293.13
PC-300- *P. pulmonarius* _5	61.42	-26.30	0.20	-5.88	311.94
PC-300- *P. pulmonarius* _6	61.63	-26.55	0.19	-5.67	321.19
PC-400_2	78.77	-27.70	-	-	-
PC-400_3	79.19	-27.93	-	-	-
PC-400_5	78.34	-27.63	-	-	-
PC-400_6	78.74	-27.94	-	-	-
PC-400- *C. versicolor* _2	70.51	-27.71	0.25	-4.74	289.89
PC-400- *C. versicolor*_3	70.33	-27.91	0.19	-6.35	376.27
PC-400- *C. versicolor* _5	70.50	-27.81	0.19	-6.49	368.28
PC-400- *C. versicolor* _6	70.93	-27.97	0.19	-7.25	368.02
PC-400- *P. pulmonarius* _1	72.36	-26.02	0.34	-4.61	214.37
PC-400- *P. pulmonarius* _2	72.50	-26.91	0.24	-7.90	303.24
PC-400- *P. pulmonarius* _3	73.49	-27.03	0.24	-7.59	306.32
PC-400- *P. pulmonarius* _4	73.14	-26.86	0.24	-6.75	310.06
PC-400- *P. pulmonarius* _5	73.36	-26.20	0.33	-6.68	223.17
PC-400- *P. pulmonarius* _6	72.58	-25.79	0.28	-6.03	257.21

Figure captions

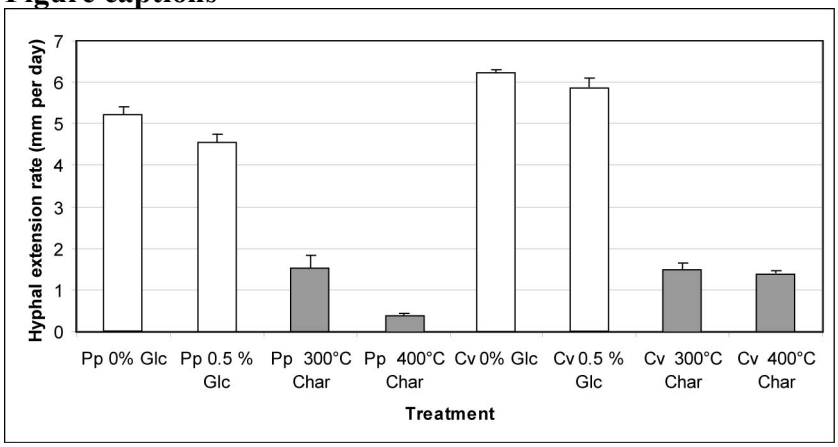

Figure 1. Charts showing comparison of mycelial extension rates of *Pleurotus pulmonarius(Pp)* and *Coriolus versicolor (Cv)* on minimal essential media (MEM) with differing carbon sources.

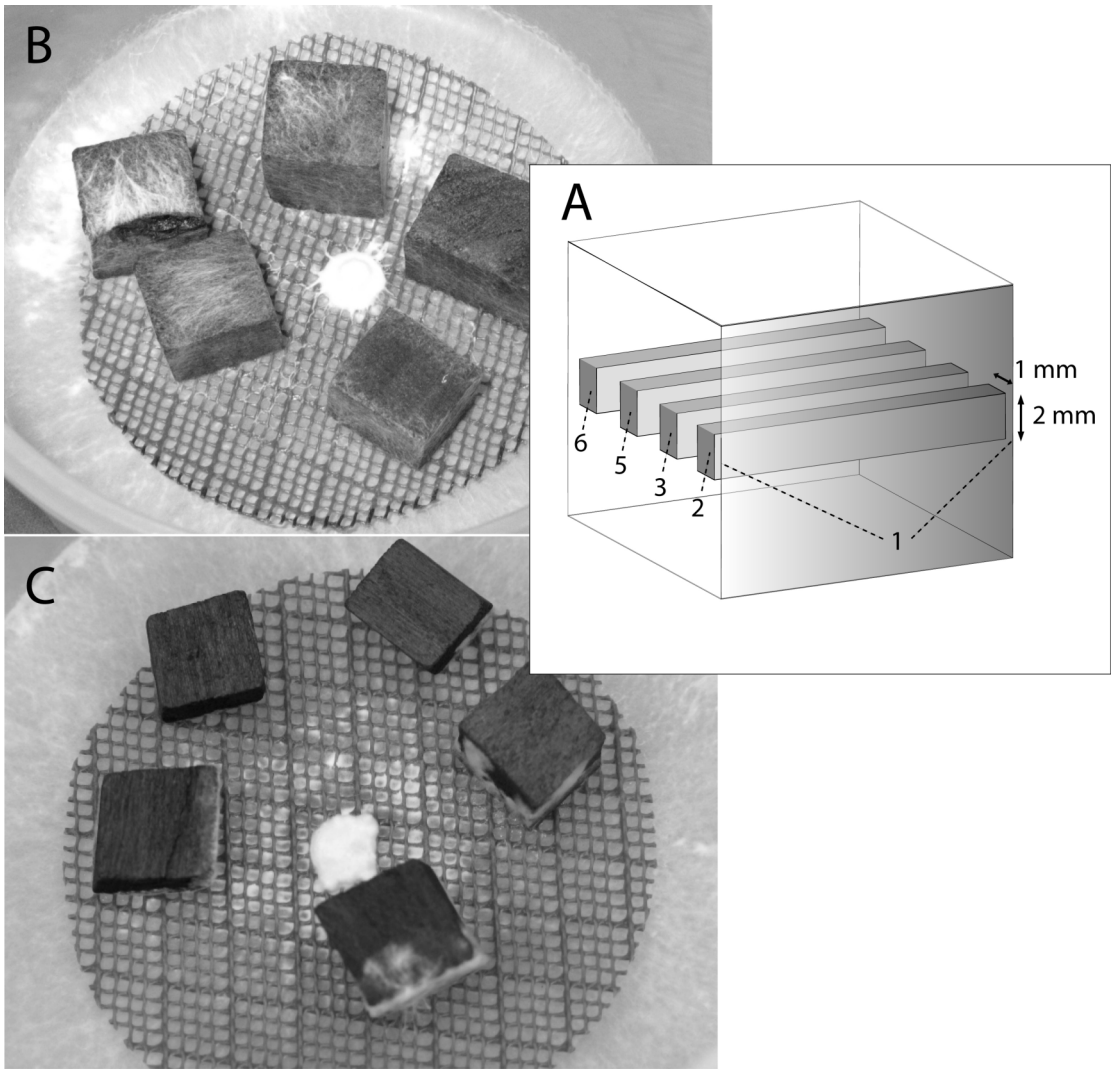

Figure 2: A. Charcoal blocks produced using furnace at 300°C inoculated with *P. pulmonarius* after 8 weeks incubation; B. Charcoal blocks produced using furnace at 400°C inoculated with *P. pulmonarius* after 8 weeks incubation; C. Schematic representation of sampling scheme for incubated and control blocks; homogenized sampled were obtained from the surface 0.5 mm (1), and at 1-2 mm and 7-8 mm depth from the top and bottom of each block (2, 3, 5, and 6, respectively). In two instances, a further sample was obtained at a depth of 9-10 mm within the block (4, not shown).

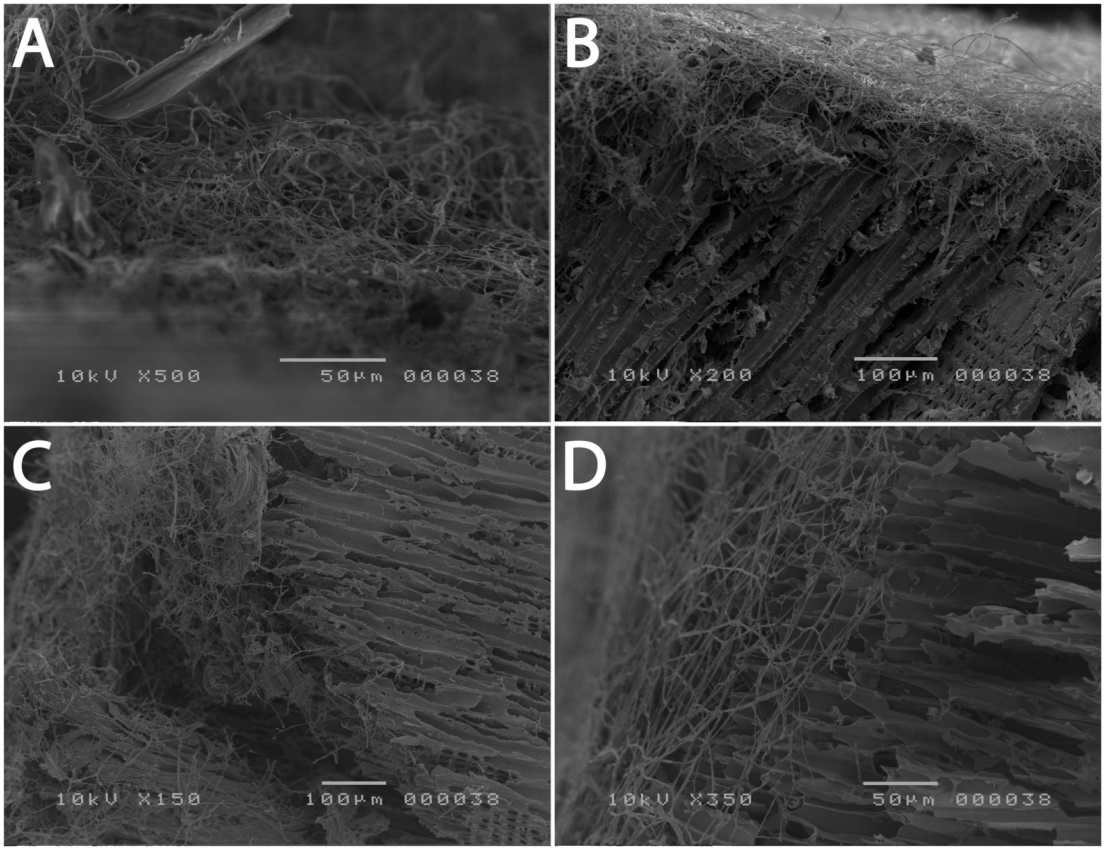

Figure 3: A: Surface colonization of pine wood by *P. pulmonarius*; B: Surface colonization of 400°C charcoal by *P. pulmonarius*; C: Surface colonization of 400°C charcoal by *C. versicolor*; D: Surface colonization of 300°C charcoal by *C. versicolor*

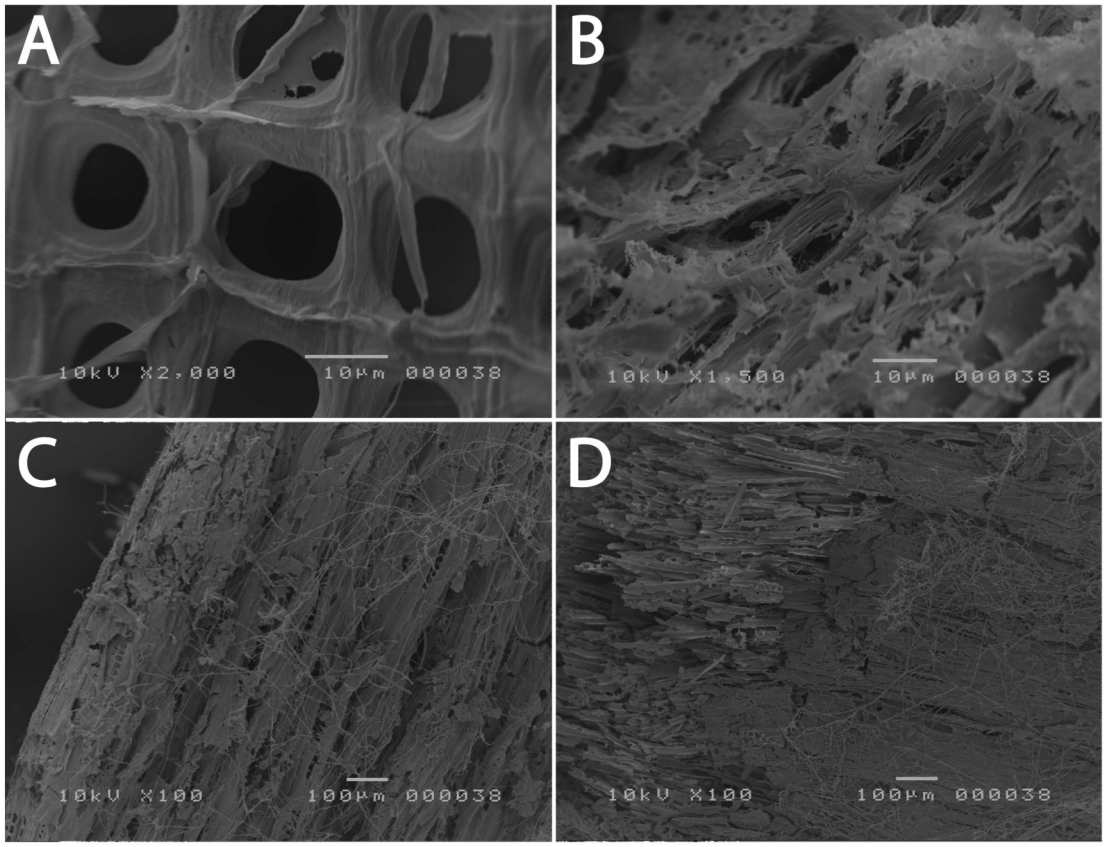

Figure 4: A: Interior structures of pine wood prior to fungal incubation, B: Interior structures of pine wood showing degradation by *P. pulmonarius* fungi following 3 months fungal incubation, C: growth structures of *C. versicolor* fungi on surfaces of fissures extending to the block interior in 300°C charcoal, D: growth structures of *C. versicolor* fungi on surfaces of fissures extending to the block interior in 400°C charcoal.

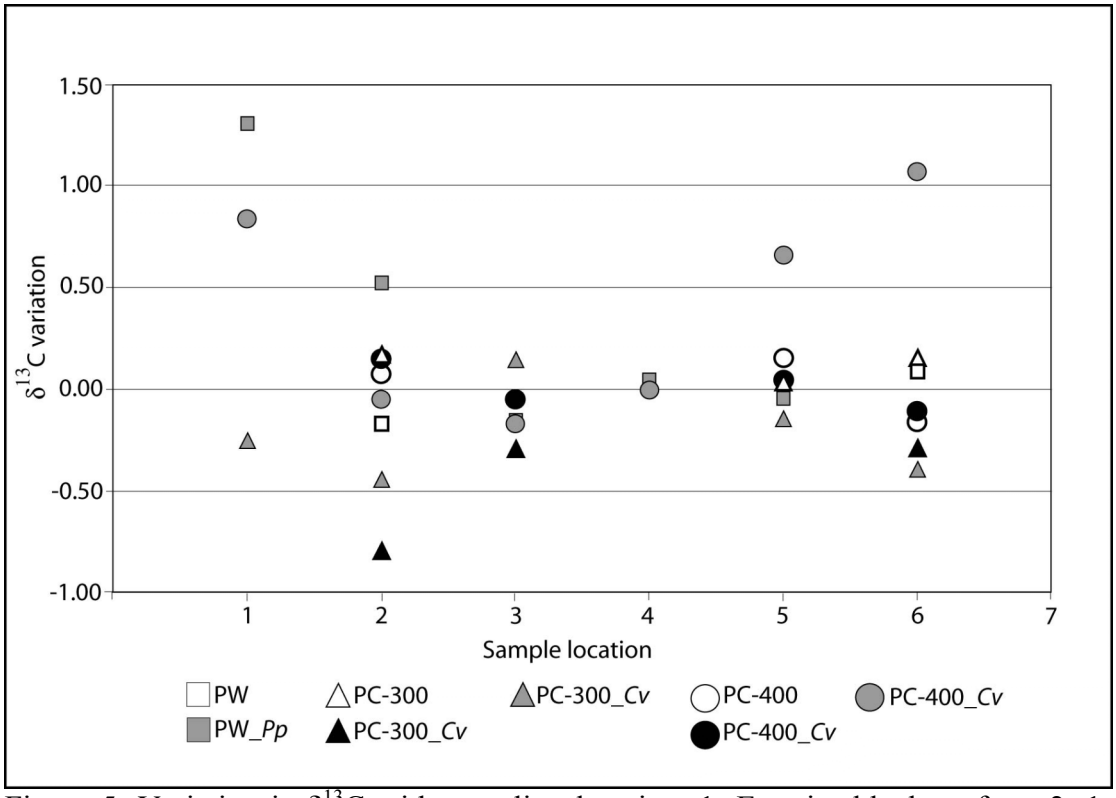

Figure 5: Variation in $\delta^{13}C$ with sampling location; 1: Exterior block surface, 2: 1-2mm depth from top, 3: 7-8 mm depth from top, 4: 9-10 mm depth from top, 5: 7-8 mm depth from base, 6: 1-2mm depth from base. PW = pine wood, PC-300 = 300°C charcoal, PC-400 = 400°C charcoal, Pp= *P. pulmonarius, Cv=C.versicolor.*

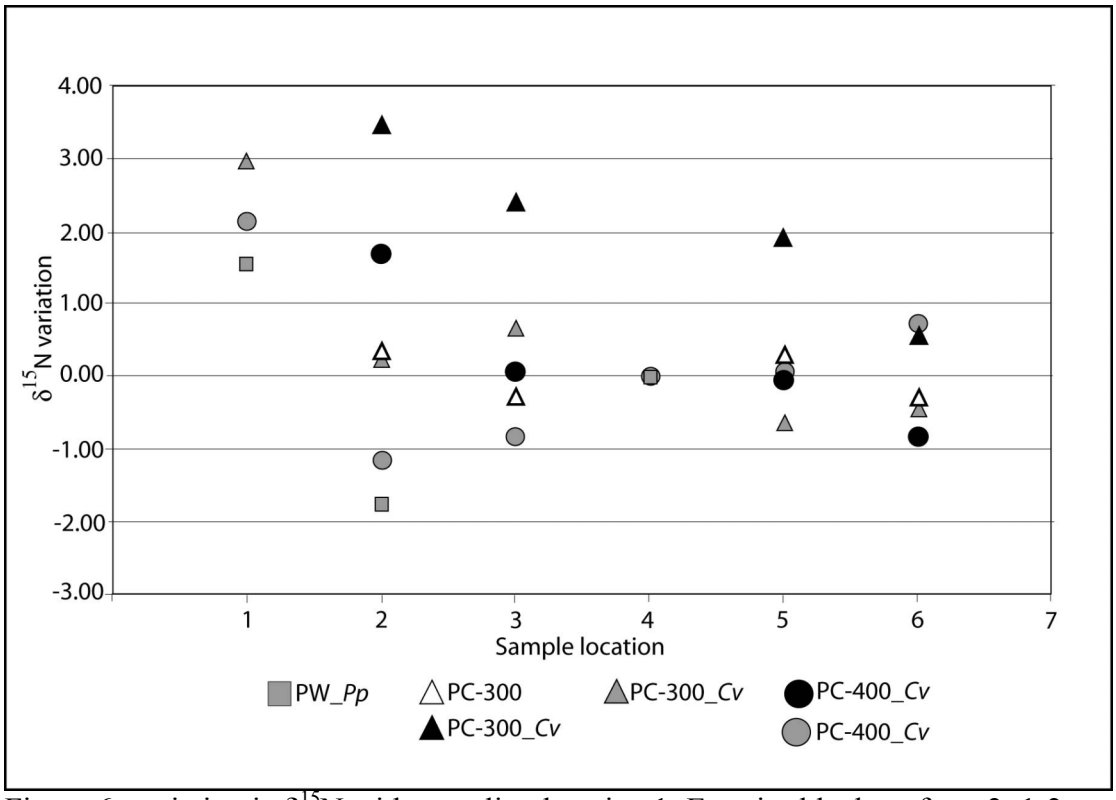

Figure 6: variation in $\delta^{15}N$ with sampling location 1: Exterior block surface, 2: 1-2mm depth from top, 3: 7-8 mm depth from top, 4: 9-10 mm depth from top, 5: 7-8 mm depth from base, 6: 1-2mm depth from base. PW = pine wood, PC-300 = 300°C charcoal, PC-400 = 400°C charcoal, Pp= *P. pulmonarius, Cv=C.versicolor.*

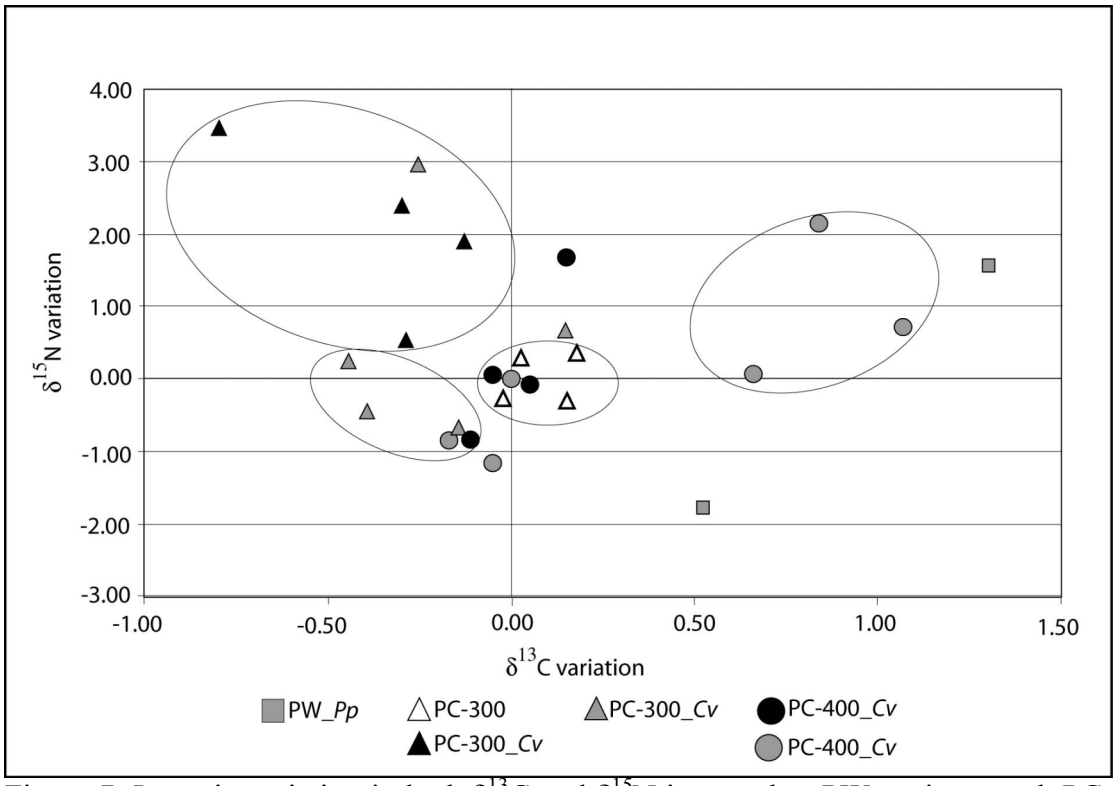

Figure 7: Isotopic variation in both $\delta^{13}C$ and $\delta^{15}N$ in samples. PW = pine wood, PC-300 = 300°C charcoal, PC-400 = 400°C charcoal. Circles show groupings of values for different substrates and fungi species, Pp= *P. pulmonarius, Cv=C.versicolor.*