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Assessing the fate and transformation of plant residues in the terrestrial environment using HR-MAS NMR spectroscopy

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Abstract

Plant litter decomposition plays a fundamental role in carbon and nitrogen cycles, provides key nutrients to the soil environment and represents a potentially large positive feedback to atmospheric CO_2. However, the full details of decomposition pathways and products are unknown. Here we present the first application of HR-MAS NMR spectroscopy on ^{13}C and ^{15}N labeled plant materials, and apply this approach in a preliminary study to monitor the environmental degradation of the pine and wheatgrass residues over time. In HR-MAS, is it possible to acquire very high resolution NMR data of plant biomass, and apply the vast array of multidimensional experiments available in conventional solution-state NMR. High levels of isotopic enrichment combined with HR-MAS significantly enhance the detection limits, and provide a wealth of information that is unattainable by any other method. Diffusion edited HR-MAS NMR data reveal the rapid loss of carbohydrate structures, while two-dimensional (2-D) HR-MAS NMR spectra demonstrate the relatively fast loss of both hydrolysable and condensed tannin structures from all plant tissues studied. Aromatic (partially lignin) and aliphatic components (waxes, cuticles) tend to persist, along with a small fraction of carbohydrate, and become highly functionalized over time. While one-dimensional (1-D)^{13}C HR-MAS NMR spectra of fresh plant tissue reflect compositional differences between pine and grass, these differences become negligible after decomposition suggesting that recalcitrant carbon may be similar despite the plant source. Two-dimensional ^{1}H–^{15}N HR-MAS NMR analysis of the pine residue suggests that nitrogen from specific peptides is either selectively preserved or used for the synthesis of what appears to be novel structures. The amount of relevant data generated from plant components *in situ* using HR-MAS NMR is highly encouraging, and demonstrates that complete assignment will yield unprecedented structural knowledge of plant cell components, and provide a powerful tool with which to assess carbon sequestration and transformation in the environment.

1. Introduction

Plant decomposition is dynamic and complex involving physical, chemical and biological processes and can be summarized as a rapid loss of the labile fractions followed by the slow degradation of more recalcitrant plant components (Latter et al., 1998; Gu et al., 2004). The ratio of labile and recalcitrant pools cannot be assumed to stay constant and this is due in part to the complexities of biopolymers such as lignin (Sanger et al., 1996; Ralph et al., 1997). Lignin is inherently recalcitrant due to its aromaticity but may also physically protect labile component structures within its polymer network, inhibiting short-term microbial degradation (Huang et al., 1998). Other recalcitrant polymers including suberin, resins and waxes may also react with secondary compounds (for example, oxidative condensation of amino acids and peptides with aromatic polymers including tannins, quinines and lignin (Rovira and Vallejo, 2002)) thus protecting nonliable constituents. The urgency for a more in-depth understanding of decomposition at the molecular-level results from the

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need to predict the effects of climate change on plant and soil carbon storage (Trumbore et al., 1996; Lal, 2004; Fang et al., 2005; Knorr et al., 2005).

Terrestrial plants are largely comprised of biomacromolecules such as lignin, carbohydrates, proteins, aliphatics/cutin, suberin and other waxes/resins that themselves have very different chemistries and thus will be degraded in the environment at differing rates. Furthermore, these components interact in complex assemblages that make predictions of decomposition processes difficult. In traditional decomposition studies, gross contents of macromolecular plant components are measured by extraction methods appropriate to the chemical characteristics of the plant constituent (Quarmby and Allen, 1986; Rutigliano et al., 1996). This type of analysis is useful but reveals little information as to the structural transformations taking place. In addition, extraction methods are not entirely specific for target biopolymers and co-extraction of other components often occurs.

A major advance in decomposition studies has been through the application of solid-state nuclear magnetic resonance (NMR) techniques such as cross polarization magic angle spinning (CP-MAS) spectroscopy that avoids the compromises of extraction and facilitates plant carbon chemistry analysis by allowing studies of intact, homogeneous plant samples and carbon constituents (Knicker and Ludemann, 1995; Knicker et al., 1996; Huang et al., 1998; McColl and Powers, 1998; Winkler et al., 2005). However, NMR is generally considered a relatively insensitive analytical technique because at natural abundance only 1% of the total carbon (^{13}C) and 0.37% of the total nitrogen (^{15}N) is NMR observable. Direct detection of ^{15}N is difficult due the low gyromagnetic ratio (the nucleus rotates relatively slowly in a magnetic field in comparison to carbon and protons) and an NMR receptivity that is only 0.022 as compared to ^{13}C (Mesnard and Ratcliffe, 2005). The complexity and multitude of components within a natural organic matter sample lead to extensive spectral overlap, especially in solid-state NMR (Hedges et al., 2000). Therefore, the transformations of minor components are difficult to monitor.

Increasing the relative abundance of ^{13}C and ^{15}N through isotopic enrichment greatly enhances NMR sensitivity and also intensifies through-bond and through-space couplings that occur between atoms that form the basis of structural identification (Knicker, 2002; Ippel et al., 2004). For example, in an experiment that measures C—C bonds, a signal will result at natural abundance when a ^{13}C nucleus (1% of the sample) is adjacent to another ^{13}C nucleus, which will only occur 0.01% of the time. Thus, introducing ^{13}C into the structures increases the sensitivity of ^{13}C–^{13}C experiments by ~10,000. Unfortunately, complex samples analyzed by solid-state NMR result in poor resolution and broad resonances rather then discrete signals. In samples highly enriched in ^{13}C, each ^{13}C nucleus splits the signals of its neighbors through dipolar (through space) and J (through bond) couplings. Thus in conventional 1-D solid-state experiments, this splitting leads to significant line broadening which in turn makes the extraction of structural information very challenging and in some cases impossible. Fortunately, the recently developed technique of semi-solid, High Resolution Magic Angle Spinning (HR-MAS NMR) can be employed to overcome challenges associated with pure solid-state NMR analyses. HR-MAS NMR allows the analysis of materials that swell, become partially soluble or form true solutions, to be analyzed at resolution close to that observed in solution-state NMR (Keifer et al., 1996; Millis et al., 1997). A solvent can be added to the analyte (in this case plant tissue) and after swelling, the components become NMR observable. Dipolar interactions that are predominant in solid materials are decreased by the addition of solvent and are averaged by spinning allowing solution-state experiments to be carried out on samples that are not fully soluble. Techniques for the study of labeled biomacromolecules in solution-state NMR are well developed. HR-MAS NMR allows the application of this vast array of NMR experiments to the study of plant components *in situ* and without chemical extraction.

The application of HR-MAS NMR to studies of plant biochemistry and physiology is in its infancy. Published papers outline how HR-MAS NMR can be used to provide detailed structural analysis that has not been possible with traditional analytical techniques. For example, detailed through-bond connectivities and assignments of structures in cuticle and algenan, two insoluble plant biopolymers, were generated by HR-MAS NMR (Fang et al., 2001; Deshmukh et al., 2003; Simpson et al., 2003). The quantitative nature of the HR-MAS NMR technique was also confirmed when it was found that the molecular mobility of a swelled cutin sample facilitated reliable quantitative analysis (Stark et al., 2000). HR-MAS NMR analysis has also been successfully applied to study the structural basis for the anti-oxidative behavior of plant extracts (Scheidt et al., 2004) and the quality of agricultural produce (Brescia et al., 2003). However, to the authors' knowledge this is the first account of HR-MAS NMR spectroscopic analysis of ^{13}C and ^{15}N isotopically enriched plant biomass.

In this study, we describe the HR-MAS NMR analysis of ^{13}C and ^{15}N enriched Lodgepole Pine (*Pinus contorta*) and Western Wheat Grass (*Agropyron smithii*). These species are commonly found in the grassland and forest regions of the Canadian Prairies and represent major carbon and nitrogen soil inputs in this region. The goal of this initial study is to monitor the transformations and loss of major biochemical components during a decomposition study and in turn better understand the processes involved in plant biomass decomposition and the potential transformation and accumulation of plant material in the environment.

2. Experimental

2.1. Plant material

Lodgepole Pine (*Pinus contorta*) and Western Wheat Grass (*Agropyron smithii*) seeds were stratified for 7 days

Table 1
Composition of hydroponic nutrient solution used for in vivo ^{15}N labeling of grass and pine plants

Nutrient	Nutrient source	Quantity (mg/L)
Nitrogen (^{15}N)	NH_4NO_3	250
Calcium	$Ca(NO_3)_2$	760
Potassium	KNO_3	122
Phosphorus	K_3PO_3	329
Sodium	H_2NaO_4P	72
Magnesium	$MgSO_4 \cdot 7H_2O$	246
Chlorine	KCl	74.76
Boron	H_3BO_3	30.9
Manganese	$MnSO_4 \cdot H_2O$	11
Zinc	$ZnSO_4 \cdot {}_7H_2O$	11.52
Copper	$CuSO_4 \cdot 5H_2O$	2.5
Molybdenum	MoO_3	5.76

The method is modified from solutions used by Jones (1983). ^{15}N was introduced into plants through isotopically labeled ammonium nitrate (NH_4NO_3), calcium nitrate ($Ca(NO_3)_2$) and potassium nitrate (KNO_3).

at 7 °C before planting in sterile quartz sand (oven dried at 110 °C and thoroughly washed with demineralised water) in an airtight plexi-glass tank within a Conviron E15 growth chamber (Controlled Environments, Asheville, NC). The seeds were exposed to 12 h of darkness (14 °C) and 12 h of light (18 °C, light intensity; 80 W m^{-2}) under a mixture of cool-white fluorescent and incandescent lights and a relative humidity of 70%. The chamber allowed automatic control of climatic conditions. ^{15}N-labeling of the plants, using the hydroponics solution outlined in Table 1, was accomplished by feeding the solution to the seeds through overhead piping in the tank. The pipes were feed through airtight holes cut in the plexi-glass at the top of the tank. The prevention of outside air getting into the tank was extremely important as the atmosphere within the plexi-glass tank was enriched with very expensive $^{13}CO_2$ gas (99% enrichment). This gas was introduced automatically (through a computer controlled valve) into the tank by the growth chamber when $^{13}CO_2$ levels fell below 100 ppm. Growth time was limited to the time it took to consume 100 L of the $^{13}CO_2$ gas which lasted ~2 months. At the end of the growth period the plants were harvested and stored at −78 °C.

2.2. Decomposition experiment

The incubation experiment was conducted as outlined in Fig. 1. The experimental design attempted to mimic natural conditions and enabled the collection of transformed and leached organic matter. Because the labeled plant material produced was very costly and in limited supply, it was not possible to bury the residues or even lay them directly on the soil surface, as this made it impossible to completely recover the biomass for NMR analysis. Instead glass funnels with borosilicate frits were submerged until flush with soil in two clay pots, one for the grass and another for the pine experiments. The soil used was sampled from areas where the native vegetation is Lodgepole Pine (a Brunisolic soil sampled near Hinton, Alberta) and Western Wheat Grass (an Orthic Brown Chernozem from South of Lethbridge, Alberta), organic carbon 23% and 2.1%, respectively (Otto and Simpson, 2005; Otto et al., 2005). The grass was separated into blades and roots and placed into different funnels while the pine needles were also isolated in separate funnels and sampled independently of the stems and roots. While living, roots are in direct contact with decomposer organisms and possess specific chemical properties that enable survival from microbial attack. It is therefore likely that the mechanisms of root decomposition will differ from that of above ground plant components. Furthermore, roots and rhizosphere comprise 10–40% of assimilated C transported during the growth of plants and exploration of the interface between plants and carbon transformation requires consideration of both above and

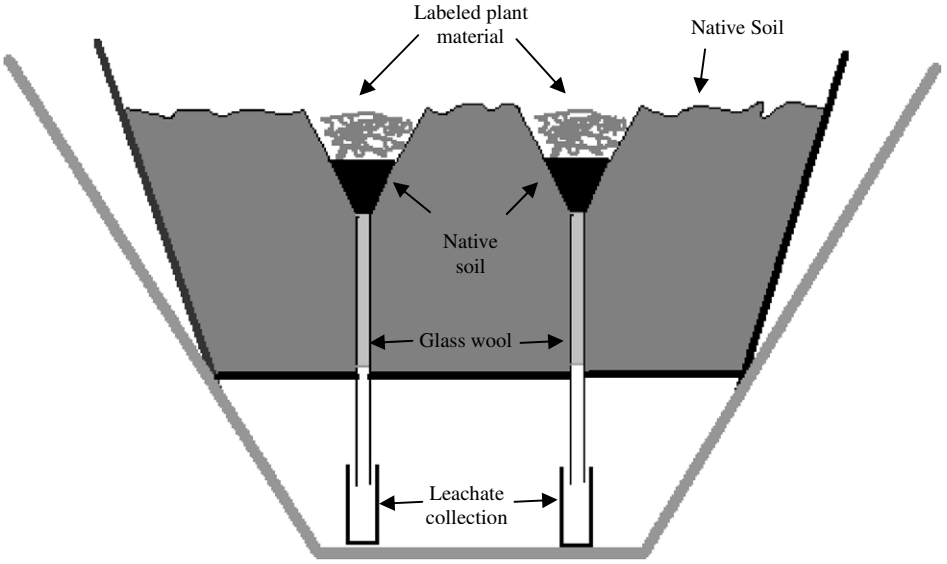

Fig. 1. Experimental setup for degradation study of labeled plant material.

below ground systems (Gorissen et al., 1995). We therefore considered their decomposition in isolation from the blades. Separation of pine needles from roots was difficult because of the presence of stems and the small volume of roots available after only two months growth. We therefore separated the pine into needles and stems/roots for the decomposition studies. The separated labeled plants (1. grass blades, 2. grass roots, 3. pine needles and 4. pine stems/roots, 1 g of each) were placed on glass frits inside designated funnels. This was to enable microbes in the soil (from surrounding surface soil and soil underneath (note the cavity beneath the fritted disk was also filled with soil see Fig. 1.)) to access the plant material. Attempts were made to place the materials directly on the soil surface and bury the materials. However only very limited amounts of labeled material were available for analysis, and it was impossible to fully recover the material, and/or distinguish the labeled material from the natural unlabelled residues that were in the soil. The soil and plants were sprinkled with water every day to mimic rain and the runoff was collected in a vial attached to the end of the funnel. Moisture levels were kept constant throughout the experiment. Runoff and plant samples were collected initially on a weekly basis and subsequently every month for a year (note analysis of the runoff will be considered in a future publication). After much deliberation it was decided due to the precious nature and limited availability of the plant material the decomposition study had to be performed indoors. We fully acknowledge that glass filtered sunlight does not completely represent natural light, and results may have differed if the experiment was carried out in the field. The apparatus was placed in an unheated area with windows such that the temperature and sunlight exposure would roughly mimic that of the outside environment. Note the average annual temperature in Scarborough Ontario is ~8 °C (where the study was carried out), while in Lethbridge, Alberta it is ~7 and ~2 °C in Hinton, Alberta. We acknowledge that the degradation rate of the plant material may increase due to the slight elevation in annual temperature. However, while it would have been possible to incubate the materials at the exact mean temperatures of Lethbridge and Alberta, physical effects, for example as a result freeze thaw cycles, would have been impossible to replicate in a controlled environment. Furthermore, given that Lodgepole pine and Western Native wheatgrass are native to the North U.S. and Canada and not just Alberta, the experiments were carried out at conditions in Scarborough, Ontario, which are closer the mean average temperature of the Northern U.S. and Canada combined. The incubation period stemmed over a twelve month period. Considering the small amount of starting material (1 g), change in porosity and state (solid—partially gel for some parts of the grass leaves), and unaccountable contributions from microbial biomass, it was not possible to obtain accurate dry mass for the labeled material at each time point during the study. Furthermore, attempts to transfer and weight the material led to losses of very precious material which was essential for NMR analysis. At the end of the study remaining material not required for NMR was weighed and back calculations indicated that >80% of all the starting materials had been decomposed during the study.

2.3. High resolution magic angle spinning (HR-MAS) NMR

Samples (~20 mg) were cut into small pieces, and placed in a 4 mm Zirconium Oxide Rotor and 60 μl of DMSO-d_6 was added as a swelling solvent. After homogenization of the sample using a stainless steel mixing rod, the rotor was doubly sealed using a Kel-F sealing ring and a Kel-F rotor cap. ^{1}H HR-MAS NMR spectra were acquired using a Bruker 500 MHz Avance spectrometer fitted with a 4-mm triply tuned ^{1}H–^{13}C–^{15}N HR-MAS probe fitted with an actively shielded Z gradient at a spinning speed of 10 kHz. ^{1}H NMR was acquired while simultaneously decoupling both ^{13}C and ^{15}N nuclei. Scans (256) were acquired with a 2 s delay between pulses, a sweep width of 20 ppm and 8 K time domain points. ^{1}H Diffusion Gated Experiments were used with a bipolar pulse longitudinal encode–decode sequence (Wu et al., 1995). Scans (1024) were collected using a 1.25 ms, 333 mT m^{-1} sine shaped gradient pulse, a diffusion time of 30 ms, 8192 time domain points and a sample temperature of 298 K. In essence the ''gate'' was optimized at the strongest diffusion filtering possible while minimizing signal loss through relaxation. As a result the more rigid components dominate the transformed spectrum while mobile components are essentially gated.

^{13}C spectra were collected in different modes, including inverse gated ^{1}H and ^{15}N decoupling, and conventional decoupling during acquisition (both ^{1}H and ^{15}N). Due to the strong signal from the labeled carbon, both approaches yielded the same spectrum. The inverse gated spectra are shown here. Scans (16 K) were acquired with a delay of 5 times that of the measured T_1 relaxation (commonly resulting in a delay of ~4 s (note: ^{13}C relaxation was fast as a result of ^{13}C–^{13}C interactions)), a sweep width of 300 ppm and 16 K time domain points. The spectra were processed with a zero-filling factor of 2 and an exponential multiplication, which resulted in a line broadening of 1 Hz in the transformed spectrum. One-dimensional ^{15}N spectra were acquired with and without DEPT enhancement. Scans (128 K) were performed with a recycle delay of 5 s, 32 K time domain points, a sweep width of 1000 ppm, and decoupling of both ^{1}H and ^{13}C during acquisition. The spectra were processed with a zero-filling factor of 2 and an exponential multiplication, which resulted in a line broadening of 10 Hz in the transformed spectrum.

^{1}H–^{13}C Heteronuclear Single Quantum Coherence (HSQC) spectra were collected in phase sensitive mode using Echo/Antiecho-TPPI gradient selection and sensitivity enhancement. Scans (8) were collected for each of the 128 increments in the F1 dimension. Two kelvin data points were collected in F2, a 1J ^{1}H–^{13}C (145 Hz) and a

relaxation delay of 2 s were employed, ^{15}N and ^{1}H were decoupled during acquisition. Similar conditions were employed for ^{1}H–^{15}N HSQC except 16 scans, 1J ^{1}H–^{15}N (90 Hz), were used with decoupling of both ^{13}C and 1 H during acquisition. For all HSQC spectra both dimensions were processed using sine-squared functions with phase shifts of 90° and a zero-filling factor of 2. Numerous additional NMR experiments were acquired but have not been shown here, including ^{1}H–^{13}C HSQC-TOCSY (2-D and 3-D), ^{13}C–^{13}C INADEQUATE, ^{1}H–^{13}C ADEQUATE, and ^{1}H–^{13}C long range HMQC. While their detailed interpretation will be the focus of future work, we would like to point out that the basic assignments are consistent with the full suite of multidimensional NMR experiments acquired.

3. Results and discussion

The 1-D ^{13}C HR-MAS NMR spectra of the four fresh samples are presented in Fig. 2. Spectral regions in Fig. 2 have been assigned (Orem and Hatcher, 1987; Wilson, 1987; Preston et al., 1994; Golchin et al., 1996; Kaiser and Zech, 1997; Guggenberger et al., 1998) and confirmed using a full range of multidimensional HR-MAS NMR experiments (data not shown). These regions, highlighted by brackets, are applicable to all ^{13}C NMR spectra and can be defined broadly as; (1) carboxylic, carbonylic and amide carbon, (2) aromatic carbon, (2a) double bonds, (3) carbohydrates including the anomeric carbon, aliphatic linkers in lignin structures, units adjacent to esters, and ethers and alcohols (such as those in cuticles and waxes), noting that some amino acids residues in proteins will resonate here, (4) methoxyl groups and (5) aliphatic carbon including that in aliphatic rings and chains and methyl groups bound to carbon. The four spectra exhibit high spectral resolution and excellent signal to noise ratio (~100 times that observed in the unlabeled material). Even in heterogeneous plant biomass it is clear to see that the resolution afforded by HR-MAS NMR allows the materials to be clearly distinguished. Due to the complexity of the data, complete assignments of the individual peaks is time consuming. However, once key components can be identified, the extraordinary resolution of HR-MAS will allow the fate of individual plant components (for example, specific lignin subunits, and linkers) to be traced, rather than simply observing the relative increase or decrease of lignin as a whole. As such, HR-MAS provides the potential

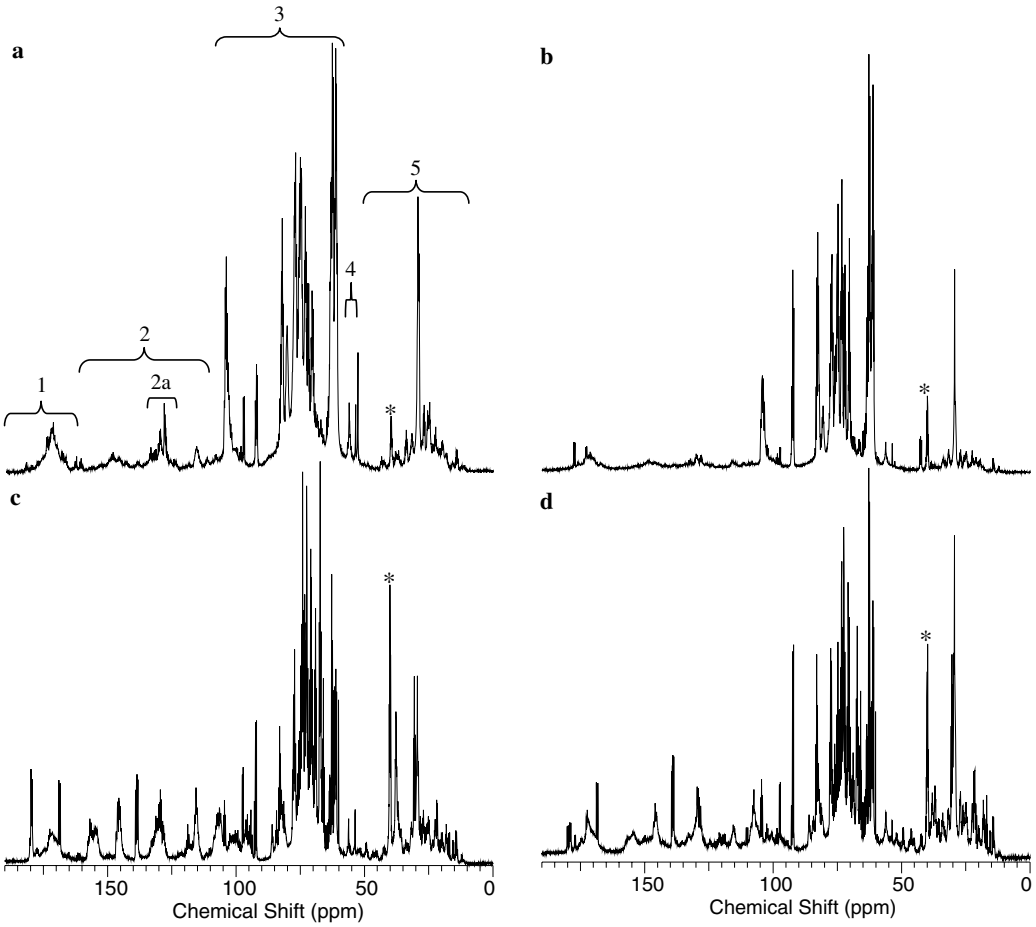

Fig. 2. 1-D ^{13}C HR-MAS NMR spectra of fresh grass blades (a), grass roots (b), pine needles (c) and pine stems/roots (d). The highlighted regions should only be used as a reference as to the predominant species in each area. Smaller contributions from other species are present in some regions. General regions are assigned in the text. *NMR solvent (DMSO $-d_6$).

to understand and monitor carbon sequestration and transformation, with precision and structural detail which until now has been impossible. In this preliminary publication the major categories of plant structures are followed over time, namely protein, aliphatic biopolymers, lignins, tannins, and carbohydrates. The excellent spectral resolution in HR-MAS permits this with increased accuracy and confidence. In select cases, the additional dispersion and structural information provided by multidimensional NMR is used to help distinguish, for example, between condensed and hydrolysable tannins, as well as identify unique nitrogen containing structures that are either selectively preserved or synthesized during humification.

3.1. Carbon transformations

After the first month of incubation, considerable changes to the plant biomass were notable. The 1-D ^{1}H HR-MAS NMR spectra for the grass blades at day zero (fresh) and after one month are shown in Figs. 3a and b, respectively. General regions highlighted by brackets are applicable to all ^{1}H NMR spectra and can be broadly defined as; (1) mainly aromatic residues. Note some amide signals in proteins/peptides also resonate in this region and in others. However evidence from many 2-D experiments indicate that while some peptides/proteins are present their signals in the 1-D spectra are swamped by the presence of more abundant species, carbohydrates, aliphatic chains, tannins etc. As such only the dominant species are noted explicitly in the 1-D assignments offered here, specific species assignments will be discussed in the text, where appropriate, (2) a mixture of signals from a numerous moieties including anomeric protons in carbohydrates, protons associated with esters, and double bonds, and protons in tannins, (3) predominantly signals from the carbohydrates, (4) signals from various substituted methylenes, and methines β to a functionality in hydrocarbons, (5) CH_2, main chain methylene in lipids, waxes, cuticle etc., (6) terminal CH_3 groups. Both spectra are dominated by carbohydrates (Fig. 1a), but with considerable contributions from aromatic species and aliphatic chains. After 1 month (Fig. 3b) the relative intensity of the aliphatic material (region 4) is much greater than that in the starting material. This is likely due to the slower decomposition of aliphatic materials such as waxes and cuticular materials (Winkler et al., 2005). Furthermore, the carbohydrate peaks display much sharper resonances (Fig. 3b) than those observed in the starting material (Fig. 3a). Sharper lines observed in NMR are often characteristic of smaller structures, and this may indicate the breakdown of the carbohydrates from large polymeric structures into smaller fragments. To test this hypothesis we applied diffusion edited experiments to the fresh and decomposed plant biomass. In diffusion edited NMR experiments small molecules are essentially gated

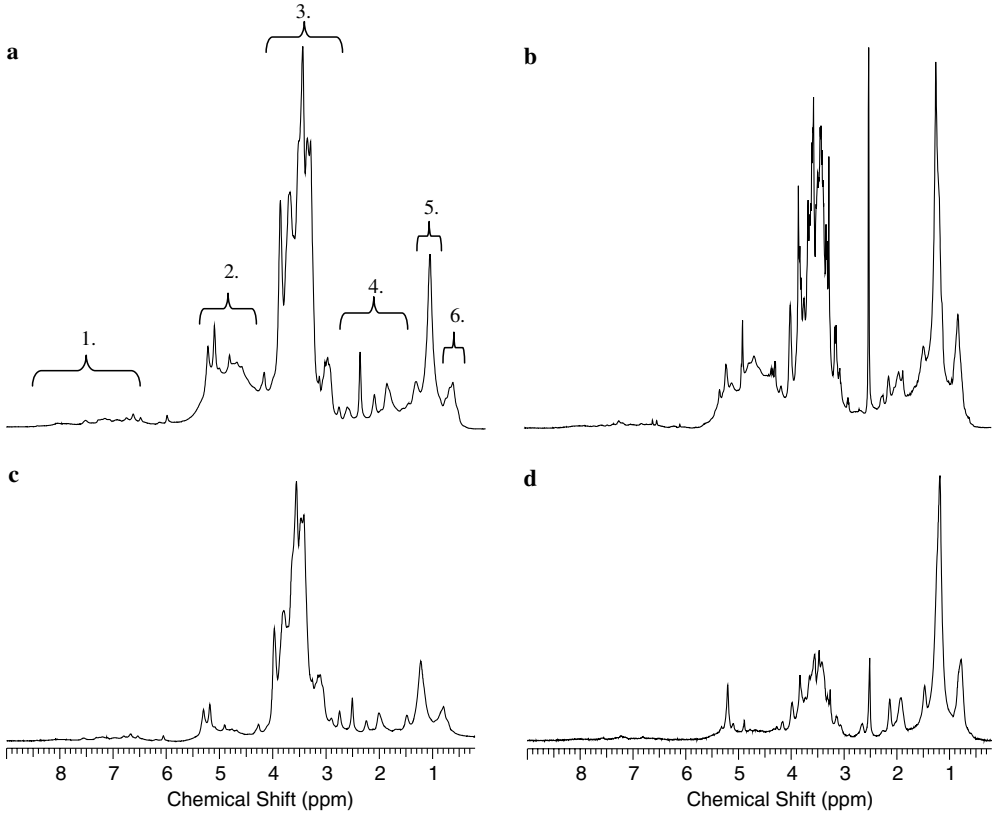

Fig. 3. 1-D ^{1}H HR-MAS NMR (a) and (b) and diffusion edited (c) and (d) spectra of fresh and one month decomposed grass blades, respectively. General regions highlighted by brackets are applicable to all spectra and are assigned in the text.

from the final spectrum but signals from macromolecules which display little translational diffusion are not gated and appear in the spectrum (Simpson et al., 2003). Figs. 3c and d illustrate diffusion edited spectra of the fresh and decomposed grass blades, respectively.

The similarity between the 1-D ^{1}H and diffusion edited HR-MAS NMR spectra of the fresh blades (Figs. 3a and c, respectively) suggests that the majority of components are macromolecular and/or rigid and exhibit little, if any translational diffusion. However, in the case of the decomposed grass blades (Figs. 3b and d) the aliphatic chains clearly dominate the diffusion edited spectrum indicating they are still preserved in rigid domains, whereas the relative intensity of the carbohydrate signals have declined suggesting a larger contribution from smaller units that have some translation diffusion. These data indicate that after just one month many carbohydrates have degraded into smaller units and are thus are highly susceptible to mineralization and rapid conversion to CO_2 (Stout et al., 1988; Kogel-Knabner, 2002).

The 1-D HR-MAS NMR spectra provide substantial detail; however, spectral overlap from the multitude of compounds makes specific structural assignments difficult. Two-dimensional (2-D) HR-MAS NMR experiments spread signals into a second dimension, thereby decreasing the overlap and providing information as to the connectivity's between nuclei in mixtures (Simpson, 2001; Hertkorn et al., 2002). ^{1}H–^{13}C Heteronuclear Single Quantum Coherence HR-MAS NMR (HSQC) is an experiment that detects H—C bonds within a structure (Ernst et al., 1987). A cross-peak in an HSQC spectrum represents the chemical shift of both carbon and proton atoms in a C—H unit. When considered together, the cross-peaks form a specific pattern or "molecular fingerprint" of a specific structure or class of structures. For example, the HSQC HR-MAS NMR spectra of fresh pine needles and pine needles after 12 months of decomposition are presented in Figs. 4a and b, respectively. Basic assignments for the major structural units of each chemical category as highlighted (regions 1–5) are; (1) C—H bonds from various aliphatic structures including fatty acids and amino acids, (2) carbohydrates, (3) anomeric carbohydrates, (4) double bonds and (5) aromatic fragments. More specific assignments are; (6) aromatic methoxyl (predominantly lignin derived), (7) cyclic hydrocarbon structures such as steroids and triterpenoids, and (8) α-protons/carbons in peptides/proteins. These assignments can be verified by investigation of the HSQC spectra of actual biopolymers representing the major structural classes, namely lignin compounds, proteins, carbohydrates, lipids and tannins (Fig. 5), as well as from other 2-D experiments such as long range ^{1}H–^{1}H, and ^{13}C–^{13}C couplings (data not shown). General assignments as to the major structural classes are given in the Figure caption (see Fig. 5).

The most dominant feature in the comparison of the spectra (Figs. 4a and b) is the near total disappearance of cross-peaks due to tannins. Tannins could not be detected by ^{1}H–^{13}C HSQC HR-MAS NMR after 6 months of incubation but were present in all spectra prior to that time (data not shown). The presence of a C_4 linkage in the tannins (Fig. 4a) suggests that the predominant form is that of condensed, nonhydrolysable tannin. This is in agreement with reports that demonstrate that gymnosperms and monocots produce condensed tannins only (Bate-Smith and Metcalfe, 1957; Haslam, 1988). There are many uncertainties as to the chemical nature and fate of tannins in soil (Lorenz and Preston, 2002). Tannins have previously been shown to rapidly disappear from black spruce needles

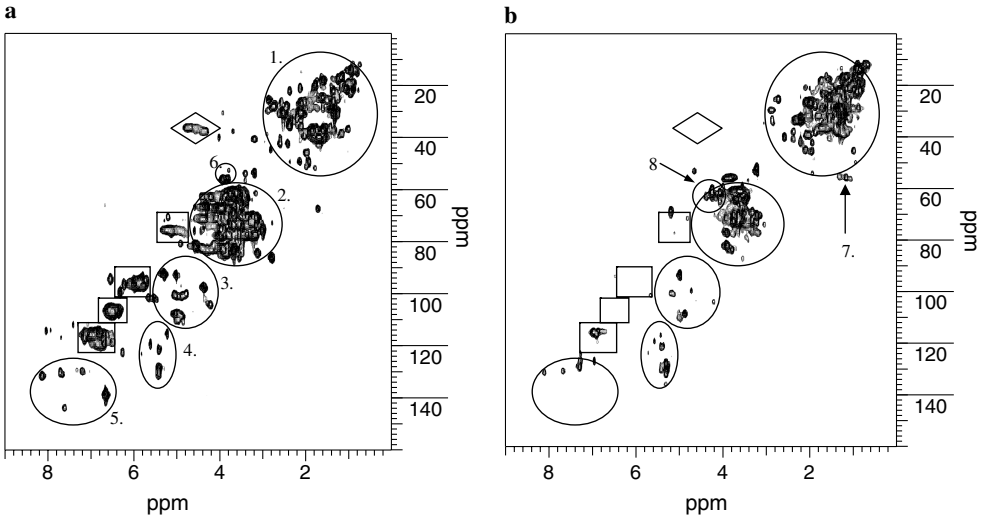

Fig. 4. HR-MAS NMR HSQC spectra of fresh pine needles (a) and pine needles after 12 months of decomposition (b). The highlighted regions 1–5 represent general assignments of the major structural classes and are assigned in the text. Cross-peaks in squares represent tannin structures whereas the diamond indicates the C_4 carbon specifically in condensed tannins (see Fig. 5). General and specific assignments have been assigned using a full range of multidimensional NMR experiments and the use of standards as outlined in Fig 5.

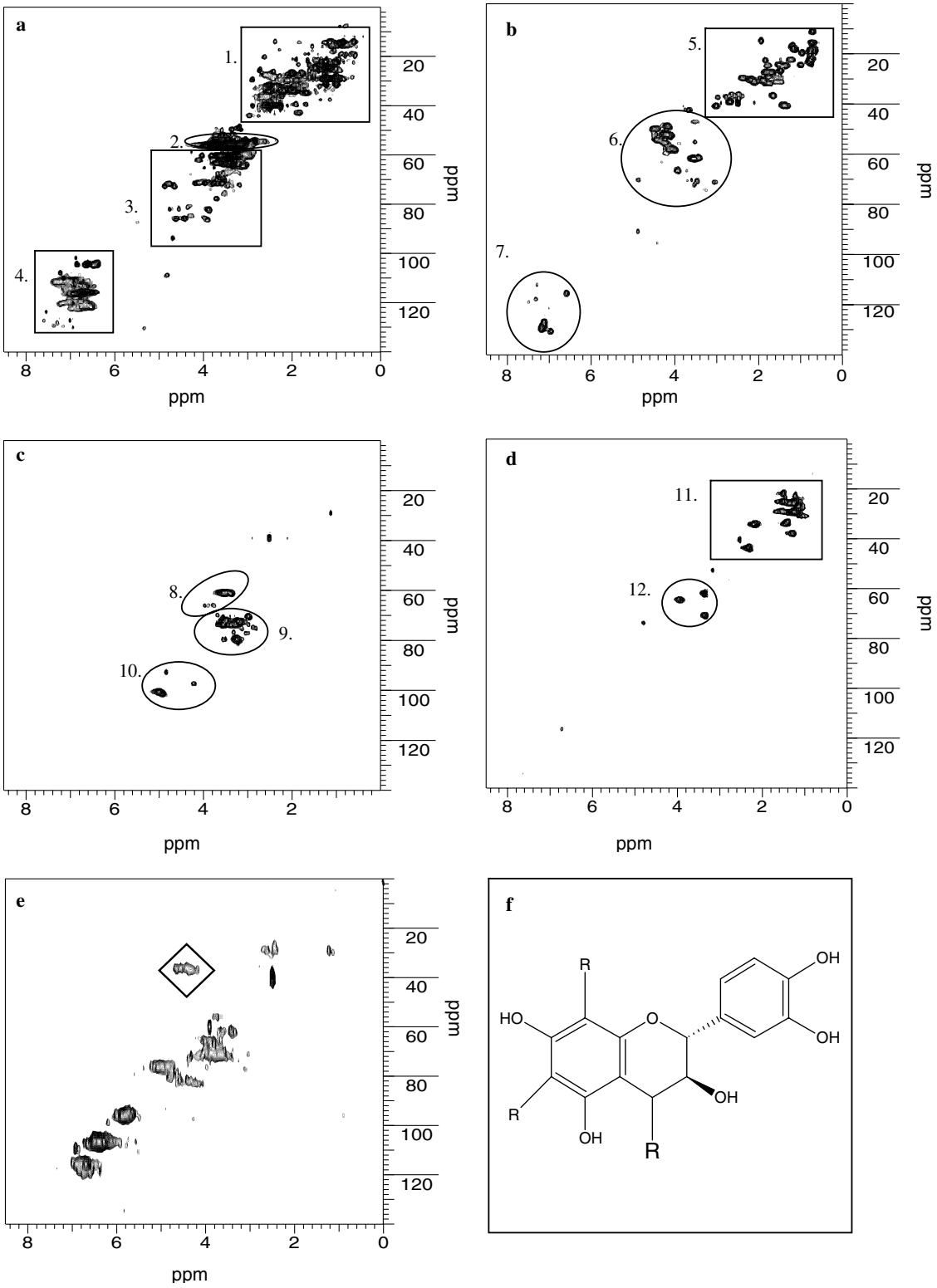

Fig. 5. HR-MAS NMR HSQC spectra of biopolymer representatives. (a) lignin (“aromatic” biopolymer), (b) Albumin Bovine Serum (protein), (c) Amylopectin (carbohydrate), (d) tomato cuticle (aliphatic biopolymer), (e) tannin (extracted from pine, the diamond highlights the C_4 linkage from condensed tannins) and (f) a common flavonoid structure that is abundant in condensed tannins (R indicates common branching in condensed tannins, the larger R is attached to the C_4 unit highlighted in E). General assignments of the major structural classes in this figure are as follows: (1) aliphatic linkers, (and aliphatic co-products extracted from wood in lignin isolation), (2) methoxyl, (3) linkers between aromatic rings, (4) aromatic rings, (5) aliphatic side chain residues, (6) amino acid α-protons in peptide chains, (7) aromatic side chain residue, (8) CH_2 in carbohydrate, (9) CH in carbohydrate, (10) anomeric units, (11) residues in main aromatic chain and (12) esters and ethers linkers.

accompanied by an accumulation of condensed tannins in the organic layer (Lorenz et al., 2000). Furthermore, nutrient cycling may be influenced by tannins ability to form complexes with proteins (Hättenschwiler and Vitousek, 2000) and by complexation and precipitation of N-containing compounds, tannins may protect proteins from microbial degradation (Almendros et al., 2000; Maie et al., 2003). As a result, proteinaceous material could escape rapid mineralization, and incorporation into microbial and plant biomass (Bradley et al., 2000). The absence of tannins in soil leachates may also be the result of adsorption to soil minerals (Kaal et al., 2005). Both the soil underneath the degrading plant material (see Fig. 1) and the leachates were recovered during the degradation experiment; these will be the focus of future studies and may provide additional information as to the binding and transformations in the soil environment.

The accumulation of cross-peaks arising from cyclic hydrocarbon structures such as steroids and triterpenoids is also notable (see Fig. 4b, region 7). Accumulation of these structures is evident in the HSQC HR-MAS NMR spectra for all pine and grass samples indicating that these compounds accumulate in the roots of plants also (data not shown). Triterpenoids, including hopanoids, are ubiquitous and occur globally in soils and sediments (Ourisson and Albrecht, 1992). Hopanoids are found in prokaryotes (Rohmer et al., 1984) and terrestrial vascular plants (Ageta et al., 1963) although the origin of the most abundant hopenoid, diploptene is still controversial (Naraoka et al., 2000). The accumulation over time of these structures in the HSQC HR-MAS NMR spectra of all of the decomposed samples underlines their recalcitrance in nature and abundance in both above and below ground biomass.

After 12 months of incubation, numerous double bonds still persist within the sample (region 4, Fig. 4b). Data from the HSQC-TOCSY HR-MAS NMR experiment (not shown) indicate that the majority of these double bonds are present in aliphatic chains with only a small proportion present in alkaloids. It has been hypothesized that, in the environment, double bonds in aliphatic chains increases reactivity (Goni and Hedges, 1990) however, it is also believed that potentially labile bonds (Largeau et al., 1986), and double bonds in algenan (Simpson et al., 2003) persist in the environment for long periods of time (Deshmukh et al., 2003). This ''preservation'' may be due to the physical structure/conformation of the biopolymer that protects the labile linkers in an aliphatic chain. Additionally it is interesting to note that α-protons/carbons in proteins and peptides are still abundant after a year (see Fig. 4b, region 8). Protein/peptide residues are discussed further later in this paper (Section 3.3).

3.2. Quantification of the degradation process

NMR analysis allows us to distinguish between the fate of component biochemical classes during decomposition. Knowledge of the relative abundance of chemical categories of plant constituents can be used in predictions as to the consequences of climate change on organic matter decomposition. For example, it has been found that different stages of soil organic matter degradation can be deduced from extract yields of components such as lignin and this information can be extrapolated to predict degradation pathways and differences in organic matter decomposition as a result of climate change (Otto et al., 2005).

The HSQC experiment provides an excellent method by which to compare major changes in sample composition, however, subtle quantitative changes are very difficult to detect from a 2-D contour plot. Therefore, more detailed quantitative considerations are best carried out from the 1-D, ^{13}C HR-MAS NMR spectra that display the highest resolution of all the 1-D spectra. A comparison of the 1-D ^{13}C HR-MAS NMR spectra of the fresh grass blades and pine needles (Figs. 6a and c, respectively) is indicative of the different types of plant biomass. However, after 12 months of incubation, these differences are less noticeable (see Figs. 6b and d) and the NMR spectra reveal some strong similarities between the degraded materials. Decomposition also results in the broadening of resonance peaks which is indicative of biopolymer functionalization or oxidation which results in a heterogeneous mixture of compounds (Wershaw et al., 1996). After 1 year the pine and grass spectra are complex and produce considerable spectral overlap making precise structural elucidation and quantification of chemical structures difficult. Resonances due to double bonds are an example of this challenge. Double bonds can be found in the polymeric lipid components of plant cuticles (Deshmukh et al., 2003) and resonate within the region representing aromatic structures (Fig. 6, ~120–140 ppm). These regions are distinguishable in the 2-D HSQC HR-MAS NMR spectra (Fig. 4, regions 4 and 5) however, it is difficult to distinguish between double bonds and aromatic structures in a 1-D spectrum and therefore we are unable to selectively quantify both components. Consequently, it is important to define regions that represent broad chemical classes such that quantification of the proportional loss or persistence of general chemical classes during decomposition can be ascertained. General assignments that can be reasonably resolved in the 1-D ^{13}C HR-MAS NMR spectrum are; 190–160 ppm—carbonyl carbon, 160–110—aromatic/double bonds, 110–58 ppm—carbohydrates, 58–54.5 ppm—methoxyl carbon from lignin and 50–0 ppm—aliphatic. There are no clear spectral boundaries that define where one group of structures starts and another ends, and thus the quantification described here is best thought of as a relative change in quantity between samples, rather than absolute quantification. Fig. 7 outlines the proportional increase and decrease of major biochemical classes as a function of time for all four samples. Most notable from these graphs is the consistent loss of carbohydrates. The initial degradation pattern is thought to reflect the ease at which microorganisms can access food and is related to the structure of plant fibers. Free carbohydrate is readily available and when consumed the micro-commu-

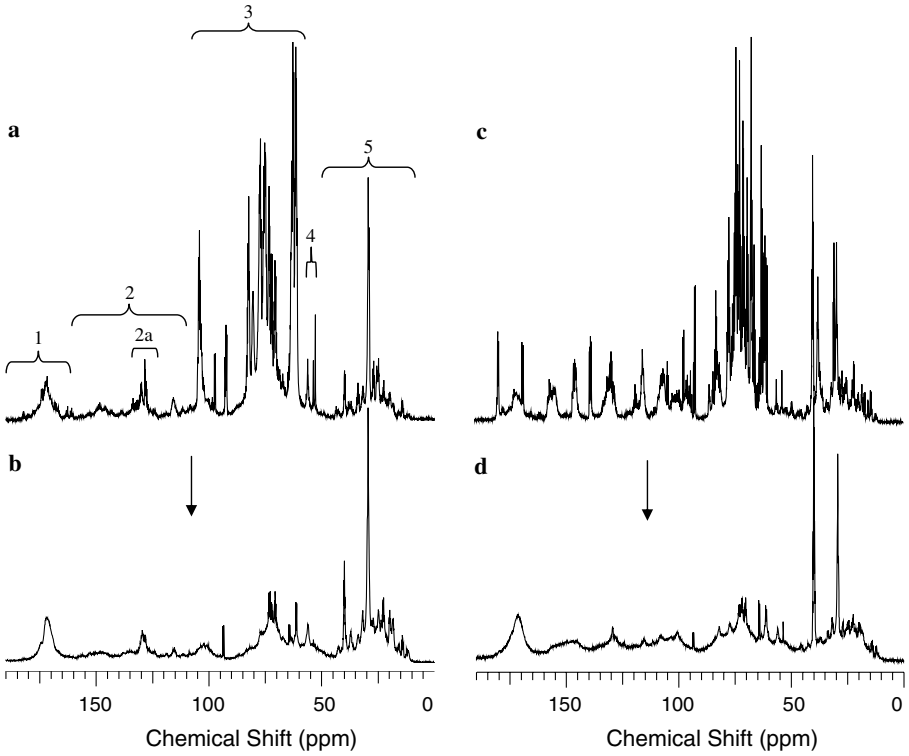

Fig. 6. 1-D ^{13}C HR-MAS NMR spectra of fresh and decomposed grass leaves and pine needles. (a) Fresh grass leaves, (b) degraded grass leaves after 12 months, (c) fresh pine needles and (d) degraded pine needles after 12 months. Regions outlined in (a) are applicable to all four spectra.

nity changes to one that can degrade the more recalcitrant components such as lignin (Berg and McClaugherty, 2003). Carbohydrates comprise approximately 75% of the fresh grass (blades and roots) and 55% of fresh pine (needles and roots/stem) and decomposition results in the loss of between 43% (grass blades) and 19% (pine roots/stem) over the 12 month period. The remaining carbohydrates are likely in less bioavailable forms such as crystalline cellulose, some hemi-celluloses (Berg and McClaugherty, 2003) and part of a heterogeneous matrix provided by lignin in which polysaccharides become embedded and cross-linked to form a recalcitrant polymer network (Ralph et al., 1997). The decomposition of carbohydrates is accompanied by a relative increase in the other four chemical categories. Stable aliphatic compounds such as cuticle and lipids are seen to accumulate during plant decomposition. Labile components of plant cuticles such as the polyesters in which waxes and fats are embedded are protected by the hydrophobic nature of cuticle (Deshmukh et al., 2003) in the same way that esters are protected in algaenan (Largeau et al., 1986). The relative increase in concentration of carboxylate functional groups is an indicator of oxidative degradation and is recognized as a first step in the biodegradation of most plant components (Wershaw et al., 1996). Methyl carbons of methoxyl groups attached to aromatic rings are a useful indicator of the progress of lignin degradation as demethoxylation is often the first step in lignin decomposition (Cain, 1980). Changes in methoxyl-C concentration are in agreement with that of aromatic components reflecting the polymeric, aromatic nature of lignin. Lignin also contains appreciable amounts of aliphatic and carboxyl carbon that may contribute to the persistence of these fractions in the decomposing plant tissues.

The initial degradation rate of the grass blades exceeds that of the roots signifying different mechanistic pathways that may be a reflection of inherent anti-microbial properties in roots. There is also a marked difference between the grass blades and pine needles with the slower degradation rate of the pine needles likely due to their resistant cuticular coatings. The variation of degradation rates between species is an important consideration in predictions of carbon transformations, especially those of globally dominant species such as grass and pine. The results demonstrate the potential of these experimental techniques to quantify transformations of chemical classes and to describe the kinetics of change during decomposition.

3.3. Nitrogen transformations

Direct 1-D ^{15}N HR-MAS NMR data were collected on all samples, but required extensive NMR time due to the lower abundance of ^{15}N. The resulting ^{15}N HR-MAS NMR spectra revealed the predominance of protonated amide-N in all the samples. This does not necessarily rule out the presence of nonprotonated nitrogen in the materials that may below detection limits. However, it does indicate that amide nitrogen is the main form of nitrogen in the plants studied and this did not change throughout the incubation experiment. To further study the protonated fraction, an inverse ^{1}H detection approach was employed

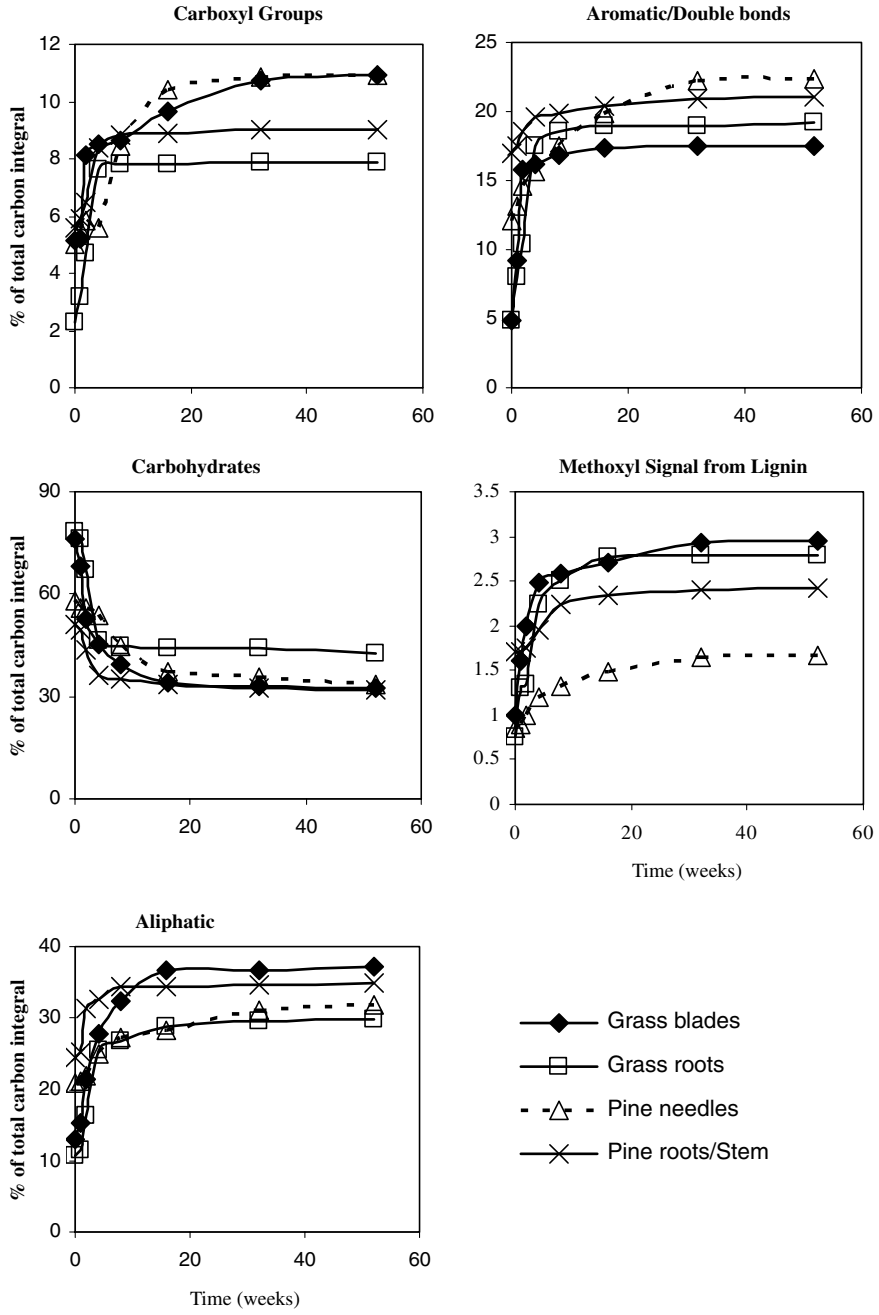

Fig. 7. Relative percentage increases and decreases of major biochemical pools as a function of time for all four plant samples.

where N is indirectly detected through its attached protons (Ernst et al., 1987). This type of experiment enables the detection of N in a much shorter time frame and also facilitates the acquisition of 2-D data (via a ^{1}H–^{15}N HSQC experiment) which describes both the ^{1}H and ^{15}N chemical shifts simultaneously.

Fig. 8 illustrates the ^{1}H–^{15}N HR-MAS NMR HSQC spectra of fresh and decomposed grass blades and roots. In all cases, the ^{15}N is in the form of amide-N, likely protein/peptide. With incubation, the amount of protein appears to decrease (1-D ^{15}N HR-MAS NMR data not shown) however, the ^{1}H–^{15}N HR-MAS NMR HSQC spectra in Figs. 8 a–c and b–d appear very similar suggesting that protein is present after 1 year. It is not clear whether this is plant protein that is physically (for example, inside intact plant cells) or chemically (for example through association with lignin) protected (Hedges and Keil, 1995; Knicker and Hatcher, 1997) or is simply "labeled" protein in microbial species which have grown on the isotopically enriched food source.

The ^{1}H–^{15}N HR-MAS NMR HSQC spectra of fresh and decomposed pine needles and roots (Fig. 9) demonstrate a different trend. The spectra of the starting material and that left after decomposition are very different. Most traces of plant proteins visible at day zero have disappeared, consistent with the breakdown of native plant proteins. In addition, new cross-peaks have appeared in the decomposed

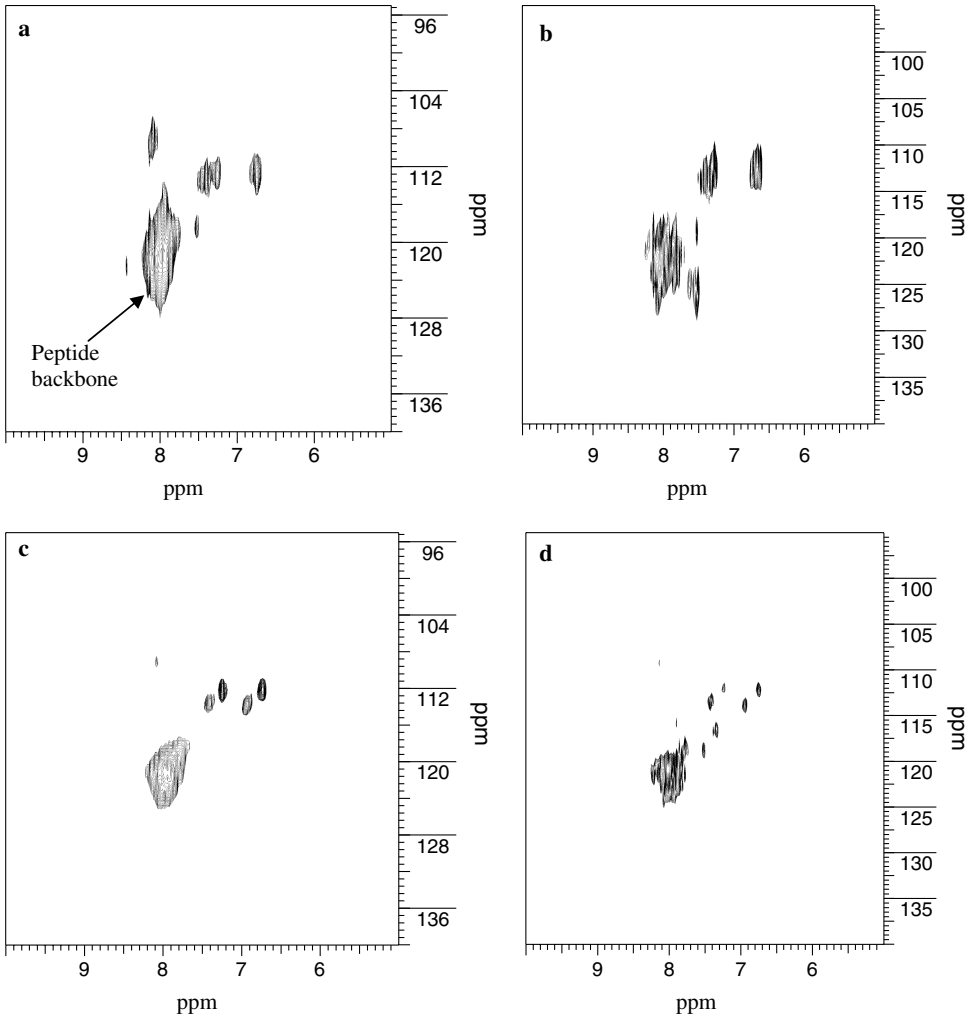

Fig. 8. ^{15}N-^{1}H HR-MAS NMR HSQC spectra of fresh and decomposed grass leaves and roots. (a) Fresh grass blades, (b) fresh grass roots, (c) decomposed grass blades and (d) decomposed grass roots.

material. Both decomposed pine needles and pine stem/roots exhibit very similar changes indicating similar degradation pathways and rates. Exact assignments are impossible with the data at hand but questions regarding their presence arise. For example, are they specific products of decomposition or are they species selectively preserved from original proteinaceous material? Or, alternatively could they be specific species synthesized from the labeled biomass by soil microbes? The presence of such structures is intriguing and work is presently under way using additional analytical approaches to try and isolate and identify these structures that may provide key structural information as to specific biological processes taking place.

4. Conclusions

HR-MAS NMR spectroscopy is applied, for the first time, to study fully labeled plant materials. High levels of isotopic enrichment combined with HR-MAS significantly enhance the detection limits, and provide a wealth of information that is unattainable by any other method. HR-MAS is used here to monitor the fate of pine and wheatgrass residues while in the presence of soil microbes. Through numerous 1-D and 2-D NMR approaches, it possible to identify specific structural units (for example the C_4 in linked polyphenols, which is indicative of condensed tannins), and determine how the various biochemical categories change structurally with time. Through the use of diffusion edited approaches additional physical information (mainly the change in size/mobility of the residues can be assessed). Furthermore, high resolution and excellent sensitivity allow accurate, quantification of the various components to be monitored over time. This study demonstrates that even with heterogeneous plant biomass excellent spectral dispersion can be obtained using HR-MAS which allows the different bio-materials to be clearly distinguished. However, due to the complexity of the data, complete assignments of the individual peaks will take considerable time. Once key components can be identified, the extraordinary resolution of HR-MAS will allow the fate of individual plant components (for example, specific lignin subunits, specific carbohydrates) to be traced, rather than simply observing the biochemical categories as a whole. As such HR-MAS provides the potential to understand

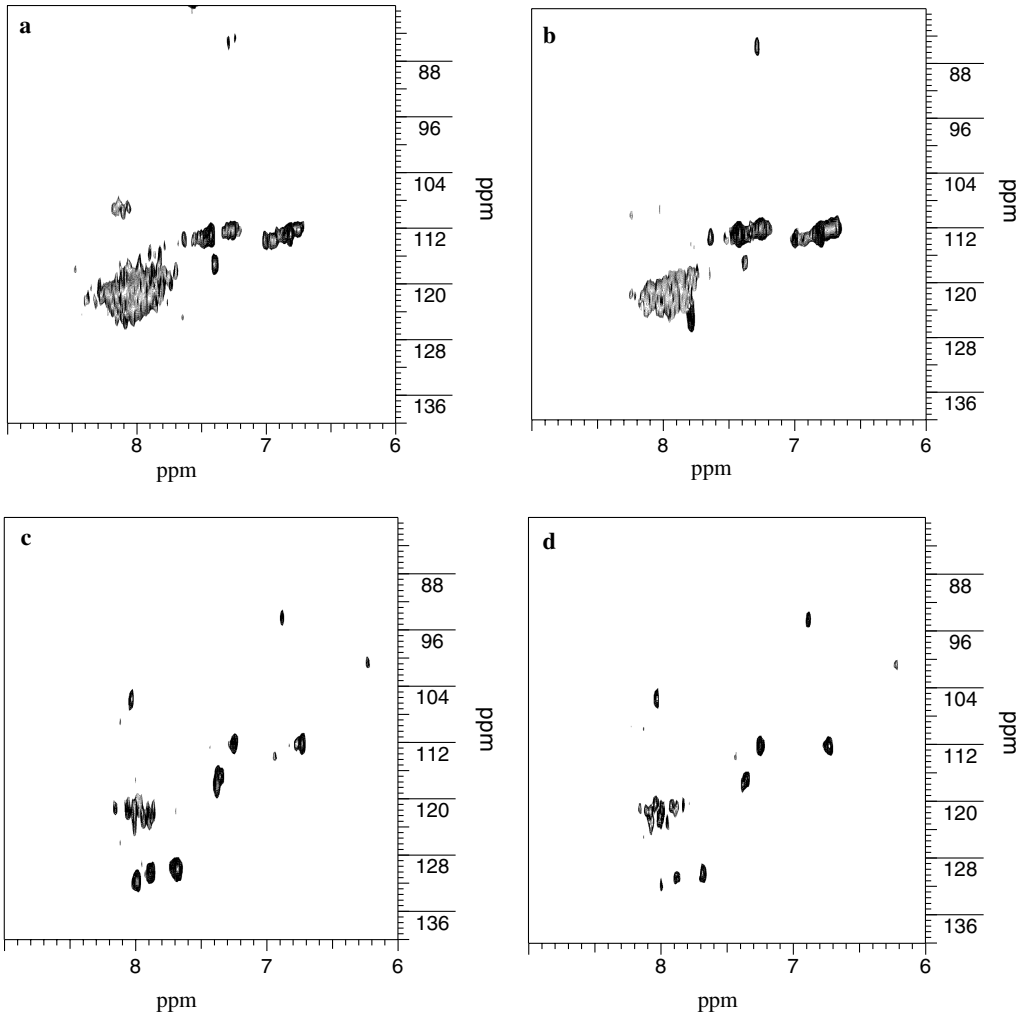

Fig. 9. ^{15}N-^{1}H HR-MAS NMR HSQC spectra of fresh and decomposed pine needles and roots/stems. (a) Fresh pine needles, (b) fresh pine roots/stems, (c) decomposed pine needles and (d) decomposed pine roots/stems.

and monitor carbon sequestration and transformation, with precision and structural detail which until now has been impossible. It is likely that HR-MAS NMR will, in the future, become a central analytical tool in unraveling the complexities of carbon and nitrogen cycling in the environment.

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