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The heterogeneous nature of microbial products as shown by solid-state ^{13}C CP/MAS NMR spectroscopy

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Abstract. Homoionic Na-, Ca-, and Al-clays were prepared from the <2 μm fractions of Georgia kaolinite and Wyoming bentonite and mixed with sand to give artificial soils with 5, and 25% clay. The artificial soils were inoculated with microbes from a natural soil before incubation. Unlabelled and uniformly ^{13}C-labelled (99.9% atom) glucose were incorporated into the artificial soils to study the effects of clay types, exchangeable cations and clay contents on the mineralization of glucose-carbon and glucose-derived organic materials. Chemical transformation of glucose-carbon upon incorporation into microbial products and metabolites, was followed using solid-state ^{13}C CP/MAS NMR spectroscopy.

There was a significant influence of exchangeable cations on the mineralization of glucose-carbon over a period of 33 days. At 25% clay content, mineralization of glucose-carbon was highest in Ca-soils and lowest in Al-soils. The influence of exchangeable cations on mineralization of glucose-carbon was more pronounced in soils with bentonite clay than those with kaolinite clay. Statistical analysis of data showed no overall effect of clay type on mineralization of glucose-carbon. However, the interactions of clay type with clay content and clay type with clay content and exchangeable cations were highly significant. At 25% clay content, the mineralization of glucose-carbon was significantly lower in Na- and Al-soils with Wyoming bentonite compared with Na- and Al-soils with Georgia kaolinite. For Ca-soils this difference was not significant. Due to the increased osmotic tension induced by the added glucose, mineralization of glucose-carbon was slower in soils with 5% clay than soils with 25% clay.

Despite the differences in the chemical and physical characteristics of soils with Ca-, Na- and Al-clays, the chemical composition of organic materials synthesised in these soils were similar in nature. Assuming CP/MAS is quantitative, incorporation of uniformly ^{13}C-labelled glucose (99.9% atom) in these soils resulted in distribution of carbon in alkyl (24–25%), O-alkyl (56–63%), carbonyl (11–15%) and small amounts of aromatic and olefinic carbon (2–4%). However, as decomposition proceeded, the chemistry of synthesised material showed some changes with time. In the Ca- and Na-soils, the proportions of alkyl and carbonyl carbon decreased and that of O-alkyl carbon increased with time of incubation. However, the opposite trend was found for the Al-soil.

Proton-spin relaxation editing (PSRE) subspectra clearly showed heterogeneity within the microbial products. Subspectra of the slowly-relaxing (long $T_1(H)$) domains were dominated by alkyl carbon in long- and short-chain structures. The signals due to N-alkyl (55 ppm) and carbonyl carbon were also strong in these subspectra. These subspectra were very similar to those obtained for microbial and fungal materials and were probably microbial tissues attached to clay surfaces by polysaccharide extracellular mucilage. Subspectra of fast-relaxing (short $T_1(H)$) domains comprised mostly O-alkyl and carbonyl carbon and were probably microbial metabolites released as neutral and acidic sugars into the extracellular environment, and strongly sorbed by clay surfaces.

Introduction

Speculation on the mechanisms responsible for the persistence of organic matter in soil have been debated in the literature for a long time. Organic structures resistant to biological degradation have long been considered as a major mechanism for this persistence. However, recent studies on the turnover of organic materials in soil demonstrate that, in addition to chemistry, the location of biopolymers within the mineral matrix of soil is a dominant factor regulating the microbially mediated decomposition processes (Van Veen & Kuikman 1990). Longer turnover times for organic materials occluded within aggregates, and those associated with clay particles, compared with free organic materials present within a soil matrix, confirm the concept that accessibility of organic materials to soil organisms decreases as a result of incorporation into soil aggregates or adsorption to clay surfaces (Golchin et al. 1995). Interactions of soil minerals with natural organics and microbes and the consequences of these interactions on the turnover rate of soil organic matter (SOM) have been the subject of several reviews and books in recent years (Oades & Ladd 1977; Huang & Schnitzer 1986; Coleman et al. 1989; Huang et al. 1995). The SOM models (e.g. Parton et al. 1987; Jenkinson 1990) also have recognised the importance of these interactions by including the impact of soil texture on the turnover of organic materials in soils . However, the effects of exchangeable cations and type of clay on the formation and turnover of SOM have not been given much attention in these studies.

Cation bridging, where multivalent exchangeable cations on the surface of the clay act as bridges between the clay and negatively charged organic substances, has often been suggested as a mechanism of adhesion (Tate & Theng 1980) that promotes the retention of carbon in soil (Muneer & Oades 1989). While there is substantial evidence to indicate that both clay microstructure and the association of organic materials with negatively charged clay surfaces are very much influenced by cations with multiple charges, the relevance of cation bridging to SOM dynamics is not clear (Oades 1995). Exchangeable cations apparently exert their primary influence on microbial growth and metabolic activities by modifying the physicochemical characteristics of microbial habitats, which in turn, influence the carbon dynamics in soil. In contrast to the indirect effects of exchangeable cations, relatively little is known about direct surface interactions between clays and organic materials through cation bridging. It is likely that in soils the indirect effects of exchangeable cations on carbon dynamics is as important as the direct effects. However, it is not possible to differentiate clearly between the direct and indirect effects.

By incorporating ^{13}C-labelled organic substrate into pure clays saturated with different cations it is possible to evaluate the effects of exchangeable

cations and the types of clay on the turnover of microbial products and metabolites synthesised as a result of utilization of the substrate. It is also possible to use solid-state ^{13}C CP/MAS NMR to characterize the organic structures of these materials synthesised under the various physical and chemical conditions induced by different exchangeable cations.

As soil microbes assimilate low molecular-weight compounds, such as sugars and organic acids, some of the substrate-carbon is respired to CO_2 to produce energy, and the rest is synthesised into either metabolites or new tissues. The metabolites are released into the extracellular environment mostly as neutral and acidic carbohydrate which always contains some nitrogen compounds. Since microbial cells and their metabolites occur spatially separate from each other, they have different proton spin relaxation times, $T_1(H)$, and it is possible to use proton spin relaxation editing (PSRE) to characterize these two components of microbial products.

Uniformly ^{13}C-labelled glucose is the most suitable substrate to study the incorporation of carbon into the microbial biomass as it is completely metabolised within several days during the incubation in soil. The rapid breakdown thus leaves no unaltered residues which may otherwise interfere with the interpretation of the chemical structures of the materials recently synthesised. The advantages of using pure clay materials are (1) the absence of paramagnetic materials that usually exist in natural soils and can potentially interfere with NMR analyses (Skjemstad et al. 1994), (2) the absence of any natural background carbon which otherwise may obscure the characterization of the materials synthesised by the soil biomass as a result of glucose utilization, and thus the lack of need for dilution of native carbon with large quantities of ^{13}C-labelled substrate.

In the present study homoionic Na-, Ca-, and Al-clays from both Georgia kaolinite and Wyoming bentonite were mixed with sand in different proportions to prepare soils with different clay contents. Unlabelled and uniformly ^{13}C-labelled glucose (99.9%) was incorporated into these soils to study (a) the influence of exchangeable cations, type of clay and clay content on the mineralization of glucose-carbon and microbial products synthesised as a result of glucose utilization, (b) the chemical structure of microbial products synthesised in soils with different exchangeable cations using ^{13}C CP/MAS NMR spectroscopy, (c) the heterogeneity of microbial products using proton-spin relaxation editing.

Materials and methods

Preparation of homoionic clays

Samples of Georgia kaolinite and Wyoming bentonite were treated with 1 M solution of NaCl to saturate the clays with Na and facilitate their dispersion. The clays were then washed with distilled water until free from salt and dispersed by gentle ultrasonic energy to separate the <2 μm fraction. The <2 μm fractions of clays were used to prepare homoionic Na-, Ca-, and Al-clays by washing the clays with aqueous solutions of NaCl, $CaCl_2$ and $AlCl_3$, respectively. After saturation of clays with different cations, samples were dialysed against distilled water until free from salt. The salt-free clay samples were freeze-dried and stored at room temperature.

Treatments

Sub-samples of Na-, Ca-, and Al-clays, from both Georgia kaolinite and Wyoming bentonite, were mixed with acid-washed sand in appropriate proportions to yield artificial soils (20 g) with 5 and 25% clay. Unlabelled glucose was applied to the soils at a rate of 5 mg C g^{-1} soil and three replicates of each soil were used for incubation. Sub-samples from pure sand, representative of soils with 0% clay, and also a control treatment receiving no glucose were incubated with the amended soils.

Sample preparation and incubation

The artificial soils were inoculated with microbes from a natural soil before incubation. The natural soil was Wiesenboden self-mulching black earth from the Waite Agricultural Research Institute and a composite sample from the A horizon of this soil (0–10 cm; 4% organic carbon; 31% clay of randomly interstratified minerals; pH = 6.25 soil/ water ratio 1:5; EC 0.12 dSm^{-1}; CEC 57.3 cmol+ Kg^{-1}) under native grass vegetation was used. The inoculum was prepared by suspending a 10 g sample of the Wiesenboden soil in 100 ml of distilled water. The prepared suspension was then allowed to stand long enough until soil particles settled down and a clear solution appeared. The inoculant for each sample was 2 ml of this clear solution together with sufficient distilled water to evenly wet the sample. The inoculated samples were subsequently air-dried.

Gravimetric water contents were determined for all soils (treatments) at -10 kPa matric potential and the required amount of carbon for each treatment was added as a glucose solution to achieve this matric potential. Nitrogen and phosphorus were provided by dissolving sufficient $(NH_4)_2SO_4$

and $NH_4H_2PO_4$ in the glucose solution to maintain a C:N ratio of 10 and C:P ratio of 80. Amendments were added to soils by a fine needle and moist samples in 50 ml containers were placed into 1000 ml cylindrical jars. A vial containing 10 ml 0.5 M NaOH to trap respired CO_2 and a vial containing deionized water to prevent desiccation were then placed in each jar and the jars sealed and incubated in the dark at 20 ± 1.5 °C. The CO_2 traps from all jars were replaced every day until day 12 and thereafter every 3 days until day 33. The amount of carbon mineralized to CO_2 in each sample was determined by titrating a 4 ml aliquot of the NaOH solution with 0.05 M HCl on a Radiometer DTS 800 Automatic Multi-Titration System.

After day 33, samples with 25% Na-, Ca-, and Al-clays from Wyoming bentonite were selected for ^{13}C NMR studies. To increase the carbon content of these samples, addition of unlabelled glucose was repeated 3 times and the period between each addition was 2 weeks. To add glucose, each sample was first air-dried and remoistened with a solution of glucose as before. Two weeks after the last addition of unlabelled glucose, replicates of each sample were mixed together and a sub-sample equivalent to 10 g on oven-dry basis was taken for incubation with uniformly ^{13}C-labelled (99.9% atom) glucose. The rate of ^{13}C-glucose carbon addition was selected so that the amount of ^{13}C nuclei remaining in the soil at the end of the incubation would be equivalent to a soil with 10% native C. It was assumed that during the incubation 70% of added substrate ^{13}C would be mineralized to carbon dioxide (Baldock et al. 1989). Two weeks after addition of ^{13}C-glucose (day 90) to Ca-, Na, and Al soils, sub-samples from these soils were removed for NMR analysis. The remaining soils were treated with unlabelled glucose and incubated until day 130, when they were removed for NMR analysis. The main aim of using ^{13}C-labelled glucose (99.9% atom) between the additions of unlabelled glucose not only was to increase the natural abundance of ^{13}C isotope but also to minimize any problems of line broadening due to J-coupling or ^{13}C-^{13}C dipolar interactions which result from adjacent ^{13}C nuclei.

Separation and preparation of fungal material

The fungal hyphae were collected from the surfaces of the soil samples incubated with unlabelled glucose. The harvested filamentous bodies were washed with water and dried at 40 °C in a fan-forced oven, and used for NMR analysis.

Solid-state ^{13}C CP/MAS NMR spectroscopy

All solid-state carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra, except Bloch decay spectra, were obtained by cross polarization and magic-

angle spinning (CP/MAS) as described by Golchin et al. (1994) using a Varian Unity 200 Spectrometer operating at 50.3 MHz. Conventional and dipolar dephased NMR analyses were performed using a contact time of 1 ms and recycle delay of 1 s. However, where the relaxation time constant $T_1(H)$ exceeded 0.14 s a longer recycle delay of 2 s was required to ensure complete relaxation of the proton spins between scans. For the dipolar dephasing experiments a 41 μs delay, during which the proton decoupler was turned off, was introduced into the pulse sequence prior to signal acquisition. In the middle of the dipolar dephasing delay time a 180° refocussing pulse was used to avoid phasing problems.

In addition to the spectra obtained for different soils, ^{13}C CP/MAS NMR spectra were also obtained for separated fungal material and a commercial *poly-D*-galacturonic acid in the same fashion. These spectra were used as references to compare with those obtained for the microbial products.

Proton-spin relaxation editing (PSRE) experiments were carried out using an inversion-recovery cross-polarization NMR pulse sequence (Newman & Tate 1991; Preston & Newman 1992). In this pulse sequence the proton magnetization was inverted by a 10.6 μs pulse (180°) and then allowed to recover for a variable interval (4.5–15 ms) before the standard cross-polarization sequence was used. In standard cross-polarization, a 5.3 μs pulse (90°) followed by a 1 ms cross-polarization contact time and 25 ms acquisition time. The delay time between pulses was 1 s.

In order to determine whether the CP/MAS technique sees all of the ^{13}C nuclei present, a Bloch decay (BD) experiment was carried out in which the spectra were obtained by direct magnetization of the ^{13}C spins, MAS, and high power decoupling. For the BD experiment a recycle delay time of 45 s was used.

Statistical analyses

The influence of clay type, amount of clay and exchangeable cations on mineralization of glucose-carbon were assessed using a factorial ($2\times2\times3$) experiment with complete randomized design (Genstat 1987). Where significant differences were obtained for the main effects, further differences between the treatments were isolated by the LSD test.

Results and discussion

Carbon mineralization

Due to low initial microbial biomass contents of the artificial soils, mineralization of added glucose was preceded with a 3 day lag in soils containing

25% Ca- and Na-bentonite (Figure 1). However, the lag period was much longer (10 days) in soil with Al-bentonite. The results suggest that mineralization of glucose in the Al-soil started after a period of adaptation to low pH and high Al toxicity. The cumulative quantities of CO_2 evolved from Ca-, Na-, and Al-soils, containing 25% clay from Wyoming bentonite and from Georgia kaolinite, over a period of 33 days are given in Figures 1 and 2. The statistical analysis of data show that there was a significant effect due to exchangeable cations on the mineralization of glucose-carbon. The losses of carbon, evolved as CO_2, were highest in Ca-soils and lowest in Al-soils, while the effect of Na was intermediate. On the basis of total carbon present initially in these soils, after 33 days of incubation the losses of carbon for Ca-, Na-, and Al-soils with 25% clay as bentonite were 63.2, 58.2 and 50.6% respectively (Figure 1). For soils containing kaolinite clay the corresponding values were 66.8, 63.2 and 61.8% (Figure 2). The results indicate that the influence of exchangeable cations on mineralization of glucose-carbon was greater in soils with bentonite clay than those with kaolinite clay. The reason for this is probably the higher cation exchange capacity (CEC) of bentonite compared with kaolinite (Table 1), because physicochemical changes induced in the soil environment as result of variations of exchangeable cations is much greater in soils with higher CEC. It is well known that exchangeable Na is associated with poor physical conditions of soils. The Na-soils, especially those with bentonite clay, dispersed strongly upon wetting resulting in a gel-type material. Therefore, the slower mineralization of glucose-carbon in Na-soils compared with Ca-soils could have been due to lack of a good aeration. However, with Ca on the exchange complex, soil structure and thus aeration and moisture relations all vary to the advantage of soil organisms, which in turn can enhance glucose-carbon mineralization. Several studies have shown the detrimental effects of acidity and Al on soil organisms (Munns & Keyser 1981; Wood & Cooper 1988). The slower mineralization of glucose-carbon in Al-soils compared with that in Ca- and Na-soils, may in part, arise from the toxicity of Al to soil organisms. This idea is supported by the observation of the long lag phase in mineralization of glucose-carbon in Al-soils (Figure 1). Zwarun et al. (1971) showed that exchangeable Al in the form of Al-saturated Wyoming bentonite decreased the number of surviving cells as compared with a Ca-saturated Wyoming bentonite.

It seems the effects of exchangeable cations on decomposition of glucose is partly through their influence on microbial growth and metabolic activities by modifying the physicochemical characteristics of microbial habitats. In contrast to the indirect effects of exchangeable cations, relatively little is known about the role of these cations in direct surface interactions between clays and organic materials through cation bridging. It is widely assumed that

Table 1. Some characteristics of the clays used in this study.

Clay Minerals	Surface area ($m^2\ g^{-1}$) Saturated cation				Na-clay		pH Saturated cation			EC ($\mu S\ cm^{-1}$) Saturated cation		
	Na		Ca		CEC	Charge density[c]	Na	Ca	Al	Na	Ca	Al
	B.E.T.[a]	EGME[b]	B.E.T.[a]	EGME[b]	$mmol_c\ Kg^{-1}$	$mmol_c\ m^{-2}$						
Wyoming bentonite	46	680	35	799	810	1.2×10^{-3}	5.3	6.4	5.1	22	50	33
Georgia kaolinite	n.d.	41	11	18	79	1.9×10^{-3}	7.0	7.5	5.8	21	35	12

[a] Nitrogen surface area as determined by B. E. T. method.
[b] Total surface area as determined by ethylene glycol monoethyl ether.
[c] Surface charge density = CEC/ Total surface area (Chorom & Rengasamy 1995).

n.d. not determined.

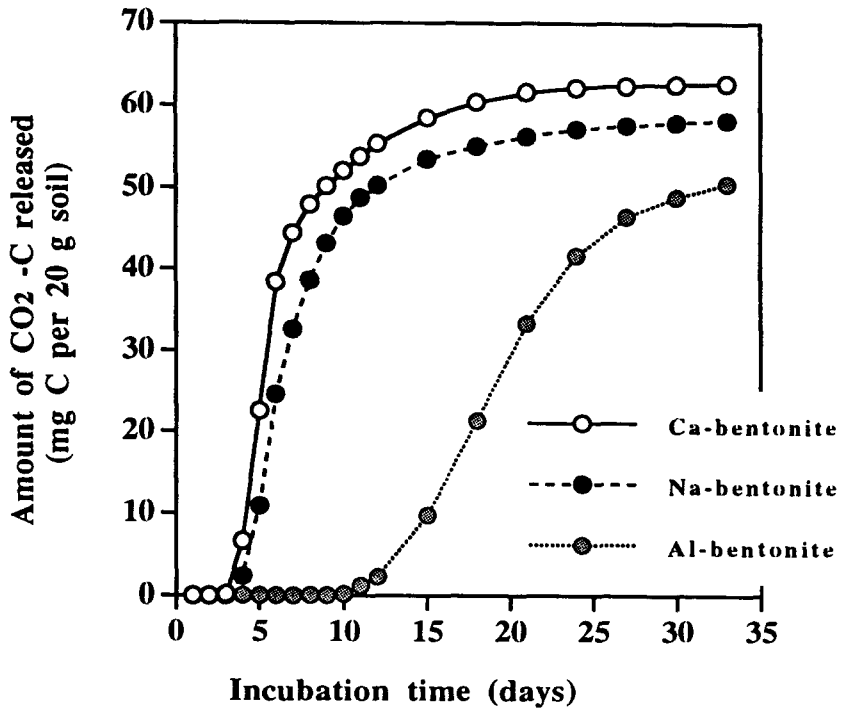

Figure 1. Cumulative CO_2 evolved from Ca-, Na-, and Al-soils containing 25% clay as Wyoming bentonite.

the high organic carbon contents of andosols are due to formation of Al-OM complexes resistant to microbial degradation (Wada & Higashi 1976; Boudot et al. 1988). The increased stability of microbial products and metabolites in the Al-soil, reflected in the NMR data as a higher carbon-13 content for that soil, is an example of such interaction and indicates the inhibitory effect of Al on carbon turnover. Boudot et al. (1986) showed that utilization of ^{14}C-labelled citrate by microbes was greatly reduced upon addition of Al. The amounts of Al used were such that toxic effects of Al species were unlikely to have caused the decrease in citrate degradation.

Statistical analysis of the data failed to show a difference in the effect of clay type on mineralization. However, for mineralization, the interactions of clay type with clay content and clay type with clay content and exchangeable cations were highly significant. In soils with 25% clay, mineralization of glucose-carbon was overall lower in soils with bentonite clay than those with kaolinite clay. While mineralization of glucose-carbon was significantly lower in Na- and Al-soils with bentonite clay than that in Na- and Al-soils with kaolinite clay, such difference was not significant in Ca-soils (Figure 3).

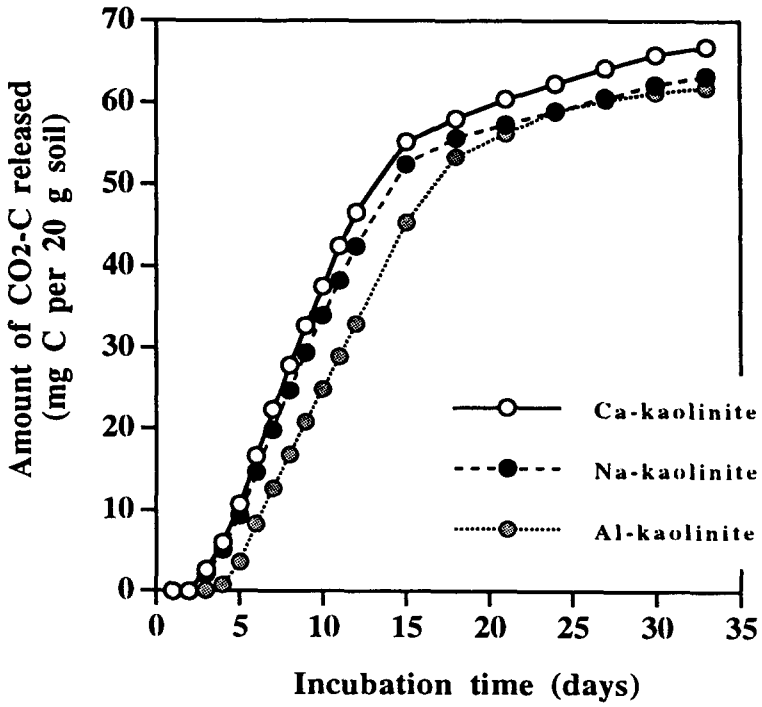

Figure 2. Cumulative CO_2 evolved from Ca-, Na-, and Al-soils containing 25% clay as Georgia kaolinite.

Several studies have implicated clay or clay related properties in the stabilization of organic residues and microbial biomass in soils (Amato & Ladd 1992). The results of this study, however, indicate slower mineralization of glucose-carbon in soils with 5% clay compared with those with 25% clay. The apparent inconsistency may be explained by the variation in osmotic tension of the samples during incubation. At water potential of −10 kPa the pure sand (0% clay) and soils with 5% clay, and in particular those with kaolinite clay, contained only one quarter the amount of water than the soils with 25% clay. Thus, the amount of glucose per unit of soil solution was four times greater in these soils compared to those with 25% clay. With respect to differences in total electrolyte concentration of these soils the most likely factor to have controlled decomposition is osmotic tension. Johnson & Guenzi (1963) showed that osmotic tension reduced CO_2evolution in a linear fashion as the electrolyte concentration of the soil increased.

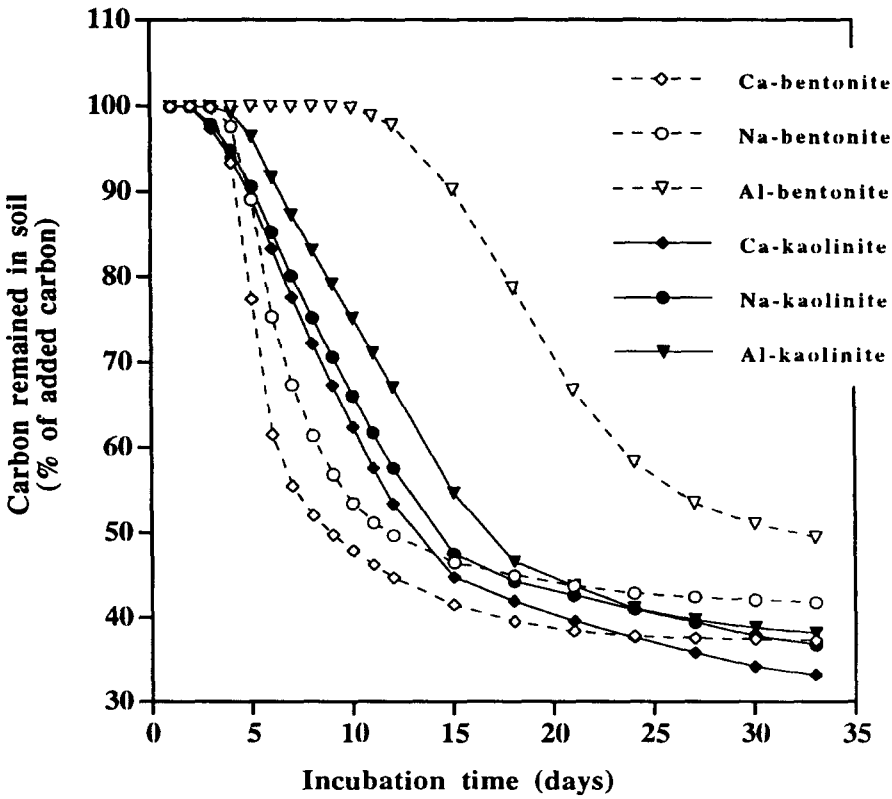

Figure 3. Amounts of carbon remaining in Ca-, Na- and Al-soils containing 25% clay as either Wyoming bentonite or Georgia kaolinite.

Conventional NMR spectra

The conventional ^{13}C CP/MAS NMR spectra obtained for Ca-, Na-, and Al-soils at days 90 and 130 of the incubation are shown in Figures 4–6. As can be seen, the spectra show good resolution and are not as broad or unresolved as those collected by Baldock et al. (1989) for ^{13}C amended soils. This indicates that signal broadening, which arises from J-coupling and homonuclear ^{13}C-^{13}C dipolar interactions between adjacent ^{13}C nuclei, has not been a serious problem in this experiment. The magnitude of signal broadening due to J-coupling is independent of magnetic field strength, however, the line width on a ppm scale is proportional to the magnetic field strength. Consequently, a 50% reduction in line width is expected for our spectra acquired at a higher field strength (50.3 MHz, 4.7 tesla) when compared with those acquired by Baldock et al. (1989) (22.6 MHz, 2.2 tesla). Furthermore, inter-

mittent use of unlabelled and ^{13}C-labelled glucose should have mixed ^{13}C and ^{12}C nuclei in a way that minimized the number of adjacent ^{13}C nuclei in microbially-derived materials.

In all NMR spectra major signals are observed for O-alkyl (60, 72 and 104 ppm), alkyl (23 and 30 ppm) and carbonyl carbon (175 ppm). The peak due to aromatic carbon (130 ppm) was small and no discrete peaks were observed at approximately 150 ppm for oxygen-substituted aromatic carbon such as those in phenolic compounds. The sharp peak at 102–104 ppm, which arose from the dioxygenated carbon linking of monosaccharide units (Oades et al. 1987), suggested that O-alkyl carbon was present in polysaccharide structures such as extracellular materials and cell wall cellulose of fungi. Many studies have shown that soil bacteria and fungi produce extracellular polysaccharides consisting of neutral or acidic sugars (Hepper 1975; Foster 1988). The cell walls of fungi also contain chitin, chitosan, cellulose and a variety of non-crystalline polysaccharides, mainly mannans and glucans that also contribute to signal intensity in the O-alkyl region. The CP/MAS NMR spectra show distinct peaks at 23 and 30 ppm for alkyl carbon. The presence of a shoulder at 14 ppm, which appeared as a discrete peak in the Bloch decay spectrum of Ca-soil (spectrum not shown), suggested that the alkyl carbon is structurally complex. Baldock et al. (1990a) concluded that peaks at 15, 23, and 30 ppm chemical shift, in NMR spectra of the clay fraction of a soil incubated with ^{13}C-glucose, arose from terminal CH_3 carbon, the CH_2 carbon adjacent to CH_3 carbon and the $-(CH_2)_n-$ carbon respectively. The close agreement between the chemical shift values observed by Baldock et al. (1990a) and those obtained for the alkyl carbon in this study indicates that the presence of $-(CH_2)_n-CH_2-CH_3$ structures in the alkyl carbon is likely. A greater contribution from the peak at 23 ppm rather than the peak at 30 ppm to alkyl carbon of these soils indicates that the majority of alkyl carbon is present in branched and/or short chain rather than long chain linear structures. Polymethylene is an important component of soil humic substances (Newman et al. 1980; Wilson 1981). However, the results of this study suggest that polymethylene is not an important component of the materials synthesised by soil organisms when utilizing a simple substrate. The alkyl structures present in the spectra were probably derived from both protein and lipid molecules of microbial cells. The presence of lipids in glucose amended soils was confirmed by extracting fatty acids from the soil samples with a non-polar solvent and characterization of the methylated fatty acids using gas chromatography-mass spectrometry (unpublished data).

In addition to other resonances, two shoulders at 56 and 62 ppm were observed in the spectra of soil samples. The absence of a well resolved resonance at 56 ppm was likely due to an overlapping with the large adjacent

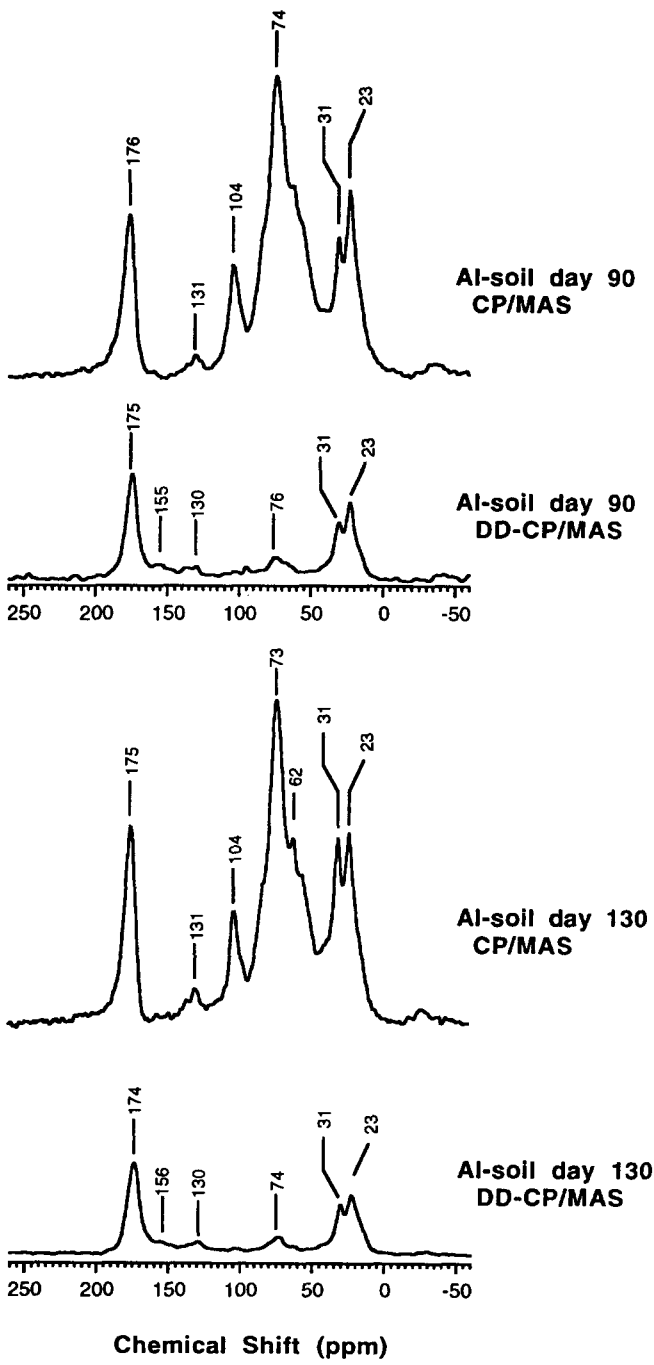

Figure 4. Conventional and dipolar dephased ^{13}C NMR spectra obtained for Al-soil at days 90 and 130 of the incubation.

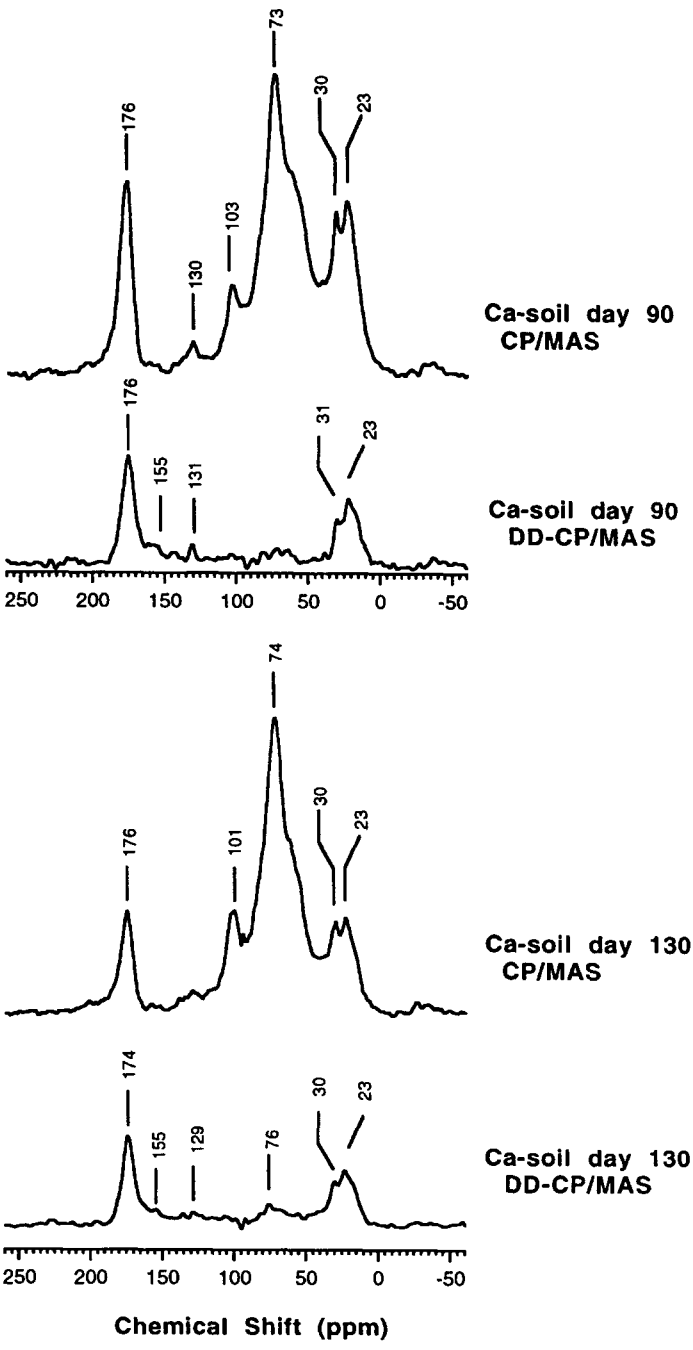

Figure 5. Conventional and dipolar dephased ^{13}C NMR spectra obtained for Ca-soil at days 90 and 130 of the incubation.

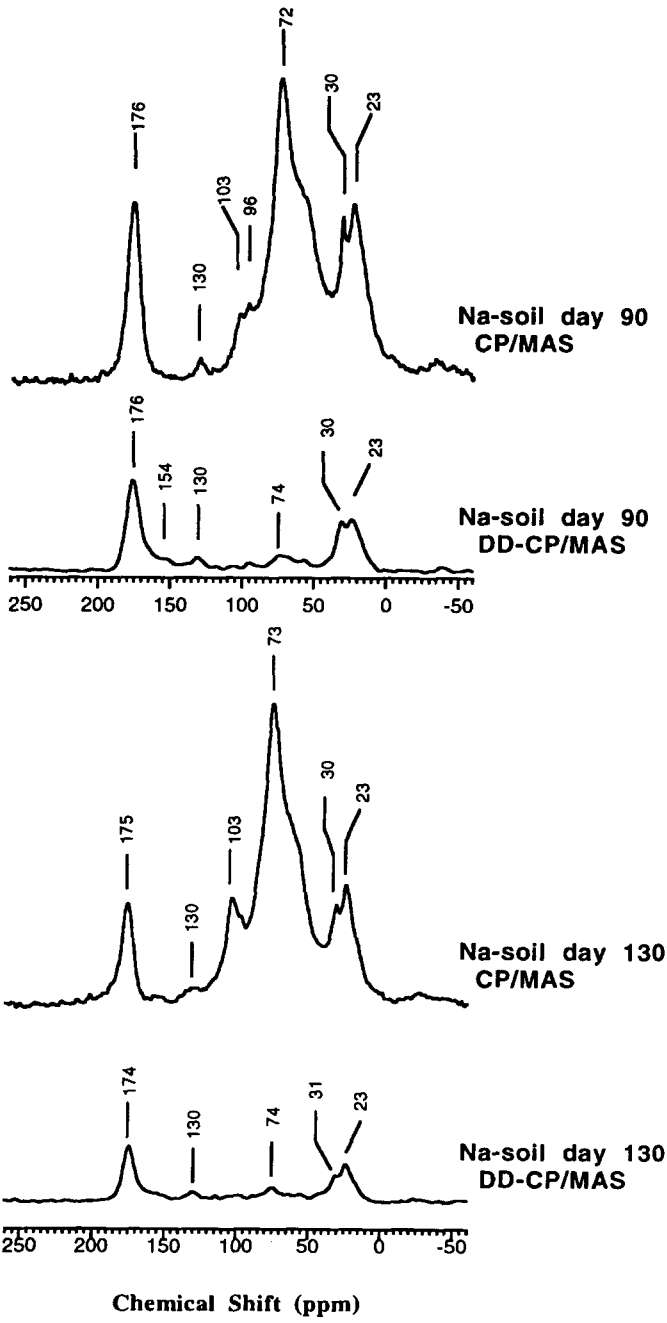

Figure 6. Conventional and dipolar dephased ^{13}C NMR spectra obtained for Na-soil at days 90 and 130 of the incubation.

O-alkyl resonance at 72 ppm (Baldock et al. 1990b). The peak at 56 ppm can be attributed to a combination of methoxyl carbon and the α-carbon in protein. As the peak at 56 ppm is broad and dipolar dephasing behaviour of the carbon associated with this peak (to be discussed latter) indicated that it was dominated by CH_2- and/or CH- carbon, it is concluded that this peak arose almost entirely from amine substituted aliphatic carbon rather than methoxyl carbon.

The ^{13}C-glucose amended soils examined in this study showed a small aromatic signal at 130 ppm (Figures 4–6). The origin of this carbon is not known but a contribution from aromatic and heteroaromatic ring carbon of amino acids and olefinic carbon is likely. In addition, the apparent absence of phenolic signals in the CP/MAS NMR spectra indicate that aromatic carbon is not the dominant type of carbon synthesised when a simple substrate such as glucose is utilized by the microbial biomass.

There was a strong resonance at approximately 175 ppm in the CP/MAS spectra which may be attributed to carbonyl carbon. Though the carbonyl carbon of carboxylic acid groups are most likely responsible for a major portion of the intensity in this region, ester and amide carbon also have chemical shifts in this range. As protein and amino acids are a major component of microbial biomass and products, the carbonyl resonance could, in part, arise from these components. When the low C:N ratio of the substrate utilized by microbial biomass is considered, some contribution from amide carbon to this region is most likely.

The proportions of different types of carbon contained in Ca-, Na-, and Al-soils, at day 90 of the incubation, are presented in Figure 7. From Figure 7 it is clear that 57–64% of carbon in these soils is present as O-alkyl carbon, indicating that polysaccharides were the most significant class of compounds in microbial products and metabolites. Next to the carbohydrates, aliphatic substances (alkyl carbon) played an important role in the chemical composition of these soils (22–24%). The amount of carbonyl carbon was lower (11–15%) and aromatic carbon occurred in the lowest quantities (2–4%). The chemical composition of the organic materials and proportions contained in these soils was very similar to that reported by Baldock et al. (1989) for microbial products and metabolites synthesised as a result of ^{13}C-glucose utilization.

Despite the differences in physical and chemical characteristics of the soils used in this study, the chemical composition of organic materials synthesised in these soils by soil biomass was virtually the same (Figure 7). However, small variations were noted in the composition of organic materials contained in these soils. The relative proportions of O-alkyl and acetal carbon were slightly higher in the Al-soil compared with the other soils. These differences

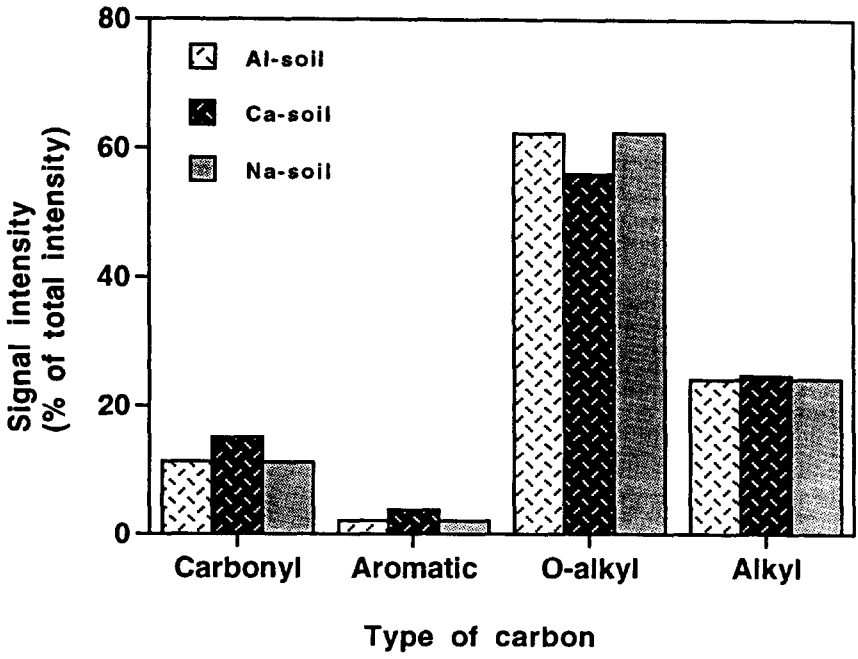

Figure 7. The relative proportions of different types of carbon in Ca-, Na-, and Al-soils at day 90 of the incubation.

probably originated from the relative abundance of fungi and bacteria in these soils. Baldock et al. (1990b), using solid-state NMR, showed that fungal materials contained more O-alkyl and acetal carbon than bacterial materials. With respect to the acidic condition of the Al-soil, fungi were expected to be dominant, because the acidity favours the growth of fungi (Alexander 1961). Furthermore, aluminium is more toxic to bacteria than to fungi (Matsuda & Nagata 1958).

A comparison of chemistry of Ca-, Na-, and Al-soils at days 90 and 130 of incubation indicates the changes brought about during decomposition. In Ca- and Na-soils, the proportions of alkyl and carbonyl carbon decreased and that of O-alkyl carbon increased with time of incubation, however, in the Al-soil the situation was reversed (Figure 8). The decrease in alkyl and carbonyl carbon in Ca- and Na-soils probably resulted from decomposition of microbial tissues rich in protein and formation of the extracellular carbohydrate under conditions in which the availability of glucose-carbon was low. This suggests that in the long-term microbial products and metabolites contribute more to O-alkyl carbon contents of soils than to alkyl carbon contents which contrasts with the decomposition of plant residues in soil where the O-alkyl carbon decomposes most rapidly and alkyl and aromatic carbon tend

to accumulate. From the results presented here it must be concluded that the alkyl carbon accumulated in soil has a different structure and a different origin from that synthesised in these experiments. The major peak for alkyl carbon of microbial products appeared at 23 ppm, while the accumulated alkyl carbon in soil shows signals at 33 and/or 35 ppm (Baldock et al. 1992) and thus must be of plant origin. Dipolar dephased behaviour of the microbial products also indicates that alkyl carbon present is predominantly in mobile structures. However, the dipolar dephasing NMR of SOM indicates that the mobile structures were lost with increasing depth in forest soil profiles (Kögel-Knabner & Hatcher 1989). Thus, if selective preservation of the alkyl carbon from microbial origin is considered as a major source for soil alkyl carbon, the alkyl carbon in the humified horizons should show a mobile behaviour with a major peak at 23 ppm instead of rigid behaviour with peak between 32 to 35 ppm when applying dipolar dephasing ^{13}C NMR. The result for the Al-soil indicated that the turnover of microbial products was retarded due to toxic effects of aluminium.

Dipolar dephased spectra

In dipolar dephased experiments, interruption in magnetization of protons for decoupling results in signals for carbons having an attached proton are lost as a function of dipolar dephasing time from the spectra. In the absence of any molecular motion, almost all signal arising from CH_2- and CH- carbons are lost if dipolar dephasing time exceeds 40 μs (Opella & Frey 1979). In the structures that undergo molecular motion, however, CH_2- and CH-carbons can still be observed when a dipolar dephasing time of more than 40 μs is used. This is because molecular motion weakens the ^1H-^{13}C dipolar interaction and thereby reduces the rate of signal decay. Due to rapid axial rotation of CH_3 groups, that drastically reduces the dipolar interaction, these groups are not dephased as rapidly as CH_2- and CH-carbons and they behave as if they were less than monoprotonated (Wilson 1987).

The dipolar dephased spectra of Ca-, Na-, and Al-soils are presented in Figures 4–6. Compared with conventional spectra, the dipolar dephased spectra showed significant decreases in intensities of signals at 72 and 105 ppm, indicating that signals from these regions were dominated by CH_2- and CH-carbon. The relatively small decrease in intensity of alkyl carbon in dipolar dephased spectra suggested that CH_2- and CH-carbon in alkyl structures were mobile. Similarly, Baldock et al. (1990b) concluded that a small decrease in intensity of alkyl carbon in dipolar dephased spectra of fungal materials was probably due to rapid molecular motion of alkyl moieties in the sample. The signal intensity of aromatic (130 ppm) and carbonyl carbon remained relatively constant in dipolar dephased spectra of glucose amended

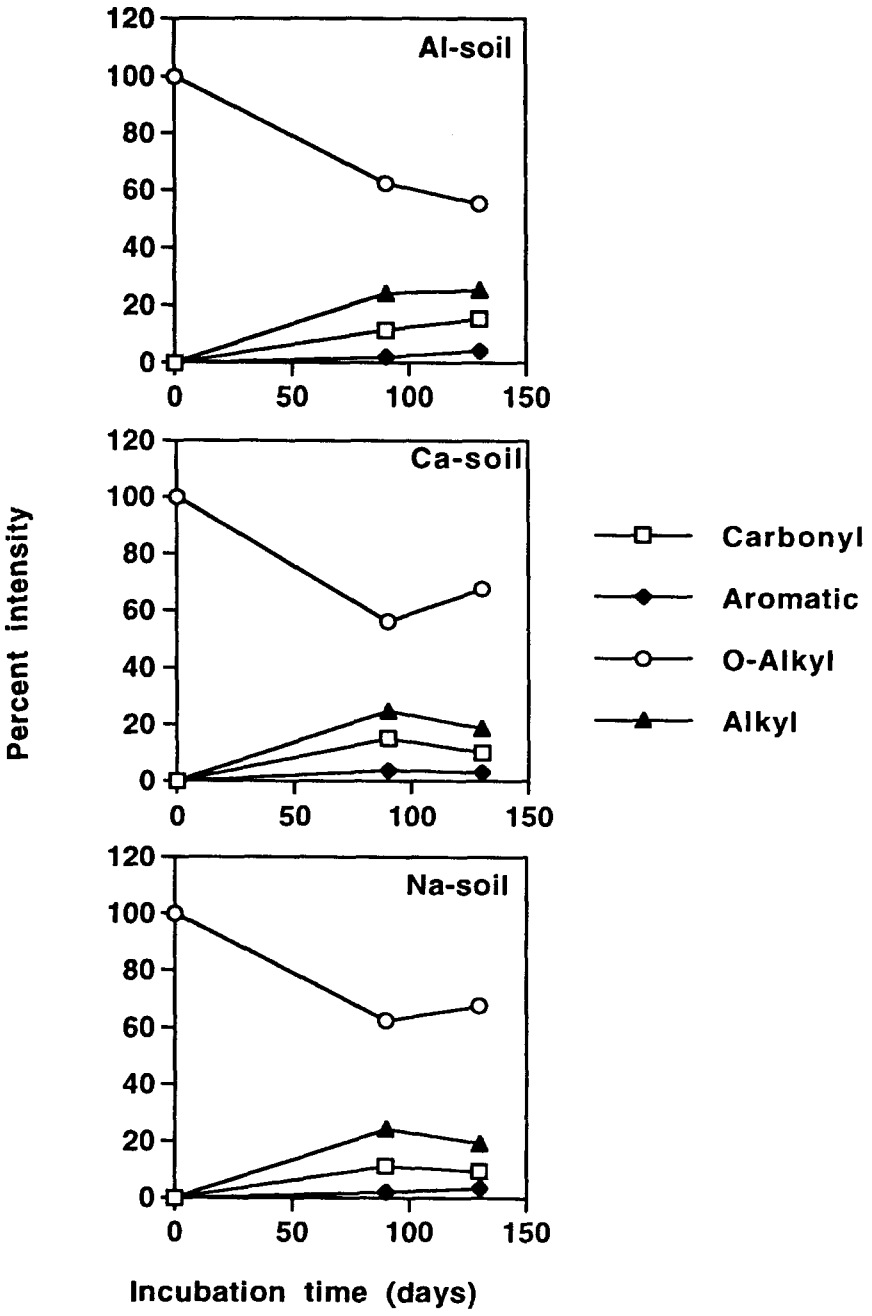

Figure 8. Changes in the proportions of carbon functional groups with time in Al-, Ca-, and Na-soils.

soils. For carbonyl carbon, this was consistent with the fact that this form of carbon was nonprotonated. The conventional NMR spectra showed no signals which could be ascribed to phenolic carbon. However, the dipolar dephased spectra exhibited small signals in the region of 145–164 ppm which could be attributed to the presence of phenolic carbon. Dipolar dephasing relatively enhances phenolic carbon compared to protonated carbon (Hatcher et al. 1989), but even under these circumstances no significant intensities assignable to phenolic carbon could be detected. It must be concluded that phenolic carbons were not present in significant amounts in the materials synthesised by soil biomass. In dipolar dephased experiments the behaviour of peaks at 55 and 62 ppm were used to identify the structures of carbon from which they originated. As discussed previously, methoxyl carbon and the α-carbon of amino acids resonate in the region of 55 ppm. Dipolar dephasing induced a large decrease in intensity of the peak located at 55 ppm, indicating that the carbon in question was predominantly composed of CH_2- and CH-carbon structures and therefore originates from amino acids.

PSRE subspectra

Soil organic matter occurs as a heterogeneous mixture of different organic compounds in which these compounds may exist as physically discrete components in spatially distinct domains. Assuming that spin diffusion equates all protons to a single proton bath for an individual molecule, protons in the domains are isolated from each other and have different spin-lattice relaxation time (T_1(H)) values, reflecting the average conditions within those domains. By using PSRE which exploits differences in proton-spin relaxation time, it is possible to generate subspectra of carbon associated with hydrogen having long and short values of T_1(H). This technique has previously been used to show structural heterogeneity of organic material in soils (Newman & Tate 1991; Preston & Newman 1992).

The PSRE subspectra of Ca-, Na-, and Al-soils incubated with ^{13}C-glucose for 130 days are shown in Figure 9. The distribution of different types of carbon in the PSRE subspectra are given in Figure 10 and shows that long and short T_1(H) subspectra have clear differences in chemical composition. The subspectra of carbon associated with fast-relaxing (short T_1(H)) protons were dominated by signals assigned to O-alkyl carbon, indicating that polysaccharides were quantitatively the most significant compounds in these fractions. The O-alkyl carbon accounted for 68–74% of total carbon, the contribution of alkyl carbon was much smaller (9–15%) and aromatic carbon occurred in the lowest quantities (4–6%). The carbonyl carbon content was higher (7–17%) in short T_1(H) fractions when compared with long T_1(H) fractions (4–10%).

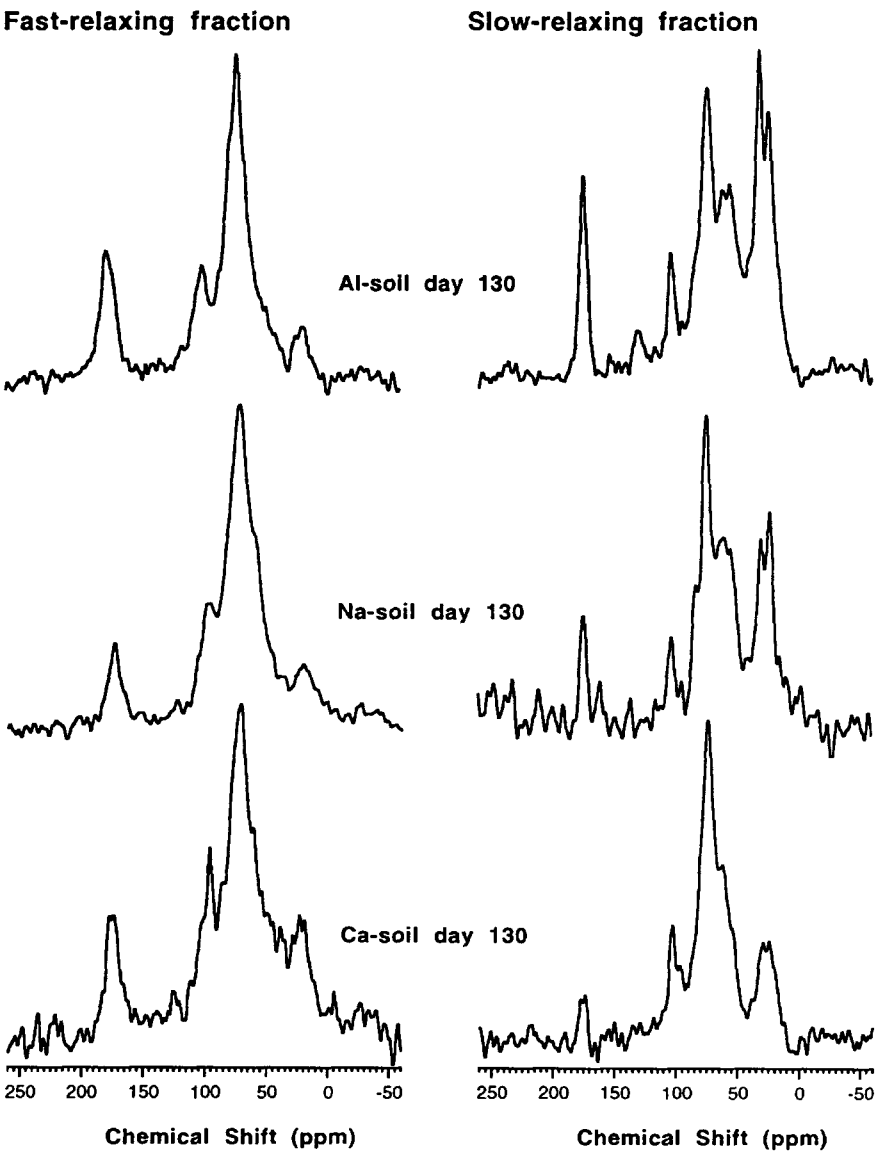

Figure 9. Solid-state ^{13}C CP/MAS NMR subspectra obtained for Al-, Na, and Ca-soils at day 130 of the incubation.

The relative proportions of O-alkyl carbon were lower (51–62%) in the long $T_1(H)$ subspectra compared with short $T_1(H)$ subspectra (Figure 10). In contrast, the alkyl carbon was an important component of organic materials contained in long $T_1(H)$ subspectra. The relative contribution of alkyl

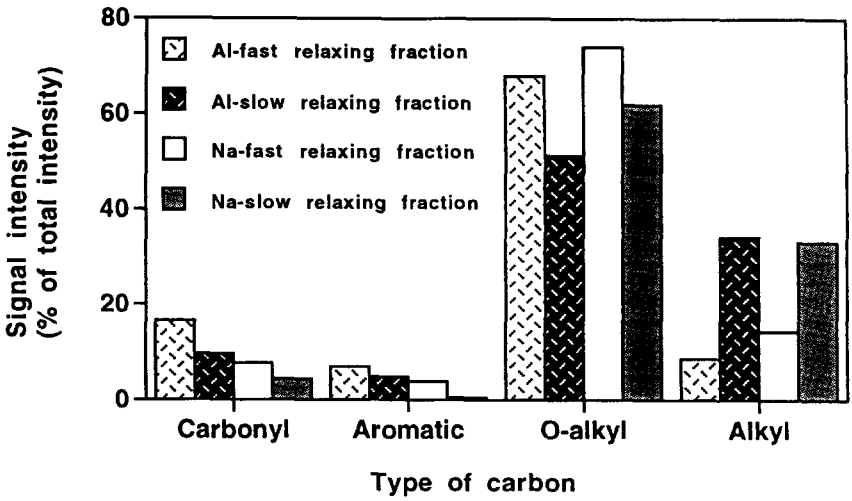

Figure 10. The relative proportions of different types of carbon in NMR subspectra of Al- and Na-soils.

resonances to total signal intensity in long T_1(H) subspectra was 33–35% which is twice the amount of alkyl carbon in short T_1(H) subspectra. The distribution of alkyl groups also appeared different in long and short T_1(H) subspectra. The long T_1(H) subspectra showed alkyl groups in long and short chain aliphatic structures with resonances centred at 23 and 30 ppm. The short T_1(H) represented highly branch alkyl material with resonances at 19 and 23 ppm. The long T_1(H) subspectra also had strong signals at 55 ppm, indicating that amino acids and protein were major components of these fractions. The long T_1(H) subspectra presented in Figure 9 are very similar to spectra obtained for fungal materials (Figure 11) and were probably microbial tissues consisting, predominantly, of chitin.

In ultrathin sections of natural soil fabrics using cytochemical techniques Foster (1988) showed that polysaccharides are secreted by soil bacteria and fungi which often fill the space surrounding the cell. These extracellular polysaccharides which were revealed as fibrils or granules after staining, showed strong association with clay particles. Oades (1984) showed that microbial polysaccharides consisted of hexoses (80%) and equal amounts of uronic acids and amino compounds. The similarity between the chemical composition of the organic materials contained in the short T_1(H) subspectra with that of uronic acids (Figure 11), suggests that these materials may well be microbial mucilages which relax quickly because of strong association with clay particles. While, the organic materials contained in long T_1(H) subspectra were cellular materials at some distance from clay surfaces.

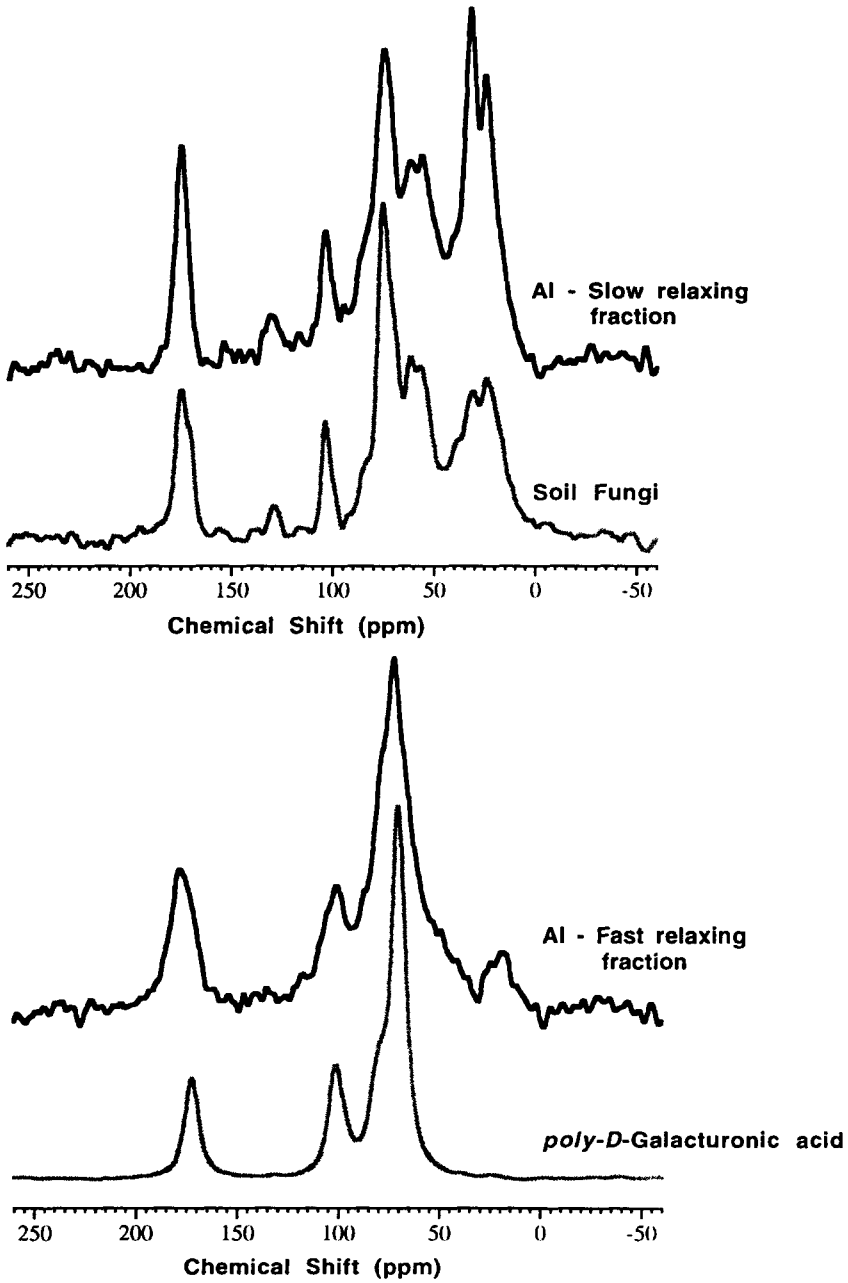

Figure 11. A comparison of the solid-state ^{13}C CP/MAS NMR subspectra of the slow and fast relaxing components of the microbial products from the Al-soil with spectra obtained for fungal material and *poly-D-* galacturonic acid.

Bloch decay spectra

In the CP/MAS NMR experiments, carbons separated from protons more than 4 bonds could be rendered invisible to NMR (Wilson 1987). Thus, ^{13}C Bloch decay spectra were aquired for the soils amended with glucose. The Bloch decay spectrum of Ca-soil after 130 days of incubation showed more signal in the aromatic region (110–140 ppm) than the ^{13}C CPMAS spectrum of the same sample (Figure 12). Two possible explanations for this discrepancy are either that all of the aromatic carbon present was not observed in the CP/MAS experiments or the Bloch decay spectrum included signal in the aromatic region derived from the Kel-F caps of the rotor. As conditions for satisfactory CP was met on the basis of $T_{1\rho}H$ experiments, a decrease in signal intensity in the aromatic region due to CP was unlikely. To test the second possibility, a Bloch decay spectrum was obtained for an empty rotor using the same experimental condition as for the Ca-soil (Figure 12). The Bloch decay spectrum obtained for the empty rotor showed a considerable contribution of signal intensity in the 70–150 ppm chemical shift region. The source of this signal intensity was from nonprotonated carbon polymers (chlorofluorocarbon polymer) contained in the Kel-F caps of the rotor. The contribution of signal intensity derived from the rotor caps in Bloch decay spectra will be more pronounced when the carbon concentration of a sample is low as was the case for our samples. The signal contribution of the Kel-F caps is considerably reduced for a CP/MAS experiment, as the Kel-F polymer contains no protons to cross polarize. As a consequence, it was assumed that the CP/MAS experiments were quantitative.

Conclusion

By conducting an uptake experiment, ^{13}C NMR spectroscopy allowed changes in the chemical structure of ^{13}C-glucose associated with its incorporation into microbial biomass to be observed. The rate and extent of mineralization of added glucose was different in soils with different exchangeable cations. However, when the added glucose was completely metabolized by soil organisms the chemistry of synthesised materials was similar in the different soils, irrespective of different physical and chemical characteristics of those soils. It is concluded that exchangeable cations exert their influence on carbon dynamics partly by controlling microbial growth and metabolic activities through modifying the physicochemical characteristics of microbial habitats. However, microbial processes and extra-cellular syntheses and thus the composition of synthesised material are not greatly affected by soil condition. The materials synthesised from glucose by soil biota were mostly

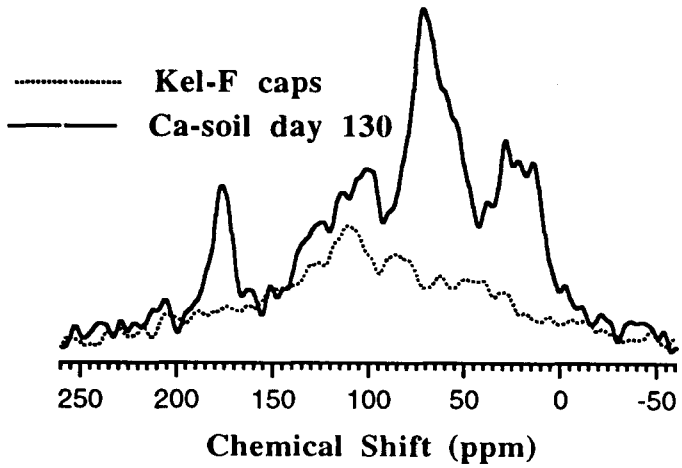

Figure 12. Bloch decay ^{13}C NMR spectra obtained for Ca-soil at day 130 of the incubation and empty rotor.

O-alkyl, alkyl and carbonyl carbon. Both phenolic and aryl aromatic carbon structures were only present in small amounts, indicating that aromatic carbon was not a dominant type of carbon synthesised by soil biomass when a simple substrate like glucose was metabolized (Baldock et al. 1989).

The present work shows that characterization of physically distinct organic components of microbial products is possible with the use of advanced NMR techniques (PSRE) which allowed the chemical composition of microbial tissues and mucilages to be determined. Fractionation by NMR techniques has a great potential for characterizing the inherent heterogeneity of soil organic matter and should be applied to the future studies of soil organic matter where structural characterization of organic components is not available by other techniques.

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