Influence of Calcium on the Decomposition of Organic Materials in Soils

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Abstract

The mineralisation of organic carbon in soils has been shown to be inhibited in the presence of calcium, added as gypsum or agricultural lime. The mechanism(s) by which calcium stabilises soil organic carbon against microbial attack was investigated in this study.

All evidence suggested that the stabilisation resulted from the formation of Ca^{2+}-organic complexes; however, an inhibition of microbial activity due to an increased electrolyte concentration could not be dispelled. The influence of electrolyte concentration on microbial activity was assessed by measuring the rate of glucose decomposition in soil amended with $CaCl_2$. Calcium concentrations comparable to those observed for gypsum amended soils did not inhibit microbial activity.

The stabilisation of organic carbon through the formation of Ca^{2+}-organic complexes was postulated to result from interactions with anionic carboxylic functional groups. An experiment in which ^{13}C-glucose was added to unamended and gypsum amended soil was performed. Carbon dynamics were followed using carbon isotope mass spectrometry and changes in the chemical structure of the substrate ^{13}C were monitored using solid state ^{13}C NMR spectroscopy.

Although gypsum addition decreased substrate mineralisation, the effect was smaller than previously noted for other soils. The ability of calcium to stabilise soil organic materials therefore appeared to vary with soil type.

The chemical structure of the residual substrate ^{13}C was not influenced by the addition of gypsum. An accurate determination of the chemical structure of the residual substrate ^{13}C contained in the samples required the determination of $T_1\rho H$ values. A poor relationship was obtained between the total CP/MAS ^{13}C NMR spectral intensity and the ^{13}C contents of the samples, presumably because of an interaction with paramagnetic species. Utilisation of glucose by the soil microbial biomass resulted in the

synthesis of alkyl, O-alkyl, and carboxyl carbon but little if any aromatic carbon. More detailed solid state ^{13}C NMR experiments were performed on particle size and density fractions separated from the soil incubated for 34 days. Again $T_1\rho H$ measurements were required to determine the distribution of carbon functionalities accurately. The residual ^{13}C accumulated in the clay fraction as alkyl, O-alkyl, acetal and carboxyl carbon. Solid state ^{13}C NMR spectra acquired for a bacterial and a fungal culture isolated from the Meadows soil indicated that the chemical composition of the residual ^{13}C in the soil samples resembled that of the fungi more closely than that of the bacteria suggesting that the microbial biomass in the soil was dominated by fungi. Dipolar dephasing experiments were also performed on all samples and provided information on the number of protons attached to and the molecular motion of each type of carbon identified.

The influence of soil type on the ability of gypsum to stabilise organic carbon against microbial attack was investigated using 9 Red-brown earths having clay contents and surface areas ranging between 7-29 % and 17-43 $m^2\ g^{-1}$ soil, respectively. The influence of gypsum on the amount of glucose ^{14}C mineralised during a 57 day incubation period varied with soil type; however, no relationship could be established between the magnitude of the decrease in substrate ^{14}C mineralisation and the clay content or surface areas of the soils.

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Statement

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of the author's knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

I consent to this thesis being made available for photocopying and loan.

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Jeffrey Alexander Baldock

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Chapter 1

Introduction and Literature Review

1.1 Introduction

The red-brown earths of the lower to mid north region of South Australia are extensively used for wheat production and represent an important group of agricultural soils. Although Piper (1938) described red-brown earths as some of the most fertile wheat producing soils in South Australia, there is an increasing awareness that the structural stability of their surface aggregates deteriorates under intensive cropping practices. Cropping systems which incorporate continuous row crop production, mechanical fallowing, removal or burning of crop residues, and the use of heavy machinery contribute to the structural deterioration of these soils.

When exposed to rapid wetting the surface aggregates of structurally degraded red-brown earths tend to slake and disperse resulting in the formation of a hard compacted surface layer on drying. Red-brown earths exhibiting such characteristics are referred to as hardsetting (Northcote 1979) and are susceptible to yield depressions as a result of restricted crop establishment and poor water use efficiency. Hardsetting soils are also highly susceptible to erosional problems during rainfall events due to low water infiltration rates and increased amounts of surface run-off. The development of cropping and management practices designed to increase the structural stability of the surface aggregates of red-brown earths is required if these soils are to regain their full agricultural potential.

Soil aggregates can be divided into two broad categories based on their size and mechanism of stabilization, macroaggregates (>250 μm diameter) and microaggregates (<250 μm diameter). Tisdall and Oades (1982) and Oades (1984) have demonstrated that the dominant binding agent

responsible for macroaggregate stability is soil organic matter. For macroaggregates ≥2mm in diameter the most important binding agent is considered to be plant roots and fungal hyphae (Tisdall and Oades 1982). As the size of macroaggregates decreases polysaccharides of plant and microbial origin become important stabilising agents as demonstrated by the large decreases in macroaggregate stability observed by selectively oxidising polysaccharides with periodate (Mehta et al. 1960, Clapp and Emerson 1965, Stefanson 1971, Baldock and Kay 1987). Owing to the transient nature of these organic binding agents in soils, they must be continually replaced to maintain levels which are sufficient to ensure the stabilisation of macroaggregates.

Tisdall and Oades (1982) and Oades (1984) have suggested that the stabilization of macroaggregates can be controlled by the adoption of adequate cropping and management practices. Cropping systems which include the production of grass crops with extensive root networks capable of supporting mycorrhizal fungi and a minimum of soil cultivation enhance macroaggregate stability; however, those which include intensive cultivations, such as a wheat-fallow system, accelerate the decomposition of these organic binding agents and reduce macroaggregate stability.

Microaggregate stability depends on the reversible dispersion and coagulation and the irreversible flocculation of individual or packets of clay particles. Dispersion and coagulation are controlled by the physicochemical properties of the soil, in particular the composition of the clay's cation exchange complex and the electrolyte concentration of the soil solution. Once coagulated the clays may be irreversibly flocculated by organic and inorganic cements. Microaggregate stability is thus relatively independent of cropping practices, at least in the short term, and can only be modified by altering soil chemical characteristics. For example, Oades (unpublished data) observed that the microaggregate stability as determined by measuring the amount of

dispersible clay was similar for soil collected from an old pasture and a wheat-fallow rotation which had been in place for more than 40 years. However, modifying soil chemical properties by adding materials capable of releasing calcium to the soil solution (eg. gypsum or agricultural lime) has been shown to reduce or control dispersion (Shainberg and Gal 1982; Shanmuganathan and Oades 1983; Rengasamy et al. 1984; Muneer and Oades 1989a,b). Using two different red-brown earths, Shanmuganathan and Oades (1983) and Muneer and Oades (1989a,b) noted a reduction in the amount of dispersible clay and an increase in the proportion of 50-250 μm diameter water-stable particles subsequent to the addition of gypsum or calcium carbonate. The reduction in dispersible clay and the subsequent formation of 50-250 μm diameter water-stable particles was attributed to the maintenance of an electrolyte concentration in the soil solution, by the dissolution of the calcium amendments, at a value greater than that required to keep the clays in a coagulated state.

Muneer and Oades (1989a,b) observed that the simultaneous incorporation of gypsum or calcium carbonate with an organic substrate into a red-brown earth increased the proportion of water stable macroaggregates and reduced the dispersion of clay particles. The induced structural stabilisation persisted longer than that due to the addition of either amendment alone. Muneer and Oades (1989a,b) also noted that the proportion of the substrate derived organic carbon mineralised to carbon dioxide by the soil microbial biomass was decreased by the addition of the calcium amendments. Therefore as well as enhancing the stability of both macroaggregates and microaggregates the simultaneous incorporation of calcium compounds with an organic substrate reduced the amount of substrate carbon mineralised to carbon dioxide. Under field conditions, the presence or addition of calcium amendments to soils may therefore reduce organic matter losses and improve the structural characteristics of red-brown earths used for wheat production in

South Australia.

The objective of this study was to determine how calcium amendments alter the extent of mineralisation of organic materials in soil. By obtaining an understanding of the mechanism(s) involved, the potential exists to modify cropping and management practices such that the turnover of soil organic matter is reduced and the structural stability of the surface aggregates is increased.

1.2 Literature Review

1.2.1 Soil Organic Carbon

The organic materials in mineral soils consist of a heterogeneous mixture of plant and microbial debris which exists at numerous stages of decomposition and can be fitted into three general categories: 1) fresh decomposable plant and microbial debris, 2) well humified biologically stable materials, and 3) organic materials associated with the living plants, soil animals and soil microbial biomass. The organic carbon associated with each of these categories will be referred to as substrate carbon, humified carbon and biomass carbon, respectively. The divisions between each of these forms of carbon is arbitrary as even the most biologically stable humified carbon is susceptible to decomposition and may therefore be viewed as a substrate. It is also difficult to quantitatively differentiate between the biomass carbon and humified carbon. For the purpose of this study these materials will be differentiated on the basis of the changes which occur to the chemical structure of an organic substrate during its decomposition. Once the original chemical structure of the substrate carbon has been completely transformed (ie. no longer resembles that of the unaltered substrate) it will no longer be referred to as substrate carbon. The portion of the residual carbon contained in the living biomass will be referred to as biomass carbon and the nonliving fraction will be referred to as humified carbon. The term soil organic matter will

be used when referring collectively to the organic materials contained in all three categories.

1.2.2 The Organic Carbon Cycle in Soil

A simple organic carbon cycle utilising the three components of soil organic matter outlined above and typical of that operating in noncalcareous soil environments is shown in Figure 1. In calcareous soils or soils with

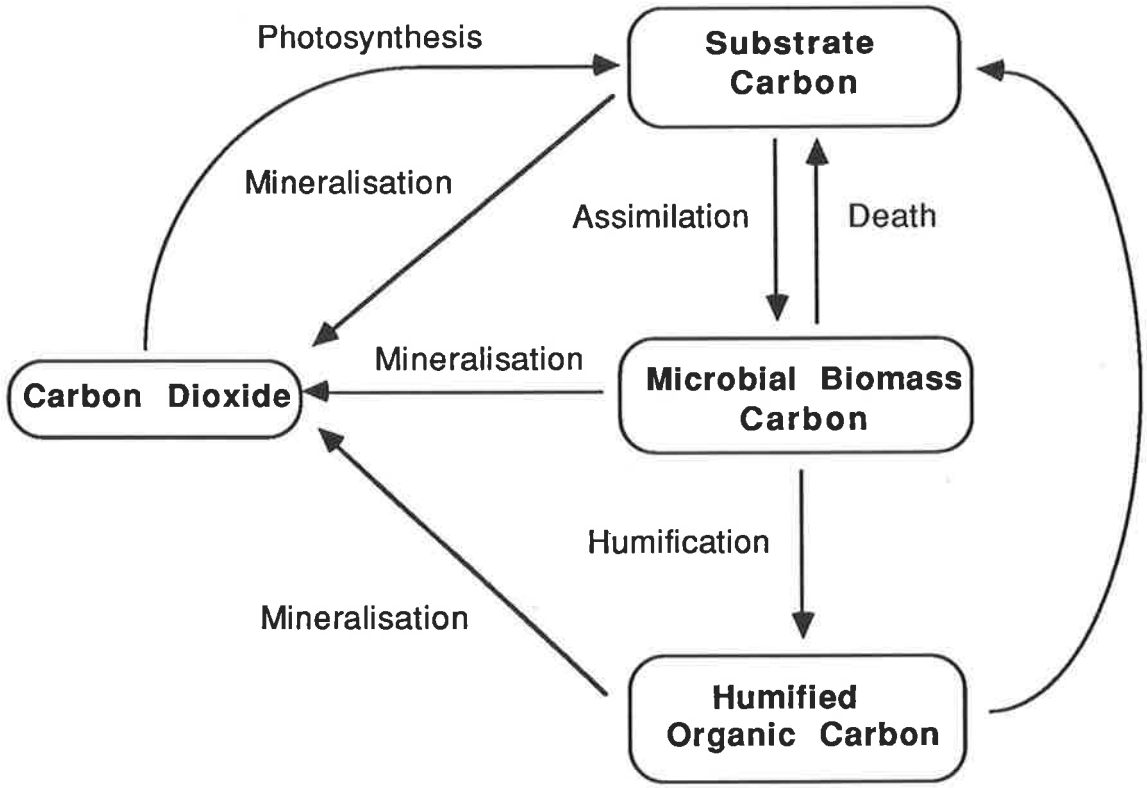

Figure 1.1: A simplified organic carbon cycle in soils.

alkaline pH values the formation of calcium carbonate must also be considered. In its simplest form the carbon cycle revolves around the fixation and regeneration of atmospheric carbon dioxide. Atmospheric carbon dioxide is fixed into organic materials by photosynthesis in photoautrophic organisms, dominantly plants and algae. During the growth of these organisms and when

they die, organic materials are deposited on the soil surface and/or in the soil matrix. These materials provide a substrate for the heterotrophic soil biomass which includes both soil animals and microorganisms. Although soil animals (eg. earthworms, mites, etc.) utilise organic materials as a substrate and may initiate the decay process, microbial decomposition assumes the dominant role (Alexander 1977) since all organic carbon, even that associated with the bodies of soil animals, ultimately passes through the soil microbial biomass. As organic substrates are decomposed by the soil microbial biomass, a portion of the carbon is utilised for the synthesis of the organic materials required by the microorganisms and a portion is returned to the atmosphere in the form of carbon dioxide. The processes by which substrate carbon is converted into microbial cellular structures or carbon dioxide are referred to as assimilation and mineralisation, respectively. Decomposition which is used to describe the disappearance of an organic substrate is therefore equal to the sum of assimilation and mineralisation. The decomposition of a substrate is normally complete long before the substrate carbon is entirely mineralised since a significant fraction of the carbon may be initially assimilated by the growing microbial biomass. However, once the original substrate becomes limiting a portion of the microbial biomass dies and the substrate carbon which it assimilated becomes a substrate for the remaining microbial biomass and is ultimately mineralised to carbon dioxide.

1.2.3 Influence of Calcium on the Soil Carbon Cycle

An influence of calcium on carbon cycling in soil was perhaps first suggested by Chizhévskii (1932) in a study which looked at the effect of adsorbed cations on the mineralisation of organic carbon. Chizhévskii observed that the amount of organic carbon mineralised from a sodium saturated soil was much greater than that mineralised from the same soil saturated with calcium. Data collected by Walksman (1932,1936) suggested

that a portion of the increased mineralisation in the presence of sodium may have resulted from an increase in the amount of water soluble organic carbon. These observations led Sokoloff (1938) to determine the influence of sulphates and chlorides of sodium and calcium on the mineralisation and solubilisation of the organic materials contained in two sandy soils (Figure 1.2). Relative to the soil treated with distilled water, the amount of carbon mineralised and solubilised was increased by adding sodium and decreased by adding calcium. In the presence of calcium, the extent of mineralisation and solubilisation were reduced to approximately two thirds of that observed for the unamended soil. The biological stabilisation of organic carbon in the

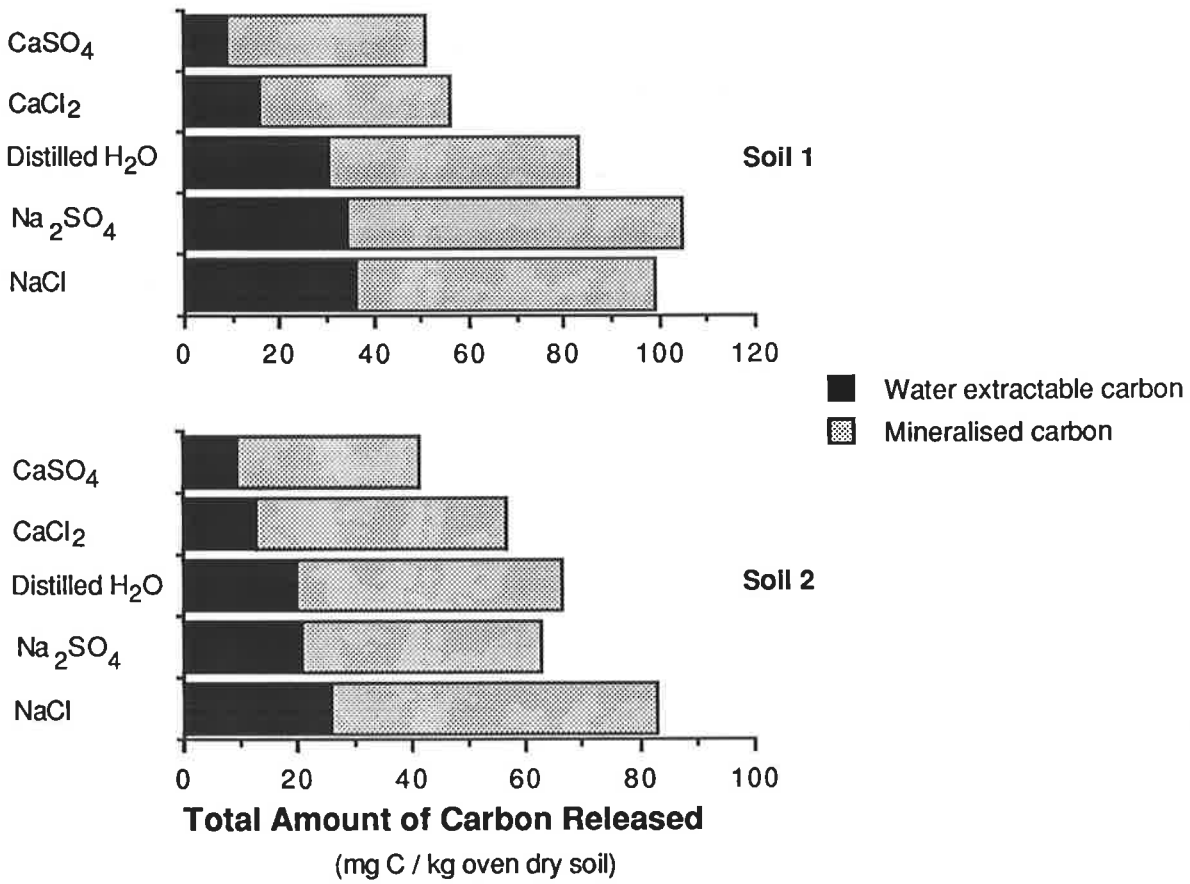

Figure 1.2: Influence of adding sodium and calcium salts to two sandy soils on the amount of water soluble and mineralisable organic carbon (Sokoloff 1938).

presence of calcium was suggested to result from the decreased amount of water soluble carbon in the soils.

An influence of Ca^{2+} cations on the biological stability of organic materials in soil was also noted by Kononova (1940) for a soil amended with plant residues. As the ratio of exchangeable Ca^{2+}/Na^{+} was decreased by a partial replacement of Ca^{2+} with Na^{+}, the amount of organic carbon mineralised increased.

In the preceding studies no mention was made of the influence which the Ca^{2+} and Na^{+} cations may have had on the coagulation and dispersion of soil clays. Addition of Ca^{2+} cations would favour coagulation while addition of Na^{+} cations would favour dispersion. A portion of the influence of Ca^{2+} and Na^{+} on the biological stability of soil organic matter may therefore have been due to an entrapment of organic materials within coagulated clays and an exposure of organic materials on the surfaces of dispersed clays. However, the increased amounts of water soluble organic materials observed in the presence of Na^{+} cations, suggested that the formation of insoluble calcium-organic complexes was involved.

Juste and Delas (1967) compared the solubility of a humic acid saturated with Al^{3+}, Fe^{3+}, Cu^{2+}, Mg^{2+} or Ca^{2+} in both water and an alkaline solution. The presence of Ca^{2+} had little influence on the solubility of the humic acid in water; however, relative to the remaining metal humates, the solubility of the Ca^{2+}-humate in the alkaline solution was much lower. Based on this observation Juste and Delas (1970) postulated that once linkages formed between Ca^{2+} and humic materials the susceptibility of humic compounds to attack by soil microorganisms may be decreased. By comparing the oxygen uptake of a soil amended with either a Ca^{2+} or Na^{+} saturated humic acid, Juste and Delas (1970) showed that the susceptibility of the humic acid to microbial attack was decreased in the presence of Ca^{2+} cations (Figure 1.3).

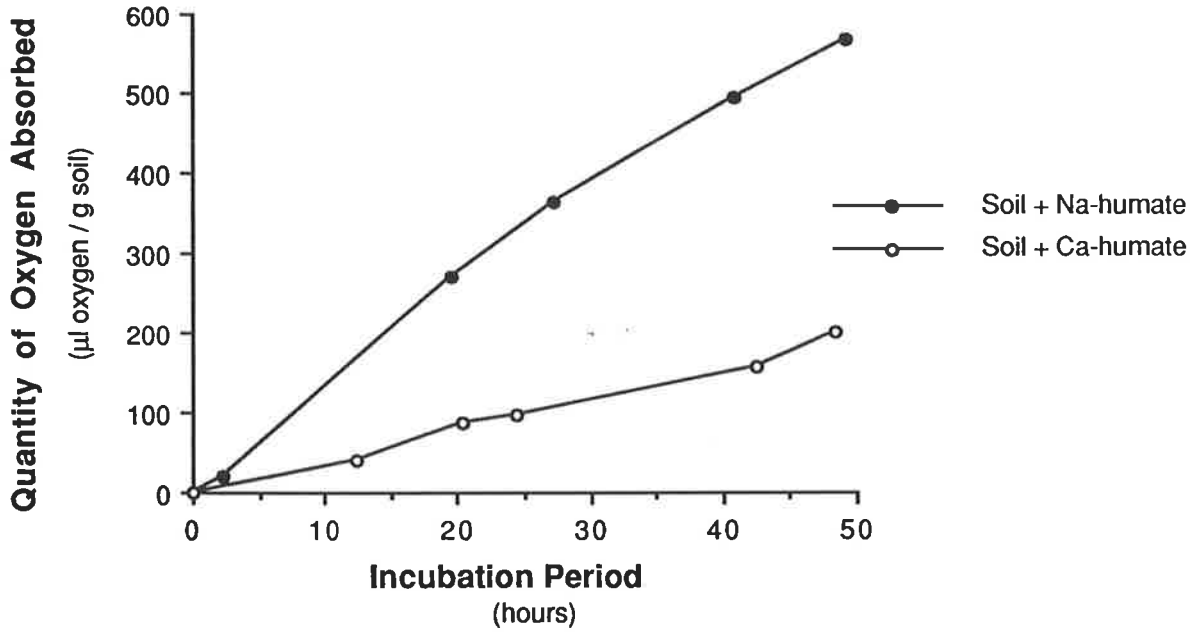

Figure 1.3: Oxygen uptake of a soil amended with either Ca^{2+} or Na^{+} saturated humic acid (Juste and Delas 1970).

In addition to a similar influence of Ca^{2+} cations on the biological stability of a humic acid, Linhares (1977) noted that the addition of $CaCl_2$ to a soil amended with a Na^{+} saturated humic acid reduced the oxygen uptake of the soil indicating that a stabilisation of the Na^{+} saturated humic acid against microbial attack occurred. Linhares postulated that the Ca^{2+} cations, originating from the added $CaCl_2$, blocked the decomposition of the humic materials by replacing Na^{+} cations and/or by forming Ca^{2+}-humate complexes at vacant sites on the humic molecule.

Juste et al. (1975) measured the oxygen uptake in a soil amended with an Al^{3+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, Cu^{2+},Ca^{2+} or Na^{+} saturated humic acid to determine if the increased biological stability of humic materials in the presence of calcium could be extended to other polyvalent cations. Relative to the soil amended with the Na^{+}-humate, significantly less oxygen was taken up by the soil amended with the polyvalent cation-humates. A similar stabilisation of organic soil components to biodegradation by the addition of Al^{3+}, Fe^{3+},

Zn^{2+}, and Cu^{2+} was noted by Martin et al. (1966, 1972), with respect to the decomposition of polysaccharides of microbial and plant origin. Juste et al. (1975) postulated that the mechanism through which polyvalent cations alter the biological stability of humic materials involves:

a) a blockage of specific sites on the humic materials which are required by the microbial biomass to initiate the decomposition process, and/or

b) a toxic effect of the polyvalent cations on the microbial biomass.

Juste et al. (1975) observed that for the Al^{3+}, Fe^{3+}, Zn^{2+}, and Cu^{2+} saturated humic materials a portion of the stabilisation undoubtably involved a toxic effect; however, no toxic effect was involved with the Ca^{2+} saturated materials. Therefore, although the biological stabilisation of soil humic materials by polyvalent cations appears to be a general phenomenon, the behaviour of the calcium saturated humic materials and the mechanisms responsible for the increased biological stability differed from those associated with the other polyvalent cations.

The influence of calcium on the decomposition of organic materials in soil has also been assessed by incubating organic substrates in pedogenically different soils containing various amounts of calcium. The mineralisation of uniformly labelled ^{14}C-glucose incubated in samples of the surface horizon of a podzol, a leached brown soil, and a rendzina was followed by Yaghi (1973). The extent of glucose ^{14}C mineralisation in the podzol, leached brown soil and rendzina was 75, 61 and 57%, respectively, indicating that as the amount of calcium carbonate in the soils increased the extent of mineralisation decreased even though the changes in soil pH would have been expected to enhance microbial activity.

Chouliaras and Jacquin (1976) incubated uniformly labelled ^{14}C glucose and maize stover in a rendzina, a brown acidic soil and the brown acidic soil amended with $CaCO_3$. The incubation procedure consisted of two 21 day incubation periods separated by a 21 day drying period. Glucose ^{14}C

mineralisation from the rendzina soil was less than that of the brown acidic soil during both incubation periods but the extent of the decrease was enhanced subsequent to the drying period. Addition of $CaCO_3$ to the brown acidic soil did not influence glucose ^{14}C mineralisation during the first incubation period; however, in the second incubation period, after the soil was dried, $CaCO_3$ addition did significantly reduce the amount of ^{14}C mineralised. The mineralisation of the maize stover ^{14}C followed the same trends as exemplified by the glucose ^{14}C apart from the fact that the extent of mineralisation was less. Irregardless of the exact mechanism responsible for the decreased substrate mineralisation noted by Chouliaras and Jacquin. (1976) in the presence of $CaCO_3$, the observation that drying the soils led to an enhancement of the effect is important since soils are exposed to numerous wetting and drying cycles under natural conditions.

The decomposition and humification of glucose and a microbial polysaccharide uniformly labelled with ^{14}C in a rendzina, an acidic mull, a leached brown soil and a podzol were studied by Tok et al. (1976). The proportion of the substrate ^{14}C mineralised during 3 weeks incubation was significantly influenced by soil type. The extent of mineralisation of the glucose and polysaccharide ^{14}C in the rendzina (57 and 18%, respectively) was much less than that observed for the podzol (75 and 29%, respectively). The acidic mull and leached brown soils gave intermediate values. This result and those collected by Yaghi (1973) and Chouliaras and Jacquin (1976) suggest that the presence of $CaCO_3$ in naturally occurring soils can biologically stabilise organic materials against microbial attack; however, they offer no direct evidence of the involvement of Ca^{2+} cations in the stabilisation process.

In addition to determining the amount of substrate ^{14}C mineralised, Tok et al (1976) also determined the proportion of the initial substrate ^{14}C which accumulated in the water soluble, alkaline soluble and insoluble humin

organic fractions associated with each of the incubated soils. The proportion of substrate ^{14}C contained in each organic fraction in the rendzina and podzol are presented in Figure 1.4. The results acquired for the acidic mull and the leached brown soil were not significantly different from that collected for the rendzina. For the glucose ^{14}C, the proportions of water soluble and alkaline soluble ^{14}C in the two soils were similar and the decreased ^{14}C mineralisation observed for the rendzina appeared to result from an accumulation of insoluble humin. For the polysaccharide ^{14}C the partitioning of ^{14}C in the organic fractions of the rendzina and podzol was very different. The largest accumulation of ^{14}C in the rendzina was found in the insoluble humin organic fraction. In the podzol the substrate ^{14}C accumulated dominantly in the alkaline soluble fraction. If it is assumed that the dominant cations present in

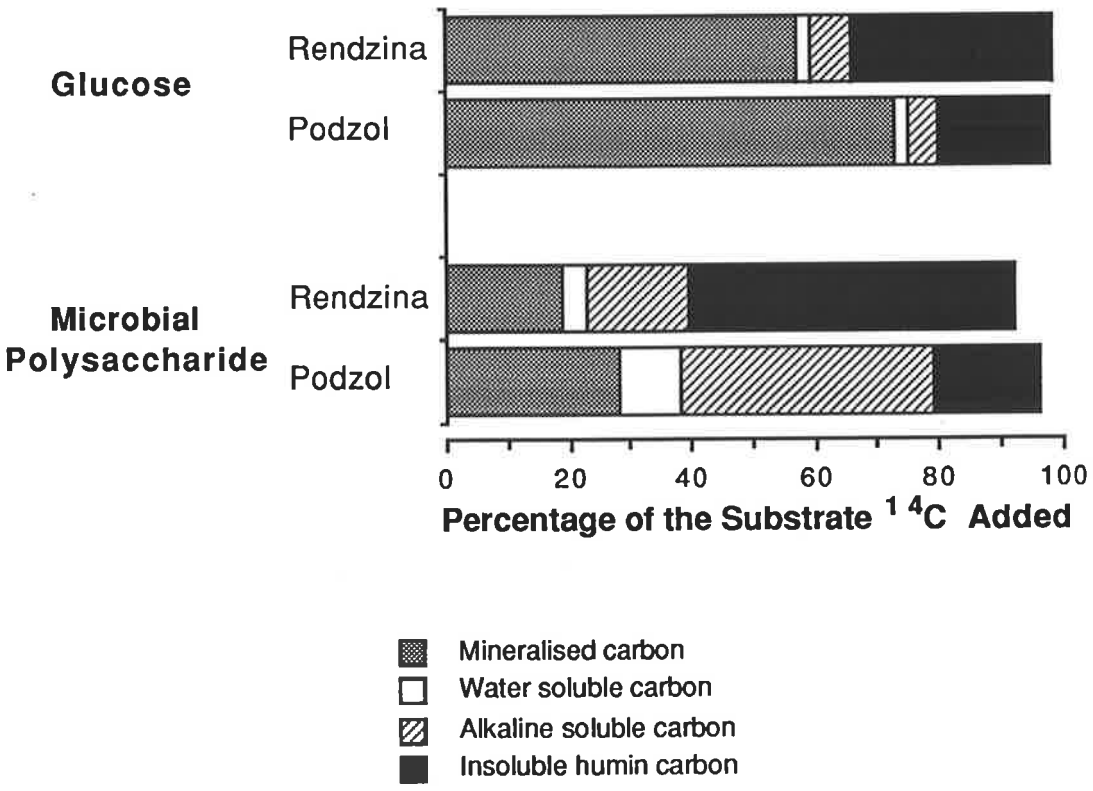

Figure 1.4: Distribution of the glucose and microbial polysaccharide ^{14}C in a rendzina and a podzol after 21 days incubation (Tok et al. 1976).

the rendzina and podzol were Ca^{2+}, and Al^{3+} and Fe^{3+}, respectively, the distributions of ^{14}C obtained by Tok et al. (1976) are consistent with the work of Juste and Delas (1967). Based on the differences in the solubility of Ca^{2+}-, Al^{3+}-and Fe^{3+}-humates in an alkaline solution observed by Juste and Delas (1967),the large accumulation of ^{14}C in the insoluble humin organic fraction and the decreased amount of ^{14}C mineralisation resulted from the formation of Ca^{2+}-organic complexes.

The influence of calcium on the solubility of soil organic carbon in alkaline solutions was also suggested by the data of Hoyos et al. (1982), Tomar et al. (1986) and Muneer and Oades (1989c). Hoyos et al. (1982) observed that when the bound organic carbon of three soils rich in $CaCO_3$ was fractionated into fulvic acid, humic acid and insoluble humin, the largest proportion of the organic carbon was contained in the insoluble humin fraction. For 9 surface soils Tomar et al. (1986) noted that the amount of insoluble humin increased with increasing exchangeable Ca^{2+} contents. Muneer and Oades (1989c) observed that the amount of insoluble humin contained in a soil incubated with glucose was significantly higher in the presence than absence of Ca^{2+} cations. Acid pretreatment (1M HCl) of the soils prior to alkaline extraction eliminated the influence of the Ca^{2+} cations on the amount of insoluble humin. These results are substantiated by the general observation that the efficiency of alkaline reagents in extracting organic materials from calcareous soils is markedly improved if the soil is pretreated with a mineral acid. The influence of the acid pretreatment presumably results from a replacement of Ca^{2+} cations in the Ca^{2+}-organic complexes with H^+ cations.

Muneer and Oades (1989a,b) studied the influence of adding calcium, as either gypsum, $CaSO_4 \cdot 2H_2O$, or lime, $CaCO_3$, to a red brown earth on the mineralisation of carbon from ^{14}C-labelled glucose in a laboratory experiment and ^{14}C-labelled wheat straw in a field experiment . The residual ^{14}C

contents of the soil used in the laboratory and field experiments are shown in Figure 1.5. Mineralisation of the glucose ^{14}C was decreased, as indicated by the higher residual ^{14}C content, by the addition of gypsum during the entire incubation period. Lime addition stimulated mineralisation initially; however,

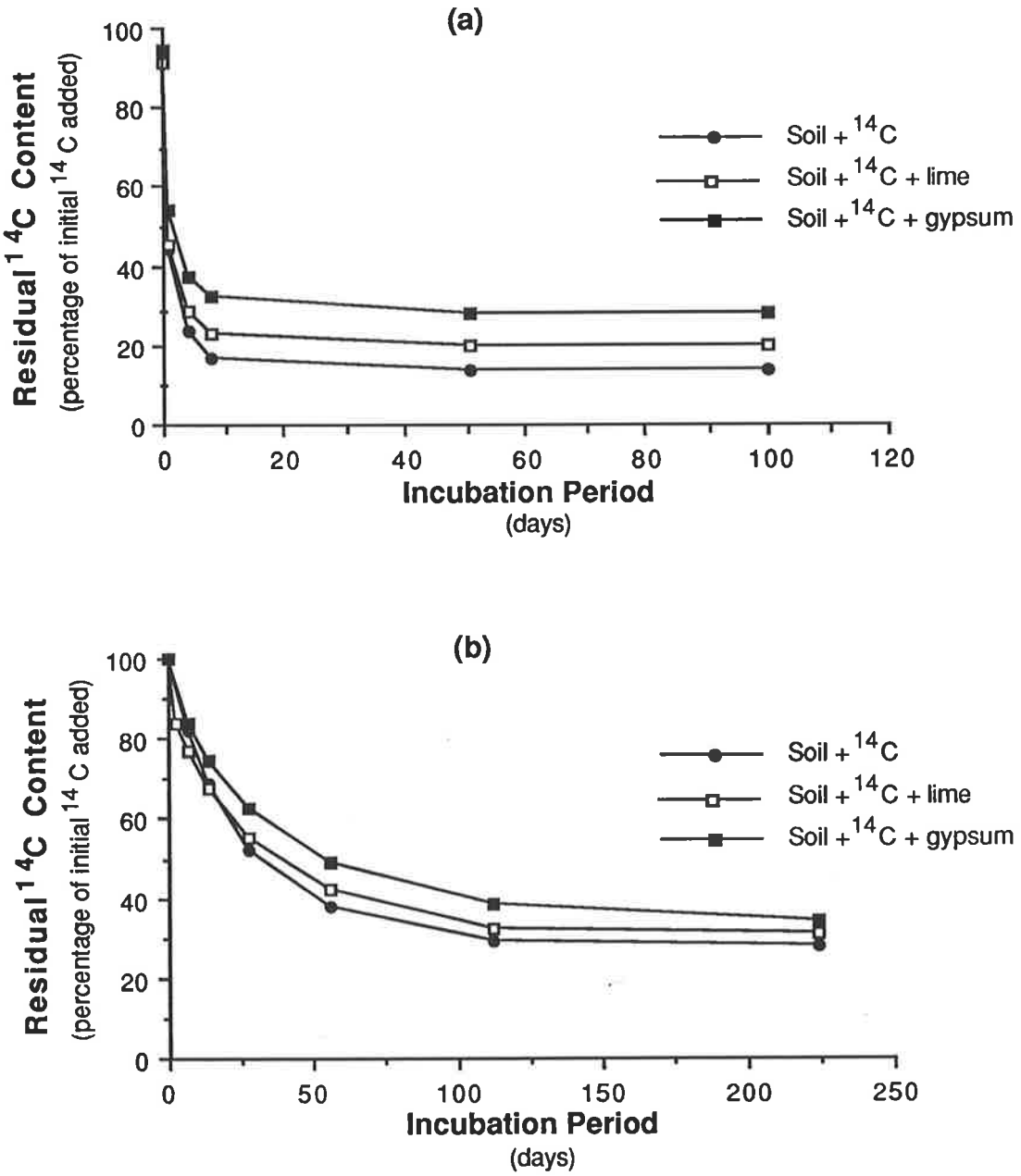

Figure 1.5: Influence of gypsum and lime addition on the proportion of residual glucose ^{14}C in a laboratory experiment (a) and residual wheat straw ^{14}C in a field experiment (b) (Muneer and Oades 1989a,b).

this effect was only transient as the final quantity of ^{14}C mineralised was less than that observed in the soil amended with ^{14}C-glucose alone. In the field experiment similar results were observed using the ^{14}C-labelled wheat straw as an organic substrate. The ability of the calcium amendments to stabilise the residual substrate ^{14}C against microbial attack was postulated to result from an interaction between the residual substrate ^{14}C and Ca^{2+} cations. The enhanced effect of gypsum over that of lime was suspected to result from its greater solubility which maintained the concentration of Ca^{2+} cations in the soil solution at higher levels.

The ^{14}C content of the microbial biomass in the soils amended with ^{14}C-labelled wheat straw and calcium was also measured using a chloroform fumigation technique (Muneer 1987). Addition of gypsum significantly decreased the amount of ^{14}C contained in the microbial biomass through out the incubation period. For the soil treated with lime a reduction in the accumulation of ^{14}C in the microbial biomass was not observed until the end of the incubation period. The decreased content of ^{14}C in the microbial biomass as a result of gypsum and lime addition was ascribed to an inability of the soil microorganisms to utilise the substrate ^{14}C which had complexed Ca^{2+} cations. However, as demonstrated by Jacquin et al. (1979) and Mallouhi and Jacquin (1985), increases in the electrolyte concentration of the soil solution can decrease biomass estimations and biomass activity. Increasing the electrolyte concentration of soils was also shown to decrease the mineralization of organic carbon from soils by Johnston and Guenzi (1963), Singh et al. (1969) and Laura (1974). Thus the decreased amount of ^{14}C incorporated into the microbial biomass in the presence of gypsum may have been due, at least partially, to a decrease in the activity of the microbial biomass induced by an increased electolyte concentration.

The mechanism(s) involved in the ability of gypsum to biologically stabilise organic materials in soil was further investigated by Muneer and

Oades (1989c). Adding glucose to a soil amended with gypsum was shown to significantly decrease the soil's electrical conductivity relative to that of the same soil containing no gypsum. The amounts of water soluble, exchangeable and EDTA-extractable calcium were also lower in the glucose amended soil than the soil to which no glucose was added. The disappearance of water soluble, exchangeable and EDTA-extractable calcium was assumed to indicate that soluble calcium was removed from solution through the formation of Ca^{2+}-organic complexes with the materials synthesised by the microbial biomass utilising the added glucose.

An influence of the presence of Ca^{2+}-organic complexes on the biological stability of soil organic materials was also shown by Gaiffe et al. (1984). By replacing a portion of the exchangeable Ca^{2+} in an organic soil with K^+ a three fold increase in the loss of organic carbon during a 25 week incubation was observed relative to that observed for the soil receiving no K^+. It was therefore concluded that replacement of the exchangeable Ca^{2+} with K^+ resulted in a decrease in the biological stability of the soil's organic carbon.

The role of Ca^{2+} in the biological stabilisation of soil organic materials is also suggested by the general observation that soils containing active calcium carbonate usually have a higher organic carbon content than adjacent noncalcareous soils developed under similar environmental conditions.

1.2.4 Interaction of Calcium with Organic Materials of Known Chemical Structure

The binding of calcium to organic molecules occurs via oxygen atom coordination. The structures of calcium complexes vary from 6- to 12-coordinate with most having a coordination number of 8. The adaptability of the calcium coordination sphere makes it possible for calcium to form complexes with organic materials with a wide range of chemical structures and also makes cross-linking a major feature of calcium's solid-state and solution

chemistry.

The ability of organic molecules to form complexes or linkages with calcium cations was suggested in the early 1900's. Sabatani (1901) postulated the formation of an unionised complex between calcium and citrate ions. Subsequent research confirmed the ability of citrate anions to complex Ca^{2+} (eg. Shear and Kramer 1927, McLean et al. 1934). By 1934 the complex formation between citrate anions and Ca^{2+} was well established; however, the ability of Ca^{2+} to form complexes with other organic anions had received little attention. In the research which followed, Ca^{2+} was observed to form complexes or linkages with numerous simple organic anions (Davies 1938a,b, Greenwald 1938, Cannan and Kibrick 1938, Topp and Davies 1940, Joseph 1946, Williams 1952) and despite variations in the experimental methods used, it was evident that the formation and stability of calcium organic complexes were a function of the nature and content of the organic anion's functional groups and the size of the ring formed in the complex. After examining the ability of 21 organic anions of varying structure to form linkages with calcium cations, Johnston (1956) found that the tribasic citrate anions were by far the most effective, and that the ability of the remaining anions fell into a series in which the order was determined by their chemical structure.The results presented by Johnston (1956) and previous researchers pertaining to the stability of the complexes formed between calcium cations and simple organic anions can be summarised as follows:

1) the most stable complexes were formed with tribasic anions and the stability decreased as the anion was changed to dibasic and then to monobasic (Cannan and Kilbrick 1938, Joseph 1946, Heinz 1951, Johnston 1956),
2) α-hydroxy substitution increased the stability of the complex (Joseph 1946, Schubert 1954),
3) stability was greatest for complexes which formed five and six membered

ring structures and decreased as the size of the ring increased (Williams 1952, Johnston 1956), and

4) the stability of the complexes formed with aromatic anions was generally less than that formed with aliphatic anions (Joseph 1946, Johnston 1956).

In addition to simple anions, Ca^{2+} cations have also been observed to form complexes with organic molecules containing polyhydroxy structures such as those found in carbohydrates. Mills (1961) provided evidence for the formation of complexes between Ca^{2+} and polyhydroxy compounds and suggested that complex formation resulted from the replacement of water molecules in the hydration sphere of the Ca^{2+} cation by several hydroxyl groups. Using ^{45}Ca, Charley and Saltman (1963) noted that Ca^{2+} cations formed a soluble relatively uncharged complex with lactose. Rendleman (1966) indicated that the requirements for the formation of a Ca^{2+}-carbohydrate complex are a group of two or more appropriately oriented hydroxyl groups or a combination of a carbonyl group with one or more properly oriented hydroxyl groups.

Although soils contain many simple organic molecules such as those discussed above, the majority of soil organic carbon is thought to be contained in larger much more complex polymeric structures. In these larger more complex molecules, in addition to the different types of oxygen containing functional groups, factors such as conformational changes induced by the complexation reactions, the density of functional groups on the molecules and molecular flexibility may influence both the ability of the molecules to complex Ca^{2+} cations and the biological stability of the resulting complexes. Changes in molecular conformation, such as those observed due to the binding of Ca^{2+} to proteins (eg. Wasserman et al. 1977), may alter the steric properties of sites where microbial enzymes operate and thus stabilise the organic molecules against microbial attack. By varying the degree of esterification of pectin, a polygalacturonic acid, Kohn and Fruda (1967) observed that increasing the

content of carboxyl groups on the molecule enhanced the formation of Ca^{2+}-pectinate complexes. Kohn and Luknár (1975) noted that for pectin molecules with a low density of carboxyl groups, soluble Ca^{2+}-pectinate complexes formed and that as the density of carboxyl groups increased the Ca^{2+}-pectinate complexes became insoluble and coagulated. The influence of molecular flexibility on Ca^{2+} complexation was investigated by Kohn and Tihlárik (1984) using 2,3-dicarboxy derivatives of starch. Relative to the more rigid structure of pectin, in 2,3-dicarboxy derivatives of starch carboxyl groups are always present as couples which can approach each other very closely. The dicarboxy derivatives of starch had a much higher affinity for Ca^{2+} than the pectin molecules with an equivalent carboxyl group content which indicates the importance of molecular flexibility and the spatial arrangement of carboxyl groups to Ca^{2+} complexation. The importance of molecular flexibility to the formation of Ca^{2+}-organic complexes was also demonstrated by Kohn and Hirsch (1986) using galactaric acid and furandicarboxylic acid. Galactaric acid, a flexible acyclic dicarboxylic acid, bound Ca^{2+} cations to a much greater extent than the rigid cyclic furandicarboxylic acid because of the ability of its two carboxyl groups to assume an optimum steric arrangement for binding.

In an attempt to examine the nature of the binding of Ca^{2+} cations to soil organic materials, Portal et al. (1977) synthesised a polyaromatic polymer from an equimolar mixture of catechol and glycol. The binding of Ca^{2+} cations to an artificial polymer was of a salt type linkage and neutralised the polymer's carboxylic charge. As observed by Juste and Delas (1967) for a Ca^{2+} saturated humic acid, the solubility of the complex formed between Ca^{2+} and the artificial polymer decreased with increasing pH, presumably due to an increased participation of phenolic and alcoholic hydroxyl groups in the complexation reactions. Changes observed in the infrared spectra of a humic acid due to the addition of Ca^{2+} cations were similar to those noted when the

dissociation of carboxyl groups was provoked by addition of a strong base (Juste and Delas 1967), and confirmed the conclusions of Schnitzer and Skinner (1963, 1965, 1967) that carboxylic groups play a dominant role in the formation of complexes between humic materials and polyvalent cations in soils.

1.3 Summary

The information reviewed, when considered collectively, gives a strong indication of the ability of Ca^{2+} cations to stabilise organic materials in soils against microbial attack. The stabilisation appears to involve the formation of Ca^{2+}-organic complexes. The most stable complexes form with anionic carboxylate oxygen. Since soil organic materials contain significant quantities of carboxylate oxygen the potential for the formation of stable Ca^{2+}-organic complexes is high provided there is a sufficient concentration of Ca^{2+} cations in the soil solution. It is postulated that the biological stabilisation of organic substrates in soils results from the formation of Ca^{2+}-organic complexes with the materials synthesised by the soil microbial biomass. The inability of the microbial biomass to utilise the carbon contained in the Ca^{2+}-organic complex resides in one or more of the following possible mechanisms:

1) a removal of water soluble organic materials from the soil solution,
2) a change in the conformation of the organic molecules bound to the Ca^{2+} cations which limits the degradative action of microbial enzymes, or
3) an interaction of the Ca^{2+}-organic complexes with soil clays

In addition to the formation of Ca^{2+}-organic complexes, the biological stabilisation of organic materials induced by the addition of gypsum to soil may be at least partially due to the influence of an elevated electrolyte concentration in the soil solution on microbial activity.

Chapter 2

The Influence of Ca^{2+} Concentration in the Soil Solution on the Activity of the Soil Microorganisms

2.1 Introduction

Materials capable of releasing Ca^{2+} cations into the soil solution (eg. gypsum and agricultural lime) are often added to leached cultivated soils to improve soil structural properties by reducing sodicity and increasing electrolyte concentration. In addition to the favourable effects which increasing the concentration of Ca^{2+} cations has on soil structural properties, decreases in the amount of carbon mineralised from native soil organic materials and organic substrates have also been noted (eg. Sokoloff 1938, Muneer and Oades 1989a,b). The influence of increased Ca^{2+} concentrations on the mineralisation of organic carbon in soil was ascribed to a decrease in the availability of the organic materials to the soil microbial biomass as the result of the formation of Ca^{2+}-organic complexes. However, as demonstrated by Jacquin et al. (1979) and Mallouhi and Jacquin (1985), increased electrolyte concentrations in the soil solution decreased the activity of the soil microbial biomass. The decreased mineralisation of carbon from soil organic materials noted by adding Ca^{2+} cations to soils may therefore have been due, at least partially, to an influence of the elevated Ca^{2+} concentration on the activity of the soil microbial biomass. The objective of this study was to determine if the addition of Ca^{2+} cations to soil at levels comparable to those obtained in the gypsum amended soils used by Muneer and Oades (1989a,b) was capable of reducing the activity of the soil microbial biomass.

2.2 Materials and Methods

The influence of Ca^{2+} cation concentration on the activity of the soil

microbial biomass was assessed by measuring the disappearance of a readily available substrate, glucose, rather than its mineralisation in a laboratory incubation experiment. Glucose was used as the substrate to ensure that the activity of the soil biomass was not limited by the substrate's chemical composition or solubility, and because it can be easily extracted from soils and quantitatively determined. It is acknowledged that the addition of glucose to the soil solution will contribute to any osmotic effect caused by the added Ca^{2+} cations. However, this problem was only present during the initial stages of the decomposition before the glucose levels began to decrease, and could not be avoided to fulfil the research objective. Adequate control samples were incorporated to account for the problem. Calcium chloride was used as the source of Ca^{2+} cations because its high solubility ensured that the desired electrolyte concentrations of the soil solution were obtained.

A composite sample consisting of approximately fifty 0.05 m diameter cores was collected from the surface 0.10 m of an old pasture developed on a red-brown earth, the Urrbrae fine sandy loam (Calcic Rhodoxeralf) (Litchfield, 1951), at the Waite Agricultural Research Institute . The sample was air dried, sieved to $\leq$ 0.002 m in diameter and thoroughly mixed. Some relevant chemical and physical properties of the soil sample collected are given in Table 2.1.

Two simple experiments were performed in which three concentrations of glucose (0, 3.5 and 7.0 mg glucose g^{-1} soil which corresponds to 0, 5 and 10 per cent on a weight of glucose-C to a weight of soil organic-C) and four concentrations of Ca^{2+} in soil solution (deionised water, 0.01, 0.05 and 0.10 M) were applied to soil samples in a 3 x 4 factorial design. A weight of air dried soil equivalent to 30.00 $\pm$ 0.02 g of oven dried soil was added to sterilised containers and wetted to a matric potential of -37.2 kPa by adding 0.276 cm^3 of the treatment solutions per gram of soil to the samples. The required amounts of glucose and Ca^{2+} cations were added to each sample in

Table 2.1: Some relevant chemical and physical properties of the soil collected.

Soil Property	Mean Value ± Standard Error
Organic Carbon (% by weight)	2.82 ± 0.03
pH (1:5 soil:water ratio)	5.81
(1:5 soil:0.01 M $CaCl_2$ ratio)	5.20
Electrical Conductivity (1:5 soil:water extract; dS m^{-1})	0.108 ± 0.010
Texture	
Sand	61.6 ± 0.8
Silt	24.3 ± 0.5
Clay†	14.1 ± 0.4

† The clay fraction was dominated by kaolinite and illite with some fine grained quartz.

the solutions used to wet the samples. All samples were placed in an incubator at 20 ± 2°C for periods of up to 125 hours. The containers were tightly capped with a screw top lid immediately after wetting to maintain the water content of the samples. The development of anaerobic conditions was lessened by manipulating the soil into a well aggregated form after 6 hours incubation and subsequently opening the containers for 15 minutes every 8 hours.

The two experiments performed differed only in the total electrolyte concentration of the Ca^{2+} solutions which were added to the soil samples, and in the number of sampling times used during the incubation. In the first experiment the electrolyte concentration of the Ca^{2+} solutions was not altered and samples were removed from incubation after 0, 3, 6, 12, 28, 36, 44, 52, 60, 72, 96 and 125 hours. In the second experiment the total electrolyte concentration of all the Ca^{2+} solutions was brought to 0.20 M with respect to the Cl^- anion by the addition of an appropriate amount of KCl and samples were removed from incubation after 0, 10, 24, 48, 72, 96 and 120 hours.

After removing samples from the incubator 20 ml of deionised water saturated with chloroform was added to the sample containers. The samples were shaken vigorously for 1 minute and stored at 4°C. The aqueous chloroform solution and low temperature storage were used to stop microbial activity. The residual glucose in the samples was extracted by four repeated washings with 40 cm^3 of the aqueous chloroform solution. The amount of residual glucose contained in all samples was determined using a colorimetric glucose oxidase method (Bergmeyer and Bernt, 1974). Absorbance values were measured using a Titertek Multisckan MCC. All residual glucose contents were calculated from a calibration line obtained from a series of glucose standards.

The efficiency of the extraction procedure used was assessed by determining the percentage of the added glucose recovered in a series of unincubated samples stored at 4°C for up to 170 hours (Figure 2.1). The mean recovery of the added glucose was 100.2 ± 1.02 per cent.

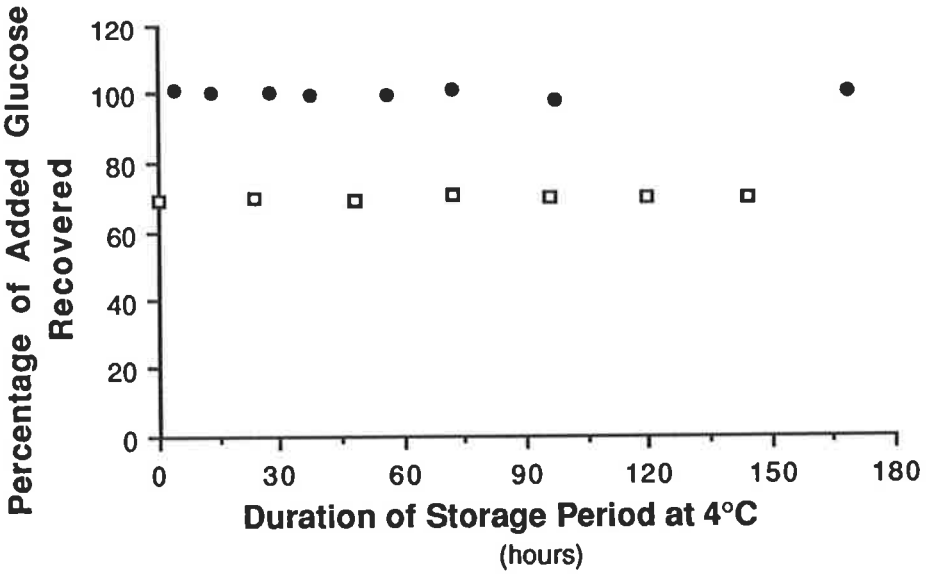

Figure 2.1: Efficiency of the procedure used to extract glucose from soil (●) and the effectiveness of the aqueous chloroform solution and low temperature storage in halting microbial activity (□).

The effectiveness of the chloroform and low temperature storage treatment at stopping microbial activity was determined by incubating soil samples amended with glucose for 24 hours and measuring their residual glucose content after storage for up to 144 hours (Figure 2.1). The absence of a significant reduction in glucose content with the duration of low temperature storage indicates the effectiveness of the procedure. The mean recovery of the added glucose was 69.6 ± 0.48 per cent.

2.3 Results and Discussion

The results of the first experiment, where the electrolyte concentration of the Ca^{2+} solutions was not equivalent, are presented in Figure 2.2. A detailed presentation of the results collected in this study is contained on the computer disk located on the back cover of the thesis. Glucose decomposition consisted of three phases, an initial lag phase, a linear phase where the rate of decomposition was independent of the amount of residual glucose, and a subsequent phase in which the rate of glucose decomposition decreased as the amount of residual glucose decreased. The linearity of the intermediate phase was confirmed by linear regression analysis (Table 2). Using a similar glucose oxidase method to monitor residual glucose contents, a linear decomposition phase was also observed by Coody et al. (1986) in a laboratory incubation study.

The shape of the relationship between the amount of residual glucose and the period of incubation indicated that the maximum rate of glucose decomposition occurred during the linear phase. The influence of the Ca^{2+} treatments on the activity of the soil microbial biomass was therefore assessed by comparing the slope of the linear phase derived for each concentration of Ca^{2+} cations (Table 2.2). As the concentration of Ca^{2+} cations increased the magnitude of the slopes decreased indicating a reduction in the rate of glucose decomposition and microbial activity. However, only the

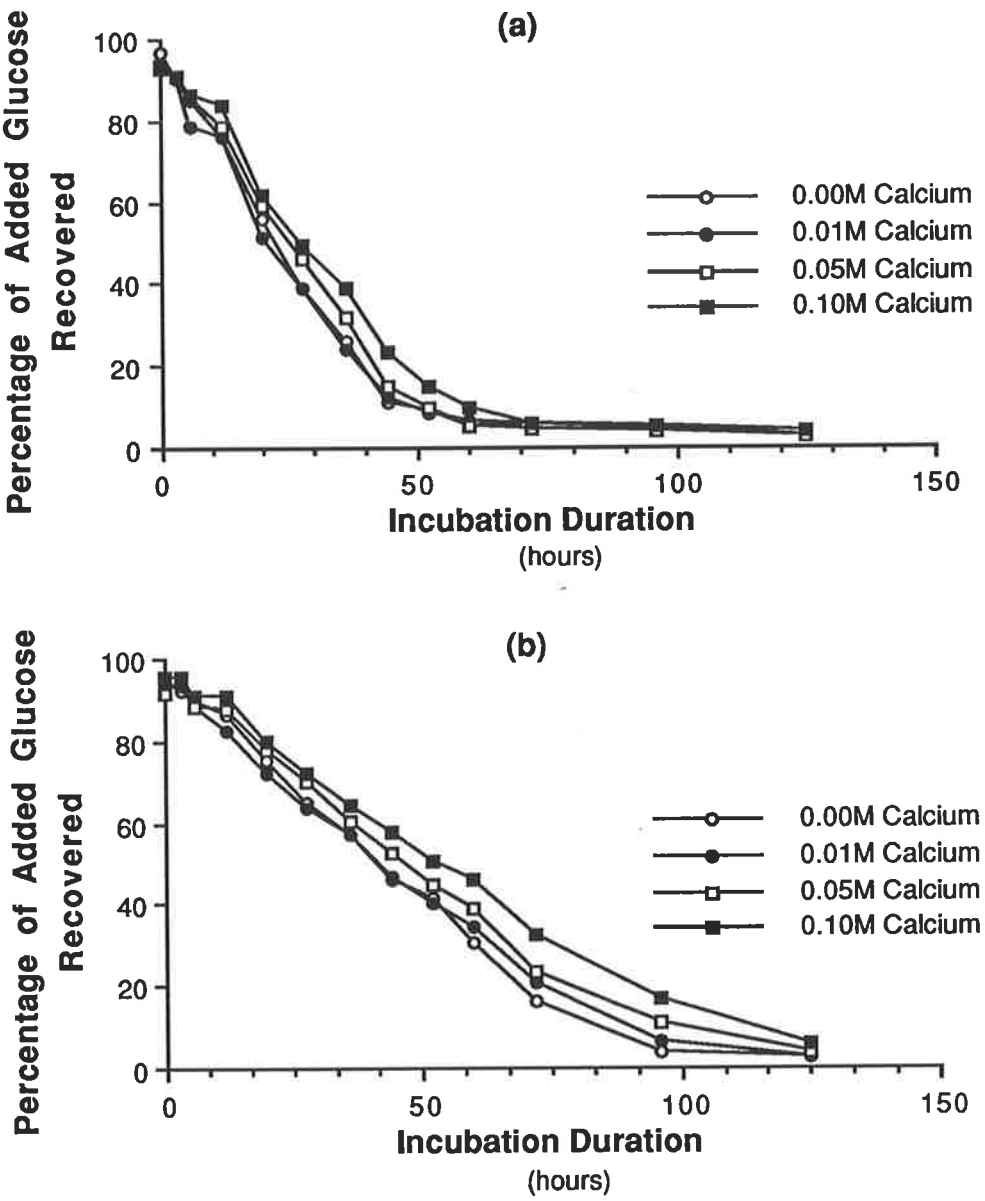

Figure 2.2: Influence of Ca^{2+} concentration in soil solutions of unequal electrolyte concentration on the decomposition of glucose: (a) 5% glucose addition, (b) 10% glucose addition.

slopes of the deionised water and the 0.10 M Ca^{2+} treatments differed significantly. The extent of glucose decomposition at the end of the incubation period was not affected by any of the Ca^{2+} treatments. Therefore, although the rate of glucose decomposition was altered by the concentration of Ca^{2+} cations, the extent of decomposition after 125 hours incubation was not.

Table 2.2: Slopes obtained from linear regression analysis of the linear phase of glucose decomposition.[ab]

Concentration of Calcium ($mol\ dm^{-3}$)	Unequal Electrolyte [c] Concentration		Equal Electrolyte [d] Concentration	
	5% Glucose Addition	10% Glucose Addition	5% Glucose Addition	10% Glucose Addition
0.00	-1.974[a]	-1.016[a]	-1.621[a]	-0.793[a]
0.01	-1.895[ab]	-0.960[ab]	-1.577[a]	-0.829[a]
0.05	-1.811[ab]	-0.925[ab]	-1.534[a]	-0.752[a]
0.10	-1.633[b]	-0.862[b]	-1.515[a]	-0.782[a]

[a] The R^2 values and probabilities associated with all regression analyses were >0.980 and <0.001, respectively.

[b] Slopes with the same superscript letters for each combination of electrolyte concentration and glucose addition are not significantly different at p=0.05.

[c] In calculating the slope values, the data from time zero to 44 hours incubation and from time zero to 96 hours incubation were used for the 5 and 10 per cent glucose additions, respectively.

[d] In calculating the slope values, the data from time zero to 48 hours incubation and from time zero to 96 hours incubation were used for the 5 and 10 per cent glucose additions, respectively.

Changes in the amount of residual glucose during incubation when the total electrolyte concentration of the Ca^{2+} solutions was equivalent (0.20 M with respect to Cl^-) are presented in Figure 2.3. This experiment was performed to determine whether the mechanism responsible for the decreased rate of glucose decomposition observed in the previous experiment was simply due to changes in the electrolyte concentration of the treatment solutions or to some direct influence of the Ca^{2+} cations on the decomposition process. The slopes of the linear phase of glucose decomposition associated with the Ca^{2+} treatments are presented in Table 2.2. These data indicate that, under conditions of equal electrolyte concentration but different Ca^{2+} cation concentration, the amount of Ca^{2+} in the soil solution had no influence on the

activity of the soil microbial biomass. The activity of the microbial biomass was therefore not inhibited by the Ca^{2+} cations, and the influence of the Ca^{2+} cations on glucose decomposition observed in the first experiment was attributed to an osmotic effect.

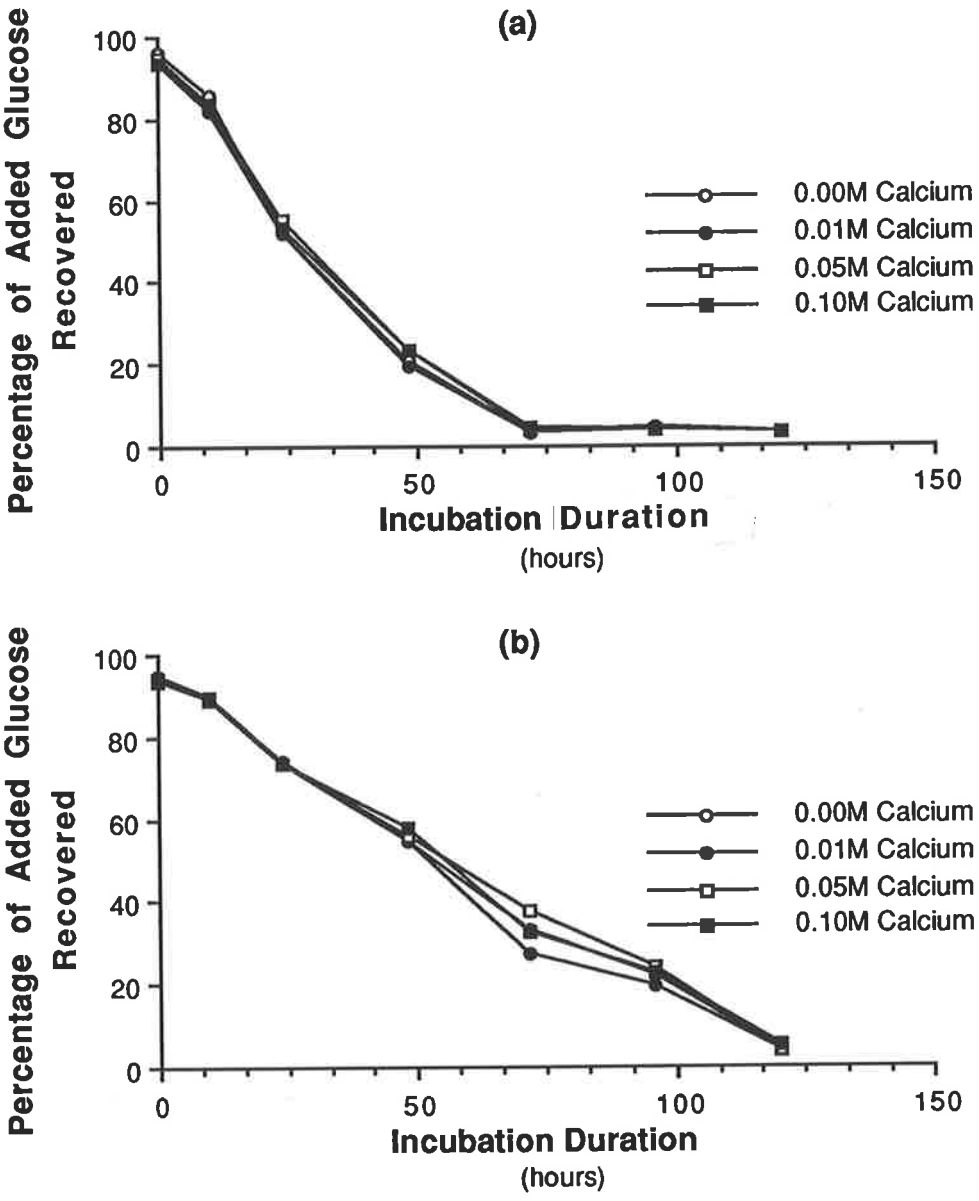

Figure 2.3: Influence of Ca^{2+} concentration in soil solutions of equal electrolyte concentration on the decomposition of glucose: (a) 5% glucose addition, (b) 10% glucose addition.

2.4 Conclusions

Increasing the concentration of Ca^{2+} in soil solution decreased the rate but not the extent of glucose utilisation by the soil microbial biomass. The reduced rate of utilisation was a function of differences in total electrolyte concentration rather than Ca^{2+} concentration. Soil amended with gypsum would have a higher electrolyte concentration than unamended soil; however, the maximum electrolyte concentration expected would be approximately 0.03 M, the solubility of gypsum. The results collected suggest that the increased electrolyte concentration induced by the addition of gypsum to a soil would not significantly influence the activity of the soil microbial biomass. Therefore, the decreased mineralisation of substrate carbon noted by Muneer and Oades (1989a,b) in a gypsum amended soil was not due to an interaction between the substrate and Ca^{2+} cations or to an influence of the increased Ca^{2+} cation concentration on the activity of the soil microbial biomass. The biological stabilisation of substrate carbon in the presence of gypsum is therefore postulated to result from an interaction between Ca^{2+} and the organic compounds synthesised by the biomass utilising the substrate, presumably various carboxylic materials which are capable of forming Ca^{2+}-organic complexes.

Chapter 3

Influence of Gypsum on the Mineralisation and Decomposition of Uniformly Labelled ^{13}C-glucose in the Meadows Soil

Carbon Balance and Solid State ^{13}C NMR Measurements

3.1 Introduction

Muneer and Oades (1989a,b) observed that the addition of gypsum or agricultural lime to a soil amended with an organic substrate increased the biological stability of the substrate carbon to microbial attack as indicated by a decrease in the proportion of the substrate carbon mineralised to carbon dioxide. The results of Muneer and Oades (1989c) and other studies reviewed in Chapter 1 indicated that the decreased mineralisation resulted from an interaction of the substrate carbon with Ca^{2+} cations. Jacquin et al. (1979) and Mallouhi and Jacquin (1985) observed that increasing the electrolyte concentration of the soil solution reduced the activity of the soil microbial biomass, which suggested that a portion of the decreased mineralisation of substrate carbon in soil amended with Ca^{2+} cations may have arisen from an influence of electrolyte concentration on microbial activity. However, the results presented in Chapter 2 established that the reduced mineralisation of substrate carbon in soils amended with Ca^{2+} cations was not due to the increased electrolyte concentration or to a direct inhibitory influence of Ca^{2+} cations on microbial activity. When considered collectively the evidence available suggests that the biological stabilisation of organic carbon in soils containing materials capable of releasing Ca^{2+} cations into the soil solution results from the formation of complexes between Ca^{2+} cations and organic materials synthesised by the soil microbial biomass.

The complexation of Ca^{2+} cations by organic materials occurs through oxygen containing functional groups (Levine and Williams 1982). The strength of the complex increases with the number of carboxyl groups involved

and with the presence of oxygen substituted carbon atoms in a position α to the carboxyl carbon (Johnston 1956). The biological stabilisation of organic carbon in soils amended with Ca^{2+} was therefore postulated to arise from the formation of Ca^{2+}-organic complexes and likely to result in an accumulation of oxygen containing functional groups, primarily carboxylic structures, in the soil's organic fraction. The objectives of this study were:

1) to follow the mineralisation and incorporation of uniformly labelled ^{13}C-glucose into soil organic matter in the presence and absence of gypsum using carbon isotope mass spectrometry, and
2) to monitor changes in the chemical structure of the substrate carbon, associated with its incorporation, in the presence and absence of gypsum using solid state CP/MAS ^{13}C NMR (cross polarisation/magic angle spinning 13C nuclear magnetic resonance) spectroscopy.

In this study carbon isotope mass spectrometry and solid state CP/MAS ^{13}C NMR spectroscopy have been used simply as experimental tools. As a result, indepth reviews of the methodology associated with these forms of analysis have not been included in the thesis. However, explanations regarding methodology and interpretation of results have been added to the text for clarification purposes, where pertinent.

3.2 Materials and Methods

3.2.1 Soil

A composite sample of the surface horizon (0-10 cm) of Meadows fine sandy loam (Rix and Hutton, 1953), an alfisol, was collected from a soil under grass with a mixed forest upper canopy. The composite sample was thoroughly mixed, sieved to ≤2 mm diameter, air dried, and stored at room temperature until required. Some relevant chemical and physical data for the collected sample are presented in Table 3.1.

Table 3.1: Some chemical and physical characteristics of the soil collected for this study.

Soil Texture [a]			Organic [c]	pH [d]	
Sand (>20μm)	Silt (2-20μm)	Clay [b] (<2μm)	Carbon (%)	Water	0.01M $CaCl_2$
64.5	26.2	9.3	2.7	6.4	5.4

Chemical composition of the Inorganic Fraction [e]

SiO_2	Al_2O_3	Fe_2O_3	Na_2O	K_2O	CaO	MgO	SO_3	P_2O_5
89.6	3.9	1.3	0.9	0.8	0.3	0.2	0.1	0.1

[a] Determined by a hydrometer method. Values are expressed in percent by weight of oven dry soil.

[b] The clay fraction contained dominantly coarse grained kaolinite with some quartz grains and a trace of illite.

[c] Determined on a LECO Carbon Induction Furnace (Model #763-000). Values are expressed in percent by weight of the oven soil.

[d] Determined using a 1:5 soil to solution ratio. Measurements were made in the supernatant after soil particles had settled.

[e] Determined by X-ray fluorescence. Values are expressed in percent by weight of the oven dry inorganic fraction.

The Meadows fine sandy loam was selected for this study because of the absence of carbonate in its surface horizon, its slightly acidic pH, and its low iron content. The absence of any carbonate species facilitated carbon balance calculations, and the acidic pH ensured that CO_2 evolution data gave an accurate measure of carbon mineralisation since the precipitation of carbonate species was unlikely. The efficiency with which solid state CP/MAS ^{13}C NMR spectra are acquired and the quantitative characteristics of the analysis are greatly reduced by the presence of paramagnetic nuclei (Preston et al. 1984 and Pfeffer et al. 1984a). Extraction of paramagnetic nuclei with chemical reagents may be required to obtain acceptable spectra (Vassallo et

al. 1987). The major paramagnetic nucleus found in most mineral soils is iron. By selecting a soil with a low iron content the collection of NMR spectra was facilitated without chemical extraction of iron.

In addition, by selecting a different soil to that used by Muneer and Oades (1989a,b) an indication of the influence of soil type on the ability of Ca^{2+} cations to stabilise organic carbon originating from an organic substrate could be obtained.

3.2.2 Treatments

Two concentrations of uniformly ^{13}C-labelled glucose (Icon, New Jersey, Cat# IC3532), 0.00 and 4.56 mg ^{13}C g^{-1} soil, and two concentrations of gypsum (AR grade $CaSO_4 \cdot 2H_2O$), 0.0 and 1.0 per cent by weight of soil, were applied to soil samples in a 2 x 2 factorial design. The rate of ^{13}C-glucose carbon addition was selected so that the amount of substrate ^{13}C remaining in the soil at the end of the incubation would be approximately five times greater than the amount of ^{13}C contained in the native soil organic carbon. It was assumed that during the incubation 70 per cent of the added substrate ^{13}C would be mineralized to carbon dioxide. The rate of gypsum addition was based on that used by Muneer and Oades (1989a,b).

3.2.3 Sample Preparation and Incubation

A bulk soil preparation was made for each treatment by adding 0.158 g of distilled water or 0.169 g of a ^{13}C-glucose solution (67.1 mg ^{13}C-glucose g^{-1} distilled water) g^{-1} of air dried soil or air dried soil amended with gypsum. The quantity of water added to each sample was sufficient to give a soil water matric potential of -33 kPa as calculated from the soil's water retention curve. The concentration of the ^{13}C-glucose solution was calculated so that the required amount of ^{13}C was added to the appropriate preparations during the wetting process. The wetted bulk preparations were thoroughly mixed and left

for 14 hours at 4°C to equilibrate.

Samples for incubation were prepared by adding the equivalent of approximately 10.0 g on an oven dry weight basis of the wetted bulk preparations to 24 mm diameter by 150 mm long test tubes. The test tubes were modified to hold two plastic vials in a manner similar to that used by Fine et al. (1986). After adding the sample to the test tube a vial containing 2.0 cm^3 of water and one containing 5.0 cm^3 of 1.0 M NaOH were placed in the lower and upper positions within the test tubes, respectively. The water was added to minimize evaporative drying of the soil surface and the 1.0 M NaOH was used to trap any carbon dioxide released from the soil during the incubation. The air above the sample was displaced with carbon dioxide free oxygen and each test tube was sealed with a rubber stopper and placed in an incubator at 22±1°C. Samples were incubated for 0, 2, 4, 6, 10, 16, 24, and 34 days. For each combination of treatment and incubation duration three replicate samples were prepared. To reduce the potential of the samples becoming anaerobic, the air space above the samples was displaced with carbon dioxide free oxygen every two days. After each incubation period the carbon dioxide traps in all samples remaining were replaced. Once the soil samples were removed from incubation they were immediately frozen in liquid nitrogen, freeze dried, and homogenised by grinding with a mortar and pestle.

3.2.4 Carbon Balance Measurements

Mineralised Carbon

The amount of carbon mineralised to carbon dioxide by soil microorganisms in each sample was determined by titrating the 1.0 M NaOH in the carbon dioxide traps with 0.05 M HCl. Titrations were performed on a Radiometer Automatic Titration System operating in the derivative mode. The volume of acid required to titrate between the carbonate-bicarbonate and bicarbonate-carbonic acid inflection points was measured. The relationship between the

amount of carbon trapped and the volume of acid required to complete the titration was established using a series of sodium carbonate standard solutions.

Six test tubes containing no soil were also included in the incubation as blanks. The amount of carbon contained in the 1.0 M NaOH traps taken from the blanks was averaged and subtracted from that obtained for each sample to account for any external contamination of the trap solutions. The total amount of carbon mineralised during the incubation was calculated as the sum of the carbon contained in all traps collected for that sample during the incubation period.

Absolute $^{13}C/^{12}C$ Ratio

The absolute $^{13}C/^{12}C$ ratio, AR, of the organic material in each sample was determined by mass spectrometry. A sample of 70-80 mg of freeze dried soil, corresponding to approximately 0.15 mmol of carbon, was placed in a silica glass tube with 150 mg cuprous oxide. The tube was evacuated to $<2x10^{-4}$ torr, sealed and heated to 650°C in a muffle furnace for 16 hours. Analysis of the residue remaining after combustion indicated that the conversion of organic carbon to carbon dioxide was quantitative. The carbon dioxide was then purified using a dry ice/ethanol bath, collected over liquid nitrogen, and sealed under vacuum ($<2x10^{-4}$ torr) in a pyrex glass tube.

A dual collector VG Micromass 602E mass spectrometer was used to determine the isotopic composition of the carbon dioxide samples. Values were obtained for the ratios of (mass 45/mass 44), δ^{45}, and (mass 46/[mass 44 + mass 45]), δ^{46}. Assuming that the variation in the mass of the carbon dioxide in the samples was only dependent on the isotopic composition of the carbon, measuring δ^{45} values would have been sufficient. However, variations in the isotopic composition of the oxygen contained in the carbon dioxide can also influence the value of δ^{45}. Therefore equation [3.1], derived by Craig (1957),

was used to obtain $\delta^{13}C$ values which were corrected for the contents of ^{17}O and ^{18}O isotopes in the sample and expressed in units of per mil deviation from a laboratory reference gas.

$$\delta^{13}C = 1.0676\ \delta^{45} - 0.0338\ \delta^{46} \qquad [3.1]$$

The laboratory reference gas was calibrated against PDB (Belemnitella americana from the Cretaceous Peedee formation of South Carolina) using the international standards NSB-18 and NSB-19 to obtain a $\delta^{13}C$ value for the sample expressed in per mil deviation from PDB. The measured AR value, AR_M, of the samples was calculated using equation [3.2], where 0.0112372 is the AR value of PDB (Galimov 1980).

$$AR_M = \left(\frac{\delta^{13}C}{1000} + 1\right)(0.0112372) \qquad [3.2]$$

The ability of the mass spectrometer to produce $\delta^{13}C$ values which accurately described the AR of a sample was assessed using a series of standards prepared by adding ^{13}C-labelled glucose to natural glucose. The amounts of labelled and natural glucose used to prepare each standard were weighed to the nearest µg. A highly significant ($p<0.001$) and linear ($R^2=0.999$) relationship was obtained between the calculated and measured AR values of the standards; however, the slope and y-intercept of the linear relationship were not equal to 1.00 and 0.00, respectively. The AR_M values were therefore corrected using equation [3.3] to obtain a sample AR value, AR_S. The values 0.9159 and 0.00267 in equation [3.3] represent the slope and y-intercept of the linear relationship between the AR_M and AR_S values of the standards, respectively.

$$AR_S = (AR_M)(0.9159) - 0.00267 \qquad [3.3]$$

Organic Carbon Content

The organic carbon content of each sample was determined using a LECO induction furnace (model# 763-000) equipped with gravimetric carbon determinator (model# 521-275). The carbon dioxide produced during sample combustion was trapped on ascarite (20-30 mesh). A more modern carbon analyser (LECO model CR 12) equipped with an infra-red detector could not be used in this study because the ^{13}C-O stretching frequency, which is used to determine the amount of carbon present, was outside the band width of the window in the detector. In the calculation of organic carbon contents corrections were made for the molecular weight of the carbon in the sample because of the high levels of ^{13}C added to some samples. The molecular weight of the carbon, MW_C, in each sample was calculated from the AR_S values according to equation [3.4].

$$MW_C = 12\left(\frac{1}{1 + AR_S}\right) + 13\left(\frac{1}{1 + AR_S}\right)AR_S \qquad [3.4]$$

The total content of organic carbon, C_T, expressed in millimoles C g^{-1} soil, in the samples was obtained using equation [3.5] in which %C represents the weight percentage of organic carbon contained in the sample on an oven dry weight of soil basis.

$$C_T = \left(\frac{\%C}{MW_C}\right)10 \qquad [3.5]$$

3.2.5 Solid State CP/MAS ^{13}C NMR Spectroscopy

Signal Acquisition

The solid state ^{13}C nuclear magnetic resonance spectra were obtained with magic angle spinning and cross polarisation from ^{1}H on a Bruker CXP 100 instrument operating at 22.6 MHz for ^{13}C. The actual pulse programs

used in this study have been described by Wilson (1987). A 3.8-6.0 μs 90° pulse width was used. Samples were packed into a boron nitride or sapphire rotor with a Kel-F cap and spun at the magic angle using a spinning speed of 3-3.5 kHz. Chemical shift values were measured with respect to external hexamethylbenzene and corrected so that they are quoted with respect to tetramethylsilane. For the series of incubated samples, conventional and dipolar dephased experiments were performed using a contact time of 1 ms and a recycle delay of 0.5 s. The same sample, 260±2 mg of freeze dried soil, was used for both the conventional and dipolar dephased experiments. In the dipolar dephasing experiments a 50 μs delay, during which the proton decoupler was turned off, was inserted into the pulse sequence prior to signal acquisition . In the absence of proton decoupling, the proton-carbon dipolar interaction acts as a powerful relaxation mechanism. Under these conditions CH_2 and CH carbon in rigid structures relax faster than nonprotonated and CH_3 carbon and CH_2 and CH carbon in structures experiencing molecular motion. The dipolar dephasing experiments were used to acquire spectra indicative of the nonprotonated, CH_3 and mobile CH_2 and CH carbon in the samples. A more detailed explanation of the dipolar dephasing technique follows in the discussion. For each analysis 10^5 scans were collected. Free induction decays (FID) were acquired in 1K of data points with proton spin temperature inversion and quadrative detection. The FID was zero filled to 4 K before application of an exponential multiplication factor which resulted in a line broadening of 100 Hz, unless otherwise specified. Fourier transformation was completed using the modified FID. A spectral width of 15 kHz was used.

The amount of sample placed in the rotor and the spectral acquisition characteristics were kept constant for all samples to collect spectra which could be compared semi-quantitatively. To perform accurate quantitative comparisons between spectra, proper spin counting experiments using an internal standard must be completed. However, since changes in the

chemical structure of the substrate carbon resulted in the production of a wide range of resonances in this study, proper spin counting experiments could not be performed using an internal standard because resonances would overlap.

Time limitations prevented the analysis of all incubated samples on the NMR spectrometer. Other than the unamended-unincubated soil, samples which did not receive any ^{13}C-labelled glucose were not analysed. For the ^{13}C enriched samples a composite sample was prepared for each combination of gypsum and incubation duration by taking 0.700±0.002 g of soil from each of the triplicate samples. The NMR signal obtained for the unamended-unincubated sample was subtracted from those obtained for all composite samples to produce spectra indicative of only the substrate ^{13}C. Subtraction of the signals was performed using the unmodified 1 K free induction decays.

Spectral Analysis

The solid state CP/MAS ^{13}C NMR spectra collected in this study are more complex than normally encountered for soils because of the presence of adjacent ^{13}C nuclei. The spectra obtained for natural glucose and the uniformly ^{13}C-labelled glucose used in this study are shown in Figure 3.1. Relative to the spectrum of natural glucose, all peaks in the spectrum of the ^{13}C-labelled glucose were broader. The signal broadening resulted from J-coupling and homonuclear dipolar couplings between adjacent ^{13}C nuclei. The rotational side bands at -64 and 190 ppm also resulted from interactions between adjacent ^{13}C nuclei and were not due to an incomplete removal of chemical shift anisotropy due to insufficient sample spinning speeds. This was demonstrated by the absence of rotational side bands in the natural glucose spectrum which was acquired using a similar spinning speed to that used to acquire the labelled ^{13}C-glucose spectrum. The origin and significance of these spectral features have been discussed by Raleigh et al. (1987).

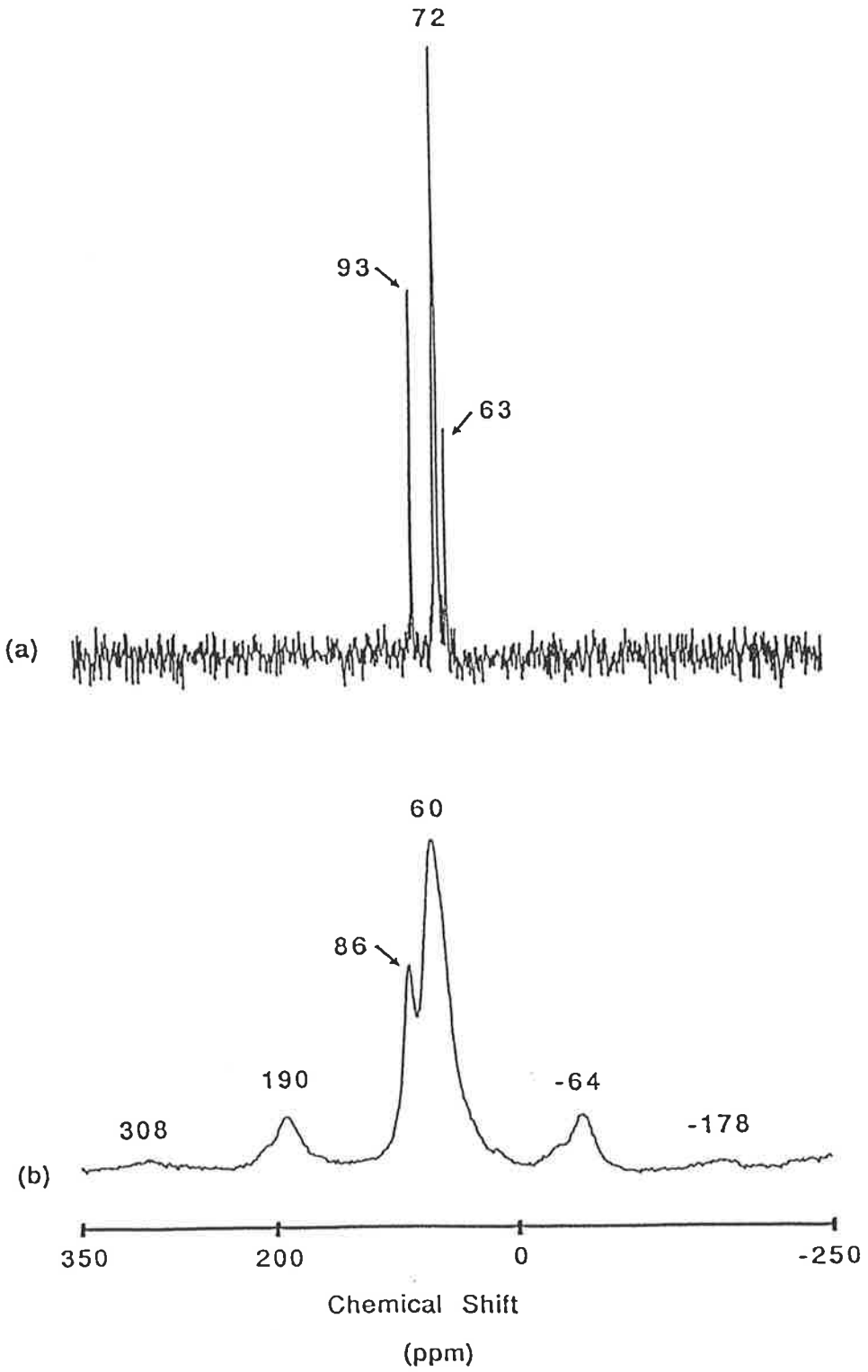

Figure 3.1: Solid state CP/MAS ^{13}C NMR spectra of (a) natural glucose and (b) uniformly labelled ^{13}C-glucose. Both spectra were collected using a contact time of 1 ms and a recycle delay time of 80 s. The number of scans collected for the natural and labelled glucose were 152 and 659, respectively. Line broadening was 2 Hz.

The solid state CP/MAS ^{13}C NMR spectra of all samples indicated that the changes in spectral features were limited to four broad regions. The chemical shift limits of the four regions are listed in Table 3.2. The general label placed on each spectral region is only indicative of the dominant form of carbon present. The assignments are approximate as there was undoubtedly some overlap of adjacent regions. The signal intensities of alkyl, O-alkyl, and carboxyl carbon were calculated as indicated in Figure 3.2.

The rotational side bands detected in the spectra represent real signals and should be included in the spectral analysis. Chemical shift values of rotational side bands are controlled by the rate at which the samples are spun and are displaced from their origin by an amount equivalent to the sample spinning speed. Since the sample spinning speed could not be kept absolutely constant in this study, the position of the rotational side bands in the collected spectra varied from sample to sample. The contribution of the rotational side bands to the alkyl and O-alkyl carbon was calculated by multiplying the high field (-64 ppm) rotational side band by a factor of two, which assumes that the low and high field side bands were of equal intensity. This assumption could not be checked for the incubated samples due to an overlapping of various signals. However, it did appear to be valid for the spectra collected for the ^{13}C-glucose and the ^{13}C-glucose amended soil after 0 days incubation.

The contribution of carboxyl carbon to the total signal was obtained from the dipolar dephased spectra because the alkyl and O-alkyl rotational side bands overlapped the region in the conventional spectra associated with carboxyl carbon. The signal intensity as collected in the dipolar dephased spectra can not be used to estimate the amount of carboxyl carbon because of the signal decay which occurs during the dipolar dephasing period. The exponential rate constant for signal decay ,T_2', of the carboxyl carbon signal in dipolar dephasing experiments is typically 125 μs (Wilson 1987) and the

Table 3.2: Chemical shift limits of the four regions into which the solid state CP/MAS ^{13}C NMR spectra were divided and the origin of the signals

Chemical Shift Limits (ppm)	Designation of the Spectral Region	Origin of Signals	
		Conventional Spectra	Dipolar Dephased Spectra
-80 - -20	High field rotational side bands	Alkyl and O-alkyl rotational side bands	No signal observed
0 - 45	Alkyl	CH_3, CH_2, CH and nonprotonated alkyl carbon	CH_3, nonprotonated carbon and CH_2 and CH carbon exhibiting molecular motion
45 -120	O-alkyl	Oxygenated alkyl, alkyl-amino, acetal, ketal and aromatic structures	Carbon of the type described in the conventional spectra which is either
150 -220	Carboxyl	Carboxyl, carbonyl, ester and amide carbon and the low field rotational sidebands from alkyl and O-alkyl stuctures	nonprotonated or exhibits molecular motion Carboxyl, carbonyl, ester and amide carbon

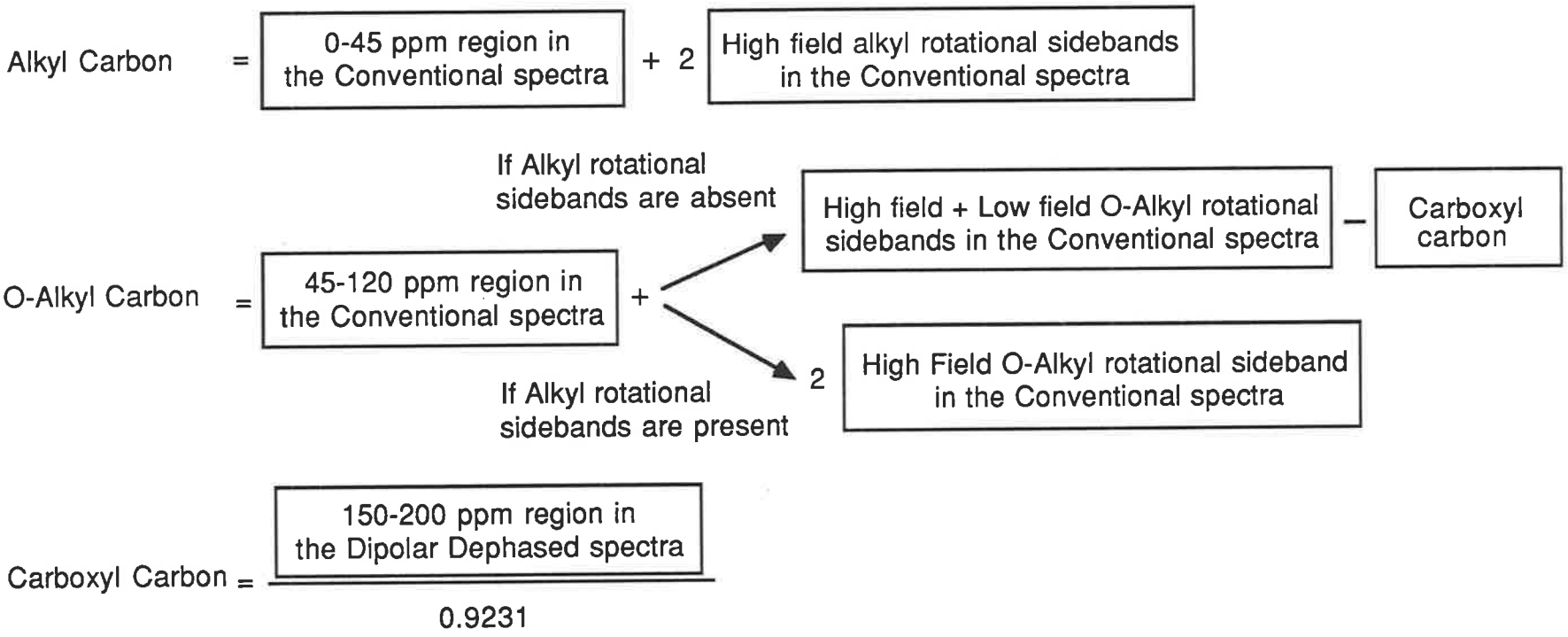

Figure 3.2: Schematic presentation of the signals in the conventional and dipolar dephased spectra used to determine the signal intensity associated with each type of carbon

decay follows equation [3.6] where I^o is the signal intensity acquired with no delay period and I^t is the signal intensity acquired after a delay period of time t_1.

$$I^t = I^o \exp -(t_1/T_2') \qquad [3.6]$$

The total contribution of carboxyl carbon was therefore calculated by dividing its signal intensity in the dipolar dephased spectra by a correction factor calculated from equation [3.6] (0.92 for a 50μs delay) to account for the signal lost by relaxation.

3.2.6 Experimental Design

All carbon balance measurements were made after each of the eight incubation periods on the triplicate samples prepared from the bulk sample of each combination of ^{13}C-glucose and gypsum treatment. The data were therefore analysed using a 2 x 2 x 8 factorial design (^{13}C-glucose x gypsum x incubation period) with three replicate soil samples per treatment combination. The residual substrate ^{13}C content data was analysed using a 2 x 8 factorial design with three replicate soil samples per treatment combination since no ^{13}C-glucose was added to the (soil) and (soil+gypsum) treatments. In both analyses all factors were considered fixed.

No comments on the statistical significance of the NMR results can be made because of the lack of replication imposed by practical experimental time constraints.

3.3 Results and Discussion

A detailed presentation of the collected results and statistical analyses is given on the computer disk located on the inside back cover of the thesis.

3.3.1 Carbon Balance Measurements

The total amount of initial carbon recovered, and the total amounts of

residual and mineralised carbon in the samples after each incubation period are shown in Figure 3.3. All values are expressed as a percentage of the total amount of carbon present in the samples initially. No significant influence of any of the treatments or the duration of the incubation period on the total

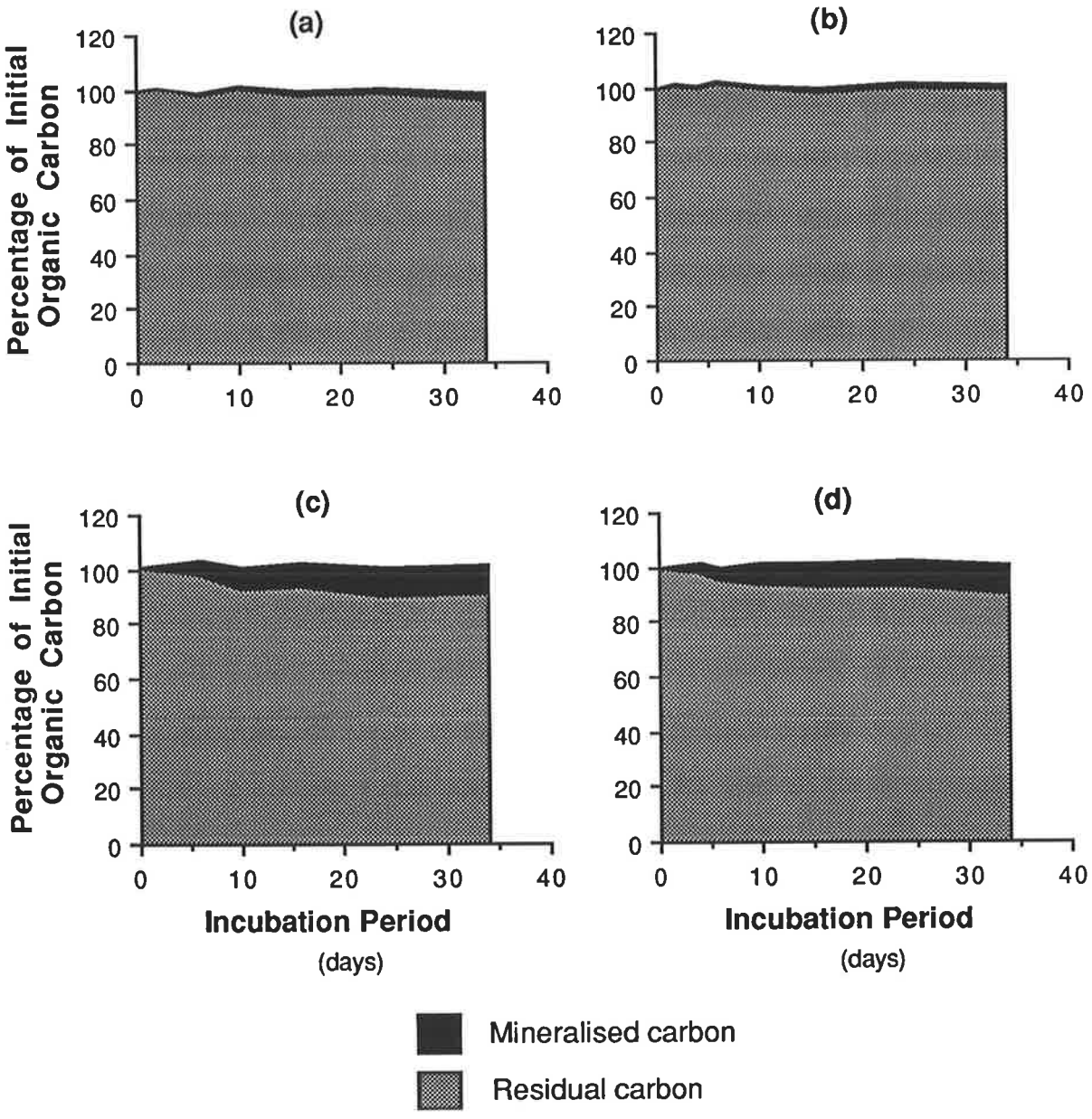

Figure 3.3: Recovery of the total organic carbon initially present in the samples after each incubation period for the (soil) (a), (soil+gypsum) (b), (soil+^{13}C-glucose) (c) and (soil+gypsum+^{13}C-glucose) (d) treatments.

amount of carbon recovered was noted, and the recovery throughout the incubation period was essentially quantitative, varying between 98.8 and 103.0 percent.

Theinfluence of gypsum and^{13}C-glucose on the total amount of carbon mineralised from the samples is shown in Figure 3.4. The total amount of carbon mineralised from the soil was enhanced by the addition of ^{13}C-glucose, which undoubtably resulted from the decomposition and utilisation of the added ^{13}C-glucose carbon by the soil microbial biomass. Gypsum addition decreased the total amount of carbon mineralised in both the presence and absence of the ^{13}C-glucose. The extent of the decreased mineralisation was small but was statistically significant. Approximately 4% less total carbon was mineralised in the presence than absence of gypsum.

The influence of gypsum and ^{13}C-glucose addition on the mineralisation of native and substrate carbon was assessed by determining the residual native and residual substrate carbon contents of the samples as the incubation progressed. The contents of native carbon, C_N, and substrate

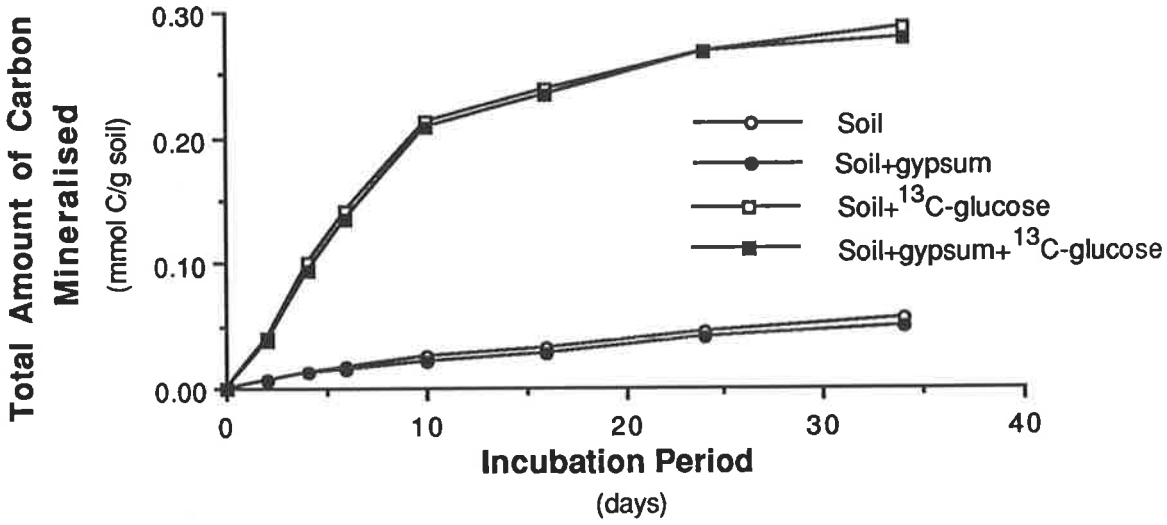

Figure 3.4: The total amount of carbon mineralised in the (soil), (soil+gypsum), (soil+^{13}C-glucose) and (soil+gypsum+^{13}C-glucose) treatments after each incubation period.

carbon, C_S, in units of millimoles of carbon g^{-1} soil, present in each sample were calculated using equations [3.7] and [3.8], respectively. Equation [3.7] is derived from the fact that the amount of native carbon expressed in millimoles will be equal to the amount of ^{12}C in the samples plus an amount of ^{13}C determined by the natural abundance of ^{13}C in the native soil. The amounts of ^{12}C and ^{13}C can be calculated using the values derived for C_T and AR_S, as defined earlier, the weight of soil in the sample, M_S, and the absolute ratio of $^{13}C/^{12}C$ in the native soil, AR_N, which was equal to 0.01094.

$$C_N = \left(\begin{array}{c}\text{Content of}\\ \text{Native } ^{12}C\end{array}\right) + \left(\begin{array}{c}\text{Content of}\\ \text{Native } ^{13}C\end{array}\right)$$

$$= \left(\frac{C_T}{(1 + AR_S)\, M_S}\right) + AR_N \left(\frac{C_T}{(1 + AR_S)\, M_S}\right) \qquad [3.7]$$

$$C_S = C_T - C_N \qquad [3.8]$$

The content of residual native carbon decreased significantly as the incubation progressed, indicating that a portion of the native organic carbon was mineralised by the soil biomass during the incubation period (Figure 3.5). The lack of any significant influence of the ^{13}C-glucose addition on the content of residual native carbon indicated that ^{13}C-glucose addition did not induce an accelerated decomposition of the native organic carbon (ie. no "priming effect" was detected). Contrary to the total carbon mineralisation data, in the presence of gypsum a small but significant increase in the amount of native carbon mineralised was noted, as indicated by a reduction in the amount of residual native carbon.

The content of residual substrate carbon decreased significantly during the incubation (Figure 3.6) as it was utilised by the soil microbial biomass, but was not influenced by the presence of gypsum. The lack of an influence of gypsum on the amount of substrate carbon mineralised, as indicated by the

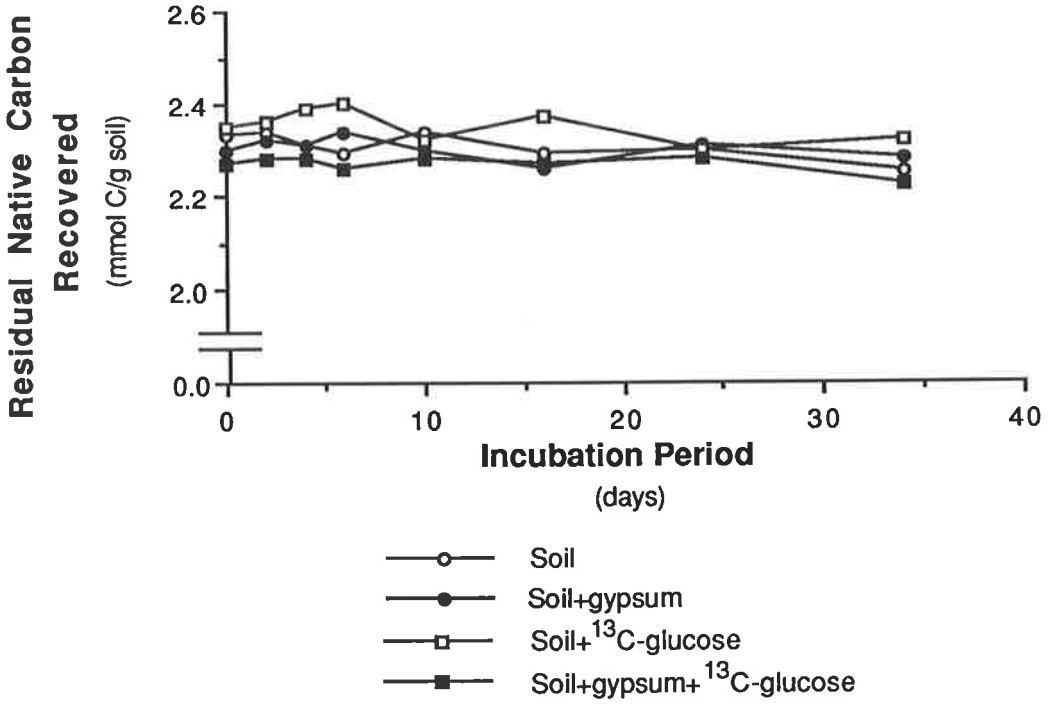

Figure 3.5: Recovery of the native organic carbon from the (soil), (soil+gypsum), (soil+^{13}C-glucose) and (soil+gypsum+^{13}C-glucose) treatments after each incubation period.

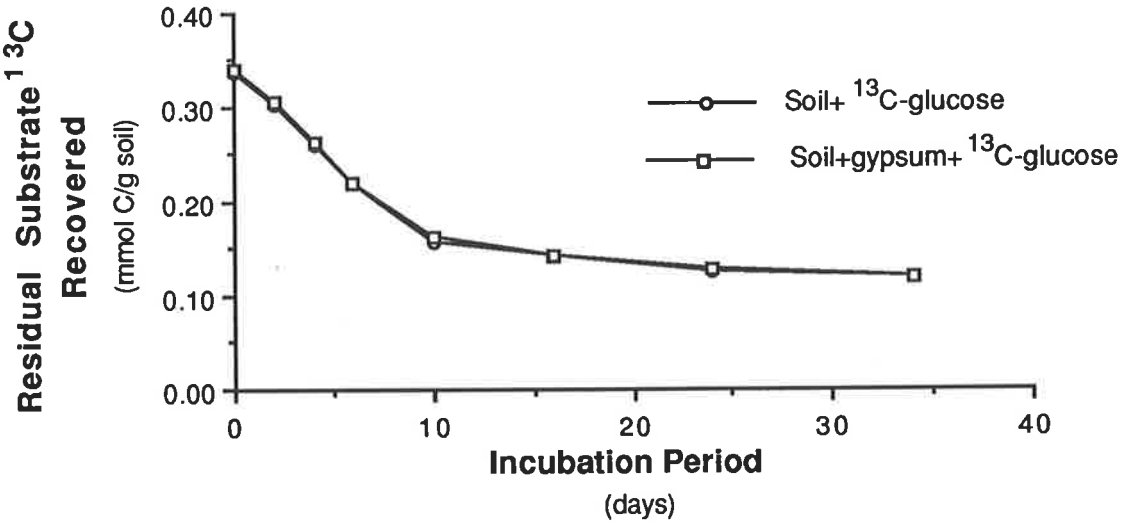

Figure 3.6: Recovery of the residual substrate ^{13}C from the (soil+^{13}C-glucose) and (soil+gypsum+^{13}C-glucose) treatments after each incubation period.

absence of an effect on the residual substrate carbon, was again contrary to the effect which gypsum addition had on the total carbon mineralisation. The inconsistency of the results, when considered in conjunction with the small magnitude of the reduction in total carbon mineralisation, suggested that gypsum addition had little if any influence on the biological stability of the organic carbon in the Meadows fine sandy loam.

After 34 days of incubation approximately 65 percent of the substrate carbon had been mineralised (Figure 3.6). A similar result was obtained by Muryama (1988) who noted that after 56 days incubation the amount of ^{13}C-labelled glucose carbon mineralised in a grey lowland soil and an ando soil was 61.5 and 67.0 per cent, respectively. The amount of glucose carbon mineralised during incubation experiments varies with soil and incubation conditions (Adu and Oades 1978, Coody et al. 1986), but generally appears to level off at values approximately equal to two thirds of the glucose carbon added.

The reduced rate of substrate ^{13}C mineralisation during the first 2 days, relative to that observed between 2 and 10 days, indicated that there was an initial lag period before the activity of the microbial biomass was maximised. Subsequent to day 2, the loss of substrate ^{13}C consisted of a rapid and slower phase. The rapid phase extended from 2-10 days and corresponded to the period when the microbial biomass was utilising the substrate and the more readily available microbial metabolites synthesised from the substrate. In the slower phase, subsequent to day 10, the microbial biomass utilised the more stable products of substrate decomposition.

An exponential function of the form $y = ae^{bx}$ was fitted to the two phases of decomposition. The resulting equation, R^2 value, and substrate ^{13}C half-life, $t_{1/2}$, of each phase are presented in Table 3.3. A seven fold increase in the half-life of the substrate ^{13}C was noted in progressing from the rapid to slower phase, indicating that a substantial change occurred in the ability of the

Table 3.3: Results obtained by fitting of an exponential decay function to the 2-10 day and 10-34 day region of Figure 3.6.

Region	Equation§	R^2	$t_{1/2}$ (days)
2-10 days	Residual $^{13}C = 106.5\ e^{-0.0814\ t}$	0.999	8.5
10-34 days	Residual $^{13}C = 51.5\ e^{-0.0114\ t}$	0.929	60.8

§ The general form of the equation used was

Residual $^{13}C = ae^{bt}$

where the Residual ^{13}C was expressed as a percentage of the initial residual ^{13}C recovered and t was the incubation period in days.

microbial biomass to decompose the substrate ^{13}C during incubation. The relationship between this observation and changes in the chemical structure of the substrate ^{13}C as assessed by solid state CP/MAS ^{13}C NMR analyses will be addressed later.

3.3.2 Solid State CP/MAS ^{13}C NMR Spectroscopy

CP/MAS ^{13}C NMR Relaxation Parameters

The opportunity existed in this study to assess the ability of the CP/MAS ^{13}C NMR analysis to view quantitatively all of the ^{13}C present in the incubated samples. Under quantitative conditions the total CP/MAS ^{13}C NMR signal intensity should be linearly related to the ^{13}C content of the samples. The relationship between the measured signal intensity and ^{13}C content of the samples incubated with the ^{13}C-glucose is presented in Figure 3.7. The absence of a linear relationship between the measured signal intensity and the ^{13}C content of the samples showed that the proportion of the total ^{13}C in the samples detected by the NMR spectrometer under constant experimental

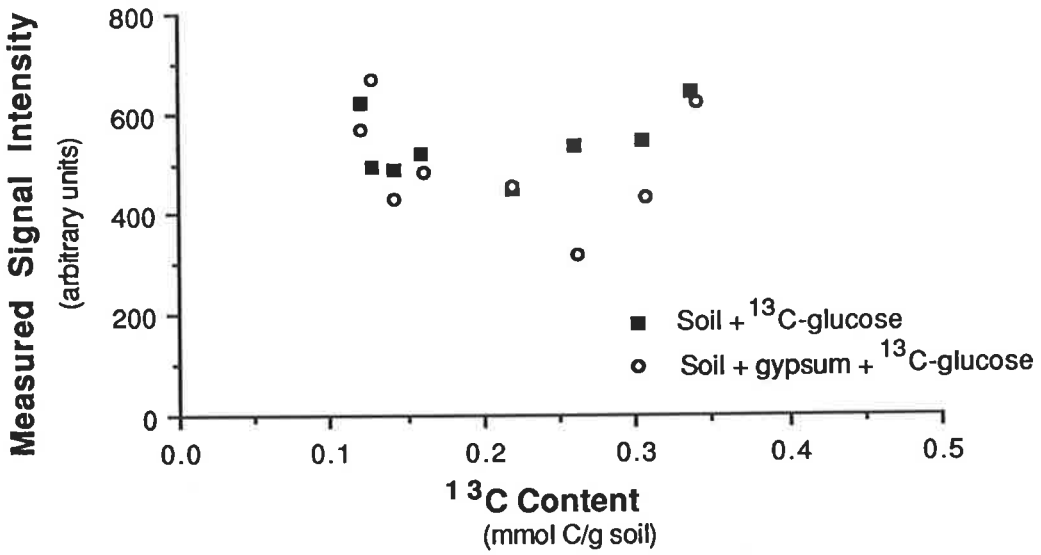

Figure 3.7: Changes in the measured total signal intensity of the solid state CP/MAS ^{13}C NMR spectra obtained from the (soil+^{13}C-glucose) and (soil+gypsum+^{13}C-glucose) treated samples expressed as a function of their ^{13}C contents. In moving from right to left the incubation time increased.

conditions changed as the incubation progressed. The shape of the relationship indicated that as the chemical structure of the substrate ^{13}C was converted from that of glucose to that associated with the microbial products synthesised by the soil microbial biomass, the proportion of the ^{13}C in the samples detected increased significantly. Although the CP/MAS ^{13}C NMR experiments were not designed to be quantitatively comparable, as the ^{13}C content of the samples decreased corresponding reductions in the signal intensity were expected. The fact that ^{13}C content and signal intensity were not linearly related could be due to a number of factors as outlined below.

In the CP/MAS ^{13}C NMR experiments used in this study, because cross polarisation was from ^{1}H, the carbon nuclei relax at the proton relaxation rate. The rate of proton relaxation is known as T_1H. To obtain quantitative signals it is essential that the delay period between pulses, the recycle delay, was

sufficiently long to allow all protons to fully relax. Hydrous and anhydrous glucose in a crystalline form have T_1H values of 40 and 77 seconds, respectively (Pfeffer 1984, Pfeffer et al. 1984b), so that very long recycle times are required to collect a quantitative signal. Since a 0.5 s recycle delay was used in this study, it is possible that the ^{13}C in the glucose did not fully relax and was not detected quantitatively. The T_1H value of the glucose may however have been substantially reduced by interaction with soil materials. Sullivan and Maciel (1982) observed that the addition of the paramagnetic metal cation Cr^{3+}, in the form of $Cr(acac)_3$, to polystyrene reduced the T_1H of the polystyrene from 1.7 s to 0.004 s. To determine whether proton relaxation was complete, the signal intensities obtained by varying the recycle delay from 0.5 to 50 s for a sterilised soil which had been amended with a ^{13}C-glucose solution and immediately freeze-dried were measured. Results indicated that the efficiency of spectral acquisition was not influenced by altering the recycle delay time (Figure 3.8). Therefore, the T_1H values of the native organic carbon

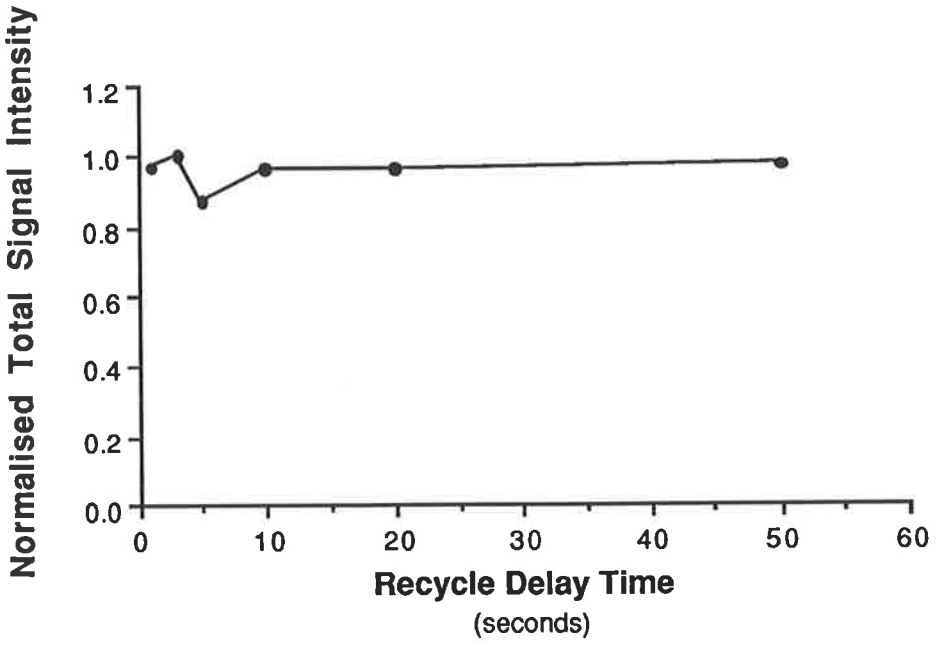

Figure 3.8: Changes in the total solid state CP/MAS ^{13}C NMR signal intensity obtained by increasing the recycle delay period from 0.5 s to 50 s.

and the glucose carbon ^{13}C were sufficiently small that a recycle delay of 0.5 s was adequate and the T_1H value of the glucose carbon was reduced from 77 s to ≤0.5 s once added to the soil.

An alternative explanation could be due to selective relaxation. In CP/MAS ^{13}C NMR pulse sequences some signal is lost during the contact time due to spin lattice relaxation in the rotating frame. The rate of spin lattice relaxation, denoted by $T_1\rho H$, may differ for different chemical structures if the structures are in physically discrete phases (Wilson 1987). Using the samples collected for the (soil+^{13}C-glucose) treatment, $T_1\rho H$ values for the total signal and for each structural type of carbon in the samples incubated for 2, 10 and 34 days were estimated by varying the contact time between 2.0 and 6.0 ms. The pulse sequence and the method used to calculate the $T_1\rho H$ values are described by Wilson (1987). The $T_1\rho H$ values calculated for the total signal and for the signal associated with each carbon type differed and increased as the incubation progressed (Figure 3.9). The $T_1\rho H$ values associated with the

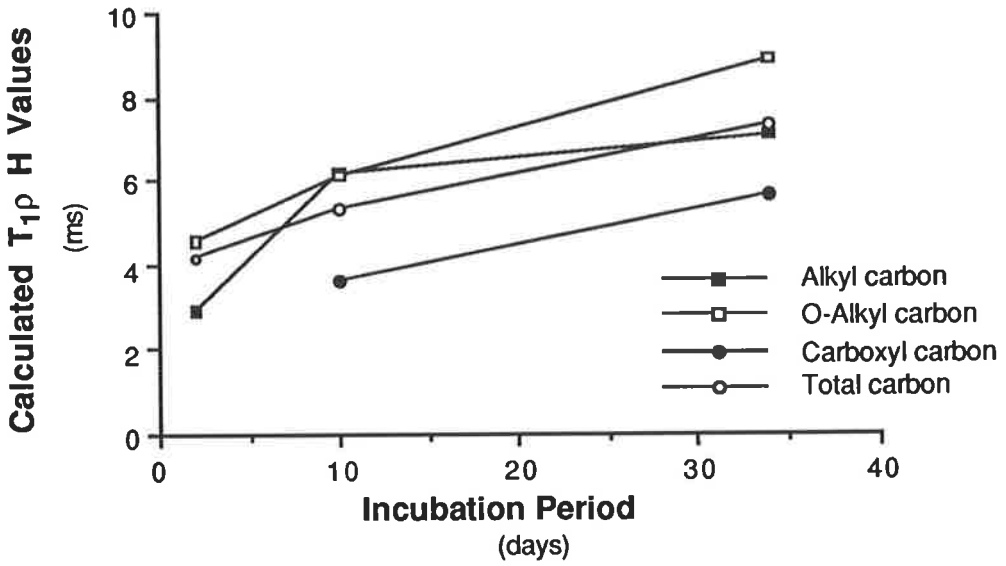

Figure 3.9: Changes in the $T_1\rho H$ values of the total signal and that associated with each type of carbon in the (soil+^{13}C-glucose) treated samples incubated for 2, 10 and 34 days.

samples from the (soil+^{13}C-glucose) treatment which were not analysed were estimated by extrapolation from the data presented in Figure 3.9. The resultant $T_1\rho H$ values calculated for the total signal intensity and for that associated with each type of carbon are presented in Table 3.4. These results indicate that as the structural chemistry of the substrate ^{13}C was altered by microbial decomposition the $T_1\rho H$ values associated with the total carbon and each carbon type increased.

The differing $T_1\rho H$ values indicate that during the 1 ms contact time the amount of signal lost due to spin lattice relaxation differed with carbon type and incubation duration. Therefore, to interpret the CP/MAS ^{13}C NMR results quantitatively the theoretical signal intensity which would have existed if no relaxation occurred during the contact time , I_o, must be calculated using equation [3.8] (Wilson 1987), where I_t is the signal intensity measured after a contact time t.

$$I_o = \left(\frac{I_t}{\exp -(t/T_1\rho H)} \right) \qquad [3.8]$$

Table 3.4: $T_1\rho H$ values determined for the total carbon signal and the alkyl, O-alkyl and carboxyl carbon signals of the (soil+^{13}C-glucose) treated samples after each incubation period.

Incubation Duration	Type of Carbon			
(days)	Alkyl Carbon	O-Alkyl Carbon	Carboxyl Carbon	Total Carbon
0	N.S.§	4.17	N.S.§	3.87
2	2.93	4.56	N.S.§	4.17
4	3.75	4.95	N.S.§	4.47
6	4.57	5.34	3.33	4.77
10	6.20	6.11	3.66	5.36
16	6.42	6.81	4.16	5.85
24	6.71	7.73	4.82	6.51
34	7.07	8.89	5.65	7.33

§ N.S. = no signal observed.

The calculated theoretical signal intensities, I_o values, for the total carbon signal expressed as a function of ^{13}C content appear in Figure 3.10. To calculate I_o values for the samples from the (soil+gypsum+^{13}C-glucose) treatment, the $T_1\rho H$ values obtained for the corresponding samples from the (soil+^{13}C-glucose) treatment were used. The results presented in Figure 3.10 indicated that accounting for the variations in $T_1\rho H$ values did not significantly improve the relationship between the signal intensity and ^{13}C content of the samples. Nevertheless it was clear that the changes in the $T_1\rho H$ values measured for each carbon type justified the applied correction procedure. All subsequent CP/MAS ^{13}C NMR data referring to the samples incubated with the labelled ^{13}C-glucose have been corrected for $T_1\rho H$ effects.

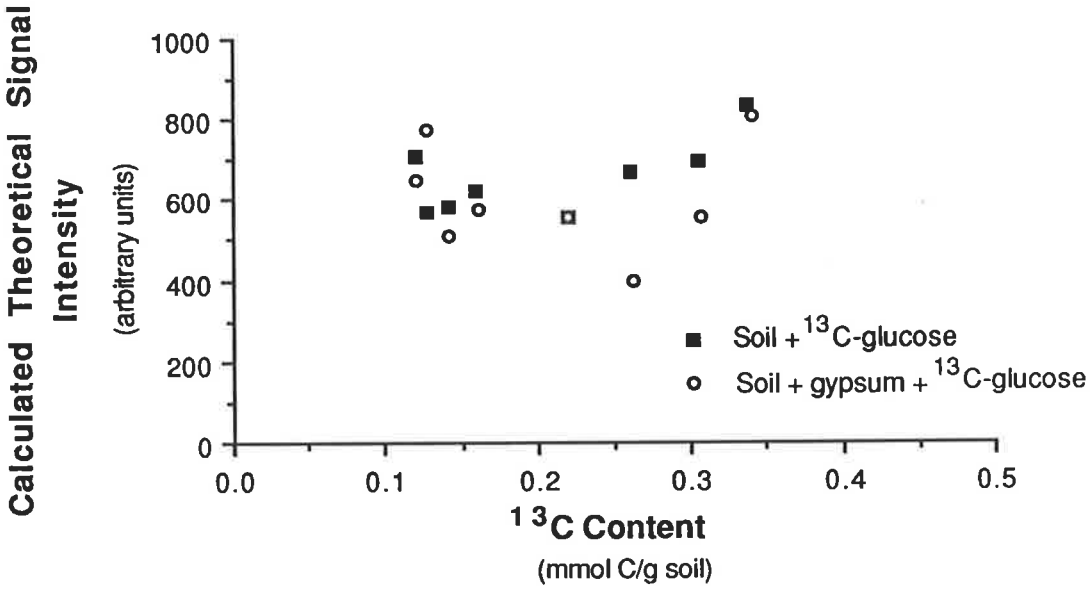

Figure 3.10: Changes in the calculated theoretical total signal intensity of the solid state CP/MAS ^{13}C NMR spectra obtained from the (soil+^{13}C-glucose) and (soil+gypsum+^{13}C-glucose) treated samples expressed as a function of their ^{13}C contents. In moving from right to left the incubation period increased.

It is postulated that the remaining discrepancy between the acquired signal intensity and the ^{13}C content of the samples resulted from a portion of the ^{13}C being rendered NMR invisible. One mechanism which may account for the observed discrepancy is the adsorption of soluble molecules containing the substrate ^{13}C onto paramagnetic mineral surfaces during the freeze drying process included in the sample preparation procedure. Initially all of the substrate ^{13}C would be contained in soluble molecules; however, as the incubation progressed the residual substrate ^{13}C would become incorporated into microbial detritus which on freeze drying would not be able to make as complete a contact with the mineral surface and its associated paramagnetics as the more soluble materials. Under such circumstances a larger fraction of the ^{13}C contained in soluble materials would be rendered NMR invisible than that associated with microbial detritus. This is substantiated by the large decrease in the T_1H value of the glucose carbon after it was added to the soil and freeze dried.

Native Unamended and Unincubated Soil

The conventional and dipolar dephased solid state ^{13}C NMR spectra collected for the native unamended soil used to prepare all of the samples are presented in Figure 3.11. In the conventional spectrum the major peaks appeared at 30 and 72 ppm indicating that alkyl and O-alkyl were the dominant forms of carbon present. Respectively, the alkyl and O-alkyl carbon accounted for 45% and 35% of the total signal. The smaller peaks appearing at 130, 150, and 175 ppm indicated that small amounts of alkyl-aromatic (7%), oxygenated-aromatic (5%), and carboxyl (8%) carbon were also present.

In the dipolar dephased spectrum only two peaks remained. The peak at 175 ppm was carboxyl carbon which remained because it was non-protonated. The second peak which extended from 10-45 ppm corresponds to the chemical shift range of CH, CH_2, and CH_3 carbon. The peak maximum

occured at 33 ppm which is indicative of CH_2 carbon in long chains. Signals from this type of carbon are not normally detected in dipolar dephasing experiments after a delay period of 50 μs unless the proton-carbon dipolar interaction is weakened by molecular motion. The influence of molecular motion on signal decay is exemplified by the relatively slow decay of CH_3 carbon signals. Even though highly protonated, the rapid rotational motion of CH_3 carbon results in a much slower signal decay than is associated with CH and CH_2 carbon. The persistence of signal at 33 ppm indicated that a significant proportion of the alkyl carbon (36%) exhibited molecular motion, more so than normally experienced in rigid solids. The origin of this molecular motion will be discussed later. The absence of a significant contribution of methyl carbon to this peak was confirmed by the absence of significant methyl

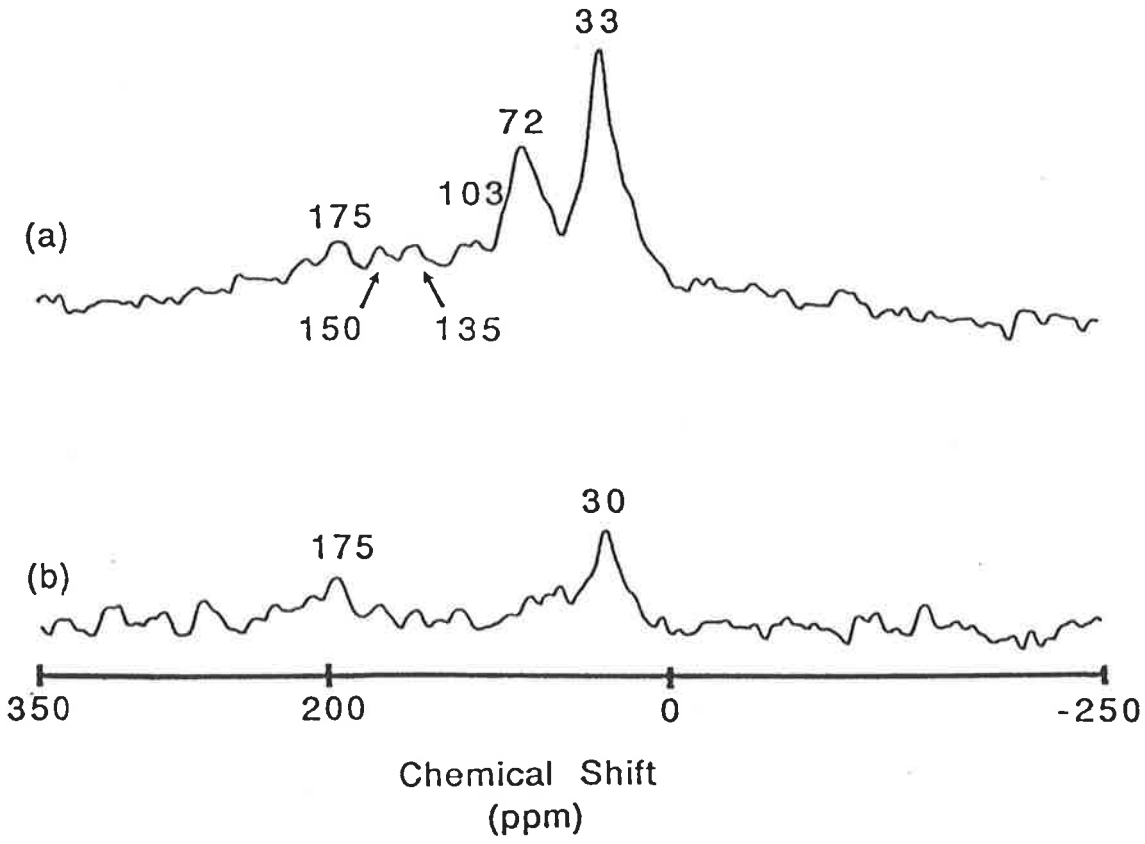

Figure 3.11: The (a) conventional and (b) dipolar dephased solid state CP/MAS ^{13}C NMR spectra collected for the unamended and unincubated Meadows fine sandy loam.

absorptions at 2959 cm^{-1} in Fourier transform infra red spectra. The resonance at 33 ppm was therefore ascribed to mobile CH_2 carbon, presumably contained in polymethylene structures.

^{13}C-glucose Amended Soil

Figures 3.12 and 3.13 show the conventional and dipolar dephased solid state spectra acquired for the samples from the (soil+^{13}C-glucose) and (soil+gypsum+^{13}C-glucose) treatments after each incubation period, respectively. The spectra acquired for the (soil+gypsum+13C-glucose) sample incubated for 24 days has not been included in Figure 3.13 because they were obtained using a different gain setting on the NMR spectrometer and were therefore not directly comparable to the other spectra. One may presume that the absence of any narrow intense signals in the spectrum indicated that the substrate ^{13}C was utilised to synthesise a wide range of structural compounds. However, it should be recalled that the presence of adjacent ^{13}C nuclei induced J-coupling which effectively broadened all resonances.

The chemical shift values of the peaks contained in the spectra acquired for the (soil+^{13}C-glucose) and (soil+gypsum+^{13}C-glucose) treated samples were not different which indicated that the presence of gypsum had little influence on the type of chemical structures contained in the residual substrate carbon. The subsequent discussion of spectral features therefore applies to both the (soil+^{13}C-glucose) and (soil+gypsum+^{13}C-glucose) samples.

The spectra acquired for the day 0 samples closely resembled those obtained for the ^{13}C-labelled glucose (Figure 3.1). The absence of any significant alkyl resonance in the day 0 samples suggested that the procedure used to remove the signal arising from the native soil organic carbon in the samples was effective. The spectra presented in Figures 3.12 and 3.13 are therefore only indicative of the changes in the chemical structure of the substrate carbon which occurred during the incubation.

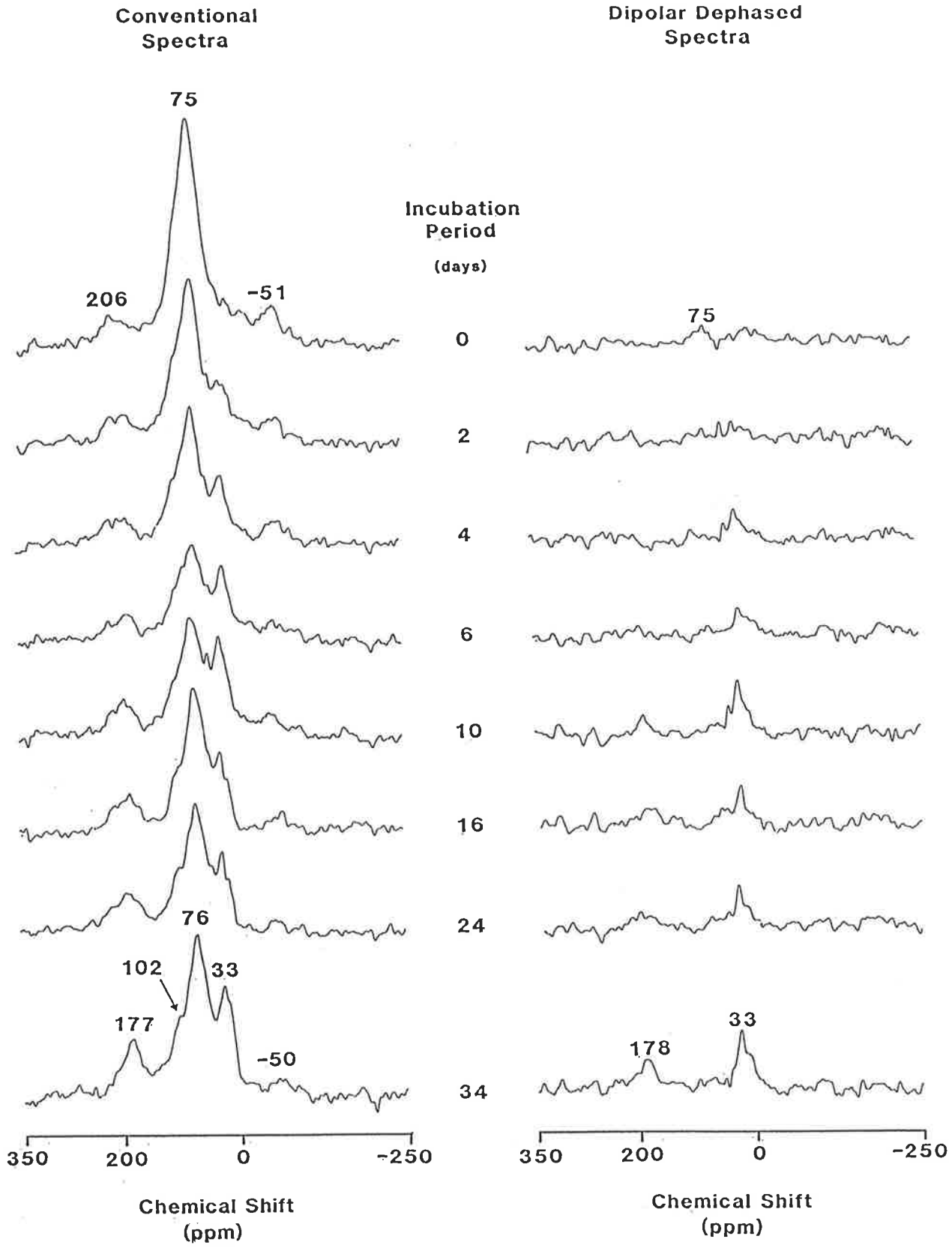

Figure 3.12: The conventional and dipolar dephased solid state CP/MAS ^{13}C NMR spectra acquired for samples from the (soil+^{13}C-glucose) treatment after subtraction of that aquired for the unamended unincubated soil.

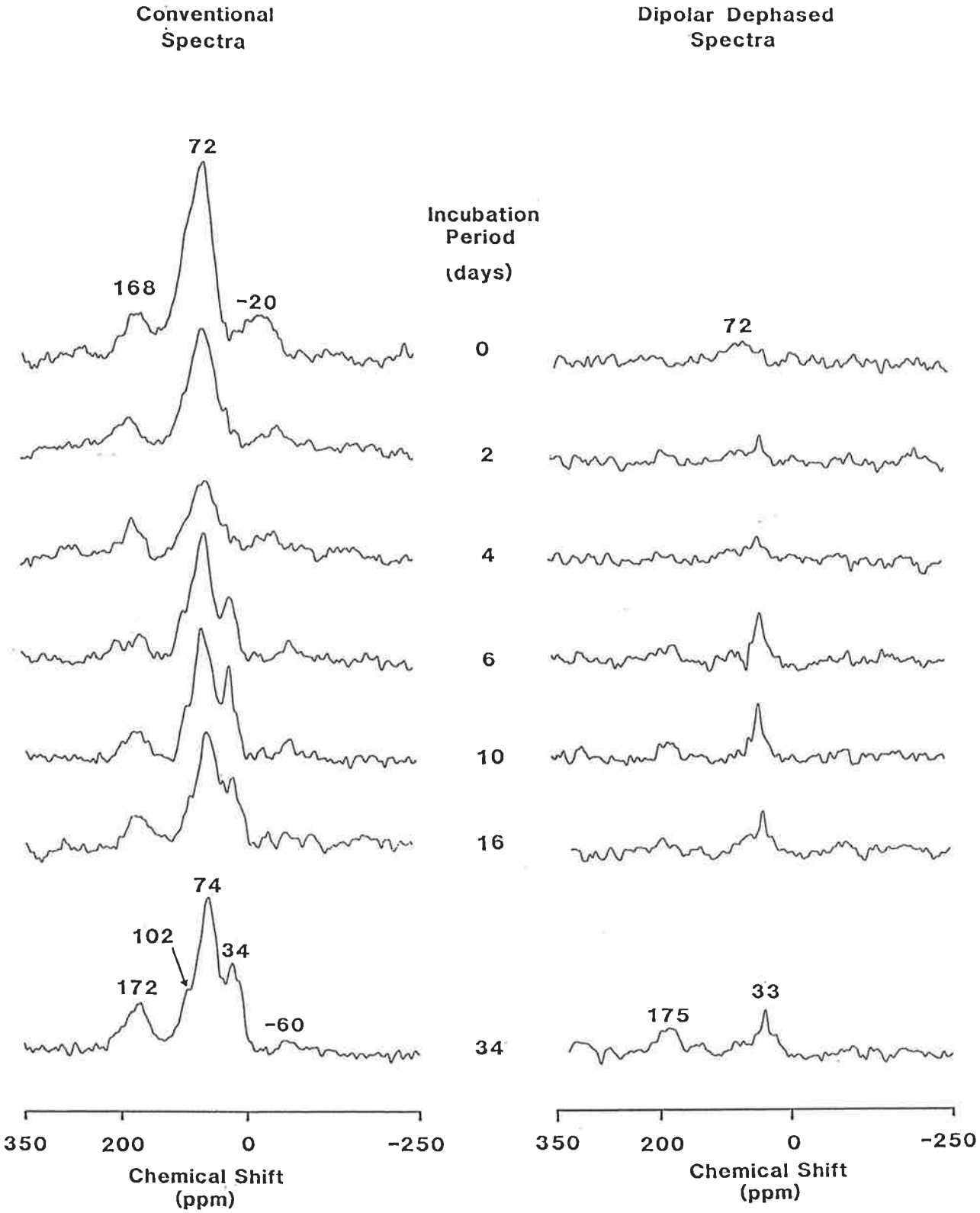

Figure 3.13: The conventional and dipolar dephased solid state CP/MAS ^{13}C NMR spectra acquired for samples from the (soil+gypsum+^{13}C-glucose) treatment after subtraction of that aquired for the unamended unincubated soil.

A reduction in the proportion of substrate carbon found in O-alkyl structures and an increase in the proportion found in alkyl and carboxyl structures was observed as the incubation progressed. The absence of any significant signal in the 120-150 ppm region suggested that few if any aromatic structures were synthesised from the substrate carbon by soil microorganisms. This observation is supported by the data of Martin et al. (1974), who noted that 92-84% of the residual carbon derived from a ^{14}C labelled carbohydrate which entered into the humic acid fraction of a soil was of a non-aromatic nature.

The proportion of the total substrate carbon contained in each spectral region was not significantly influenced by the addition of gypsum to the soil (Figure 3.14). The lack of any influence of gypsum on the chemical structure of the residual substrate carbon was consistent with the inability of gypsum to alter the amount of substrate carbon mineralised. Changes in the chemical structure of the residual substrate carbon in the presence of gypsum would only have been expected if a particular fraction of the residual substrate carbon was stabilised through an interaction with calcium.

The largest portion of residual substrate ^{13}C appeared in the O-alkyl fraction throughout the incubation. Initially the O-alkyl signal originated entirely from the added glucose. However, since glucose decomposition in soils is generally complete within 3-7 days (Chapter 2, Coody et al. 1986), the O-alkyl signal detected subsequent to day 6 must have arisen from structures synthesised by the soil microbial biomass (eg. polysaccharide structures such as cellulose and extracellular mucilaginous materials). The presence of polysaccharide structures was supported by the shoulder on the alkyl peak at approximately 103 ppm which arises from the dioxygenated carbon linking monosaccharide structures (Oades et al. 1987). The absence of a peak at 103 ppm in the dipolar dephased spectra demonstrated that this resonance arose from acetal structures which suggested the presence of cellulosic materials.

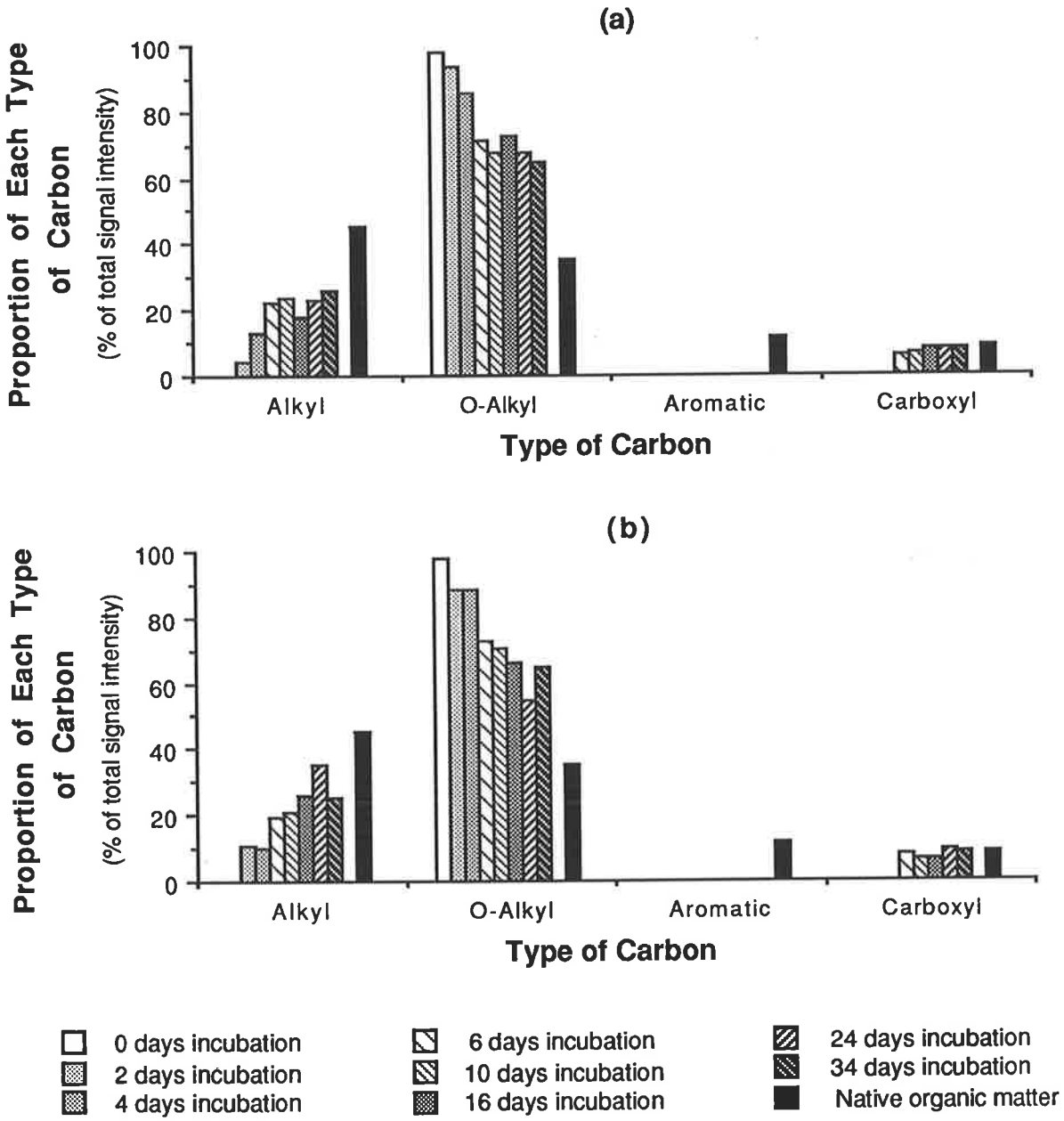

Figure 3.14: Distribution of the residual substrate ^{13}C within the alkyl, O-alkyl and carboxyl carbon fractions during the incubation for the (soil+^{13}C-glucose) (a) and the (soil+gypsum+^{13}C-glucose) (b) treatments.

The appearance of alkyl resonances indicated the ability of the soil microorganisms to synthesise alkyl materials from a carbohydrate substrate. The synthesis of alkyl carbon was initiated within the first two days. The most

rapid increase in the proportion of alkyl carbon occurred during the first six days of incubation when the soil microorganisms was actively growing and utilising the added substrate. Subsequent to day 6, the proportion of alkyl carbon increased slowly to a value of approximately 25% of the total residual substrate ^{13}C.

The rapid appearance of alkyl carbon could be a result of the synthesis of lipid molecules in microbial cell membranes. The lipid component of microbial cell membranes consists of fatty acid and phospholipid structures (Gottschalk 1986). The membrane structure is stabilised by hydrogen bonding between the hydrophilic heads and the alkyl chains exist as structures in the membrane wall. After death, any order in the membrane wall may be reduced so that alkyl chains may exist in some non-rigid semi-solid phase which could explain why a portion of the alkyl carbon remained in the dipolar dephased spectra even though it was highly protonated. Approximately 47% of the alkyl carbon signal was detected in the dipolar dephased spectra of the sample incubated for 34 days.

No signal from carboxyl carbon was detected until day 6 of the incubation. This result was surprising, since the first products of glucose decomposition contain carboxyl structures (eg. pyruvate and acetyl coenzyme A). However, since these materials are used to synthesise numerous cellular components (Gottschalk 1986), in an actively growing microbial population they would have a transient existence and may have occurred at concentrations too low to be detected by the NMR spectrometer. The appearance of carboxyl carbon coincided with the completion of the decomposition of glucose and was suspected to occur once the soil microbial biomass began to utilise the less readily available products of glucose decomposition.

Comparison of Old and New Soil Organic Carbon

The spectra acquired for the unamended native soil and the soil amended and incubated with ^{13}C-glucose for 34 days can be used to compare the chemical nature of the older native soil organic carbon and the recently synthesised new soil organic carbon. These materials were found to differ in their respective contents of alkyl, O-alkyl and aromatic carbon (Figure 3.14).

The older native organic material contained proportionally more alkyl and less O-alkyl carbon. Similar increases and decreases in the respective contents of alkyl and O-alkyl carbon have been noted during the decomposition of peat (Preston et al. 1987, Hammond et al. 1985) and spruce, pine and beech litter (Zech et al. 1987, Wilson et al. 1983b); however, no comments could be made as to whether the increased proportion of alkyl carbon resulted from the synthesis of alkyl structures by the microorganisms or a selective preservation of the alkyl structures present in the initial materials. The present study clearly demonstrates the ability of the soil microorganisms to synthesise alkyl carbon structures as it metabolised O-alkyl carbon. The similar dipolar dephasing behaviour of the alkyl carbon in the old and new soil organic carbon suggests that the materials were similar.

The absence of a significant aromatic carbon signal in the spectra of the new soil organic matter indicates that little if any aromatic carbon was synthesised by soil microorganisms utilising the added ^{13}C-glucose. Since the microorganisms did not appear to synthesise aromatic structures, the aromatic signal detected in the spectra acquired for the unamended native soil is considered to arise from lignin residues derived from plant materials. However, a slow synthesis and selective preservation of aromatic materials cannot be dismissed as a possible source of the aromatic carbon in the older native soil organic carbon.

3.3.3 The Relationship Between Biological Stability and Chemical Structure

The progressive changes in the chemical structure of the residual substrate carbon towards that of the native soil organic carbon is demonstrated in Figure 3.14. The most dramatic changes were confined to the first 6-10 days incubation, during which time the half life of the residual substrate carbon increased by a factor of seven . The formation of alkyl and carboxyl structures appears to have stabilised the residual substrate carbon against microbial decomposition. Whether the biological stabilisation was directly related to the observed changes in the chemical structure of the newly synthesised organic materials or was indirectly induced by an adsorption of these materials onto mineral surfaces remains questionable. Studies such as that by Paul and van Veen (1978) and Haider and Martin (1975) have demonstrated that polymeric materials especially of alkyl and aromatic nature are more slowly decomposed by the soil microbial biomass than O-alkyl materials. However, these materials, especially when they contain carboxyl structures, are readily adsorbed on mineral surfaces. Oades et al. (1987) and Schnitzer et al. (1988) have clearly demonstrated the ability of soil clays to adsorb alkyl materials, and Theng et al. (1986) have postulated that the recalcitrant nature of alkyl materials in soils is the result of adsorption on clay surfaces and in the interlayer spaces between clay particles. It is therefore expected that the initial changes in biological stability of the residual carbon derived from organic substrates in soils result from an alteration of the chemical structure of the substrate carbon, in particular, a loss of carbohydrate components and a synthesis of alkyl and carboxyl structures. Any further stabilisation results from the ability of the soil to protect the residual substrate carbon by adsorption onto mineral surfaces and/or entrapment within soil aggregates in positions inaccessible to soil microorganisms or their enzymes, rather than from a further alteration of chemical structure.

3.4 Conclusions

The use of uniformly labelled ^{13}C-glucose as an organic substrate enabled the incorporation of substrate carbon into soil organic matter in the presence and absence of gypsum to be monitored quantitatively, using carbon isotope mass spectrometry to obtain carbon balance measurements, and qualitatively, using solid state CP/MAS ^{13}C NMR spectroscopy to determine the distribution of substrate carbon in various chemical structures.

Carbon balance measurements indicated that no "priming effect" of glucose addition on the decomposition of the native organic carbon was noted, and that based on residual substrate ^{13}C contents, the rate of substrate ^{13}C mineralisation could be separated into two distinct regions having half-life values of 8.5 and 60.8 days.

The solid state CP/MAS ^{13}C NMR data showed that the utilisation of glucose by the soil biomass resulted in the synthesis of alkyl, O-alkyl and carboxyl carbon but little if any aromatic carbon. The conversion of carbohydrate carbon to aromatic carbon by microbial activity is therefore not considered to be a dominant mechanism in the accumulation of aromatic structures in the soil used. All large changes in the chemical composition of the substrate carbon were virtually complete after 10 days incubation, which corresponded to the time when the largest change in the half-life of the substrate carbon was observed. Subsequent to day 10 as the mineralisation of the substrate continued, albeit at a much reduced rate, no significant changes in the chemical structure of the substrate carbon were noted.

The addition of calcium to the soil as gypsum had little if any influence on the mineralisation of glucose carbon to carbon dioxide. The inability of gypsum to protect the residual substrate carbon against attack from the soil microbial biomass in the Meadows fine sandy loam, when considered in conjunction with previous results, suggests that the mechanism of biological stabilisation must somehow involve the mineral fraction and/or

physicochemical properties of the soils and is not due strictly to the formation of Ca^{2+}-organic complexes. It is postulated that the mechanism involved includes the binding of the Ca^{2+}-organic complexes to the charged surfaces of clay particles in the soil such that the Ca^{2+} cations act as a bridge binding the organic materials to the clay surfaces.

Chapter 4

Solid State CP/MAS ^{13}C NMR Analysis of Particle Size and Density Fractions of the Meadows Soil Incubated with Uniformly Labelled ^{13}C-glucose

4.1 Introduction

In solid state CP/MAS ^{13}C NMR experiments the amount of signal detected and the spectral quality (signal:noise ratio) both improve as the concentration of ^{13}C nuclei in the sample increases. Fractionation procedures based on particle size and particle density have been used to improve the quality of solid state CP/MAS ^{13}C NMR spectra acquired for the native soil organic carbon in mineral soils by concentrating the carbon into specific fractions (Skjemstad et al. 1986, Oades et al. 1987,1988). The objective of this study was to fractionate the Meadows fine sandy loam incubated for 34 days with the uniformly labelled ^{13}C-glucose (Chapter 3) on the basis of particle size and density to determine:

1) the distribution of the residual substrate ^{13}C in the isolated fractions using carbon isotope mass spectrometry, and
2) the chemical composition of the residual substrate ^{13}C in the isolated fractions using solid state CP/MAS ^{13}C NMR spectroscopy.

As a result of the increased concentration of ^{13}C in the samples isolated by the fractionation procedure more detailed solid state CP/MAS ^{13}C NMR analyses were performed than those presented in Chapter 3.

Although the objectives of this study did not directly address the overall objective of the thesis, valuable information related to the chemistry and dynamics of substrate decomposition in soils was obtained.

4.2 Materials and Methods

4.2.1 Soil

Meadows fine sandy loam (Rix and Hutton 1953), an Alfisol, was incubated for 34 days in the absence (control) and presence (treated) of uniformly labelled ^{13}C-glucose (ICON, New Jersey Cat.# IC3532). Since the addition of gypsum did not alter either the biological stability or chemical composition of the residual substrate ^{13}C, the control and treated samples used in this study were obtained by combining the soil from the (soil) and (soil+gypsum) treatments and the (soil+^{13}C-glucose) and (soil+gypsum+^{13}C-glucose) treatments described in Chapter 3, respectively. The procedures involved in the collection, preparation and incubation of the soil, as well as some relevant chemical and physical data pertaining to the collected sample, have also been described in Chapter 3.

4.2.2 Fractionation Procedure

The soil was dispersed by adding approximately 20 g of soil to 50 cm^3 of deionised water in a 150 cm^3 beaker and sonifying the suspension for 5 minutes using a Branson Sonifier (Model 250) operating at 50 percent of maximum output. Ice was packed around the beaker during sonification to reduce sample heating. The dispersed sample was passed through a 53 μm sieve and then separated in to a >2 μm diameter fraction and a ≤2 μm diameter fraction by gravitational sedimentation in deionised water. The ≤2 μm diameter fraction was dialysed against deionised water and freeze dried. The organic debris and coarse particles retained on the 53 μm sieve were added to the >2 μm diameter fraction. The >2 μm diameter fraction was separated into a light and heavy fraction by centrifugation in a sodium polytungstate, $Na_3WO_4 \cdot 9WO_3 \cdot H_2O$ (Semetu, Falkenried 4, D-1000 Berlin, FRG), solution of density 2.0 Mg m^{-3}. The light fraction was separated from the supernatant by filtration through a piece of Nylex 5TI having 5 μm openings. The polytungstate filtrate was added back to the centrifuge bottles. The

residue was resuspended and centrifuged and the supernatant removed and filtered. This process was repeated until a clear supernatant was obtained after centrifugation. The light fraction was washed with deionised water and dried at 45°C. The residue remaining in the centrifuge bottles, the heavy fraction, was washed five times with deionised water and dried at 45°C.

Using the above procedure the following three fractions were isolated quantitatively from the control and treated soil:

1) clay fraction (≤2 μm diameter particles)
2) light fraction (>2 μm diameter particles with a density ≤2.00 Mg m^{-3})
3) heavy fraction (>2 μm diameter particles with a density >2.00 Mg m^{-3})

A more extensive fractionation than that described could not be utilised because of the limited amount of soil available.

4.2.3 Mass Balance and Carbon and Nitrogen Balance Measurements

Mass balance measurements were performed by weighing the initial amount of soil dispersed and the material collected in each isolated fraction. For the unfractionated soil and each fraction, total carbon contents were measured using a LECO induction furnace (model# 763-000) equipped with a gravimetric carbon determinator (model# 521-275), the absolute ratio of $^{13}C/^{12}C$ was determined as described in Chapter 3 and total nitrogen contents were measured using a Kjeldahl digestion procedure (Bremner and Mulvaney 1982). The amounts of native carbon and residual glucose ^{13}C were calculated from the measured total carbon contents using the mass spectrometer data according to Chapter 3. The soil contained no detectable inorganic carbon so that all subsequent references made to the carbon contents of the soils or fractions refer to organic carbon contents.

4.2.4 Solid State CP/MAS ^{13}C NMR Spectroscopy

Signal Acquisition

Conventional and dipolar dephased solid state CP/MAS ^{13}C NMR spectra were acquired as described in Chapter 3 for the clay and light fractions of the control and treated soil. For the heavy fractions of the two soils only conventional spectra were acquired because of their very low carbon contents. The conventional and dipolar dephased spectra collected for the unfractionated soils were presented in Chapter 3.

In the conventional solid state CP/MAS ^{13}C NMR pulse sequence the intensity of the ^{13}C signal is enhanced by a transfer of energy from ^{1}H nuclei to ^{13}C nuclei during the contact time. The energy transfer and subsequent signal enhancement requires a finite time referred to as the cross polarisation time, T_{CH}. The exact value of T_{CH} varies with the type of carbon and depends on factors such as molecular motion and the proximity of the ^{13}C nuclei to ^{1}H nuclei (Wilson 1987). During the contact time, as the ^{13}C signal develops, the signal also begins to decay as a result of spin lattice relaxation in the rotating frame. The rate of relaxation is denoted as $T_1\rho H$ and, like T_{CH}, its exact value can vary with the type of carbon. To obtain quantitative results from CP/MAS ^{13}C NMR experiments it is essential that the following conditions are satisfied:

1) the rate of signal development for each type of carbon must be much faster than its rate of signal decay,ie. $T_{CH} << T_1\rho H$,
2) the $T_1\rho H$ values of the different types of carbon must be determined to account for differences in the rates of signal decay during the contact time, and
3) the delay period inserted between pulses, the recycle delay, must be sufficiently long to allow the signal to decay completely.

That the third condition was met for the samples used in this study was demonstrated in Chapter 3. The magnitude of the $T_1\rho H$ values and the relationship between T_{CH} and $T_1\rho H$ were determined for each type of carbon in the clay and light fractions by measuring the signal intensities in spectra

collected by varying the contact time between 0.01 ms and 10.0 ms. Low carbon contents prevented completion of such analyses for the carbon contained in the heavy fractions.

In dipolar dephased CP/MAS ^{13}C NMR experiments a delay period in which the proton decoupler is turned off is inserted into the pulse sequence before signal acquisition. In the absence of proton decoupling, proton-carbon dipolar interactions act as a powerful relaxation mechanism which causes the signal arising from CH_2 and CH carbon in rigid structures to decay faster than that of nonprotonated and CH_3 carbon and CH_2 and CH carbon in structures experiencing a considerable amount of molecular motion such as those in a gel or semi-solid state. As the duration of the delay period, referred to as the dipolar dephasing time, increases the extent of signal decay increases. The rate at which signals decay as the dipolar dephasing time increases is denoted by T_2' and can provide additional information on the chemical structure and molecular motion of the carbon in a sample. The T_2' value of each type of carbon in the samples was determined by acquiring spectra using dipolar dephasing times ranging from 1 μs to 200 μs.

Spectral Analysis

The total signal intensity and the proportion contributed by each type of carbon were determined by integration of spectral regions. Resolution was sufficient to delineate six spectral regions. The regions identified and their chemical shift limits were as follows: alkyl (10-45 ppm), O-alkyl (45-90 ppm), acetal (90-110 ppm), aromatic (110-140 ppm), phenolic (140-160 ppm) and carboxyl (160-200 ppm). The labels assigned to each region are considered to be only indicative of the dominant type of carbon present. Spinning side bands originating from homonuclear dipolar interactions between adjacent ^{13}C nuclei (Raleigh et al. 1987) were included in the spectral analysis of the samples isolated from the treated soil as indicated in Chapter 3.

4.3 Results and Discussion

A detailed presentation of the results collected in this study is contained on the computer disk located on the back cover of the thesis.

4.3.1 Mass Balance Measurements

Recovery of the soil material used in the fractionation procedure was quantitative and the distribution within the isolated fractions was similar for both the control and treated soils (Table 4.1). The similarity in the yields of the fractions from the control and treated soils indicated that the amount of dispersion induced by the ultrasonic treatment was constant for both soils. The amount of material collected in the clay fraction was equivalent to the clay content of the soil as determined by a hydrometer method which incorporated both a mechanical and chemical dispersion (Gee and Bauder 1986). The ultrasonic treatment used therefore appeared to disperse the soils completely.

Table 4.1: Mass balance data collected for the control and treated soil. All values are expressed as a percentage of the initial weight of soil fractionated.

Fraction	Control Soil	Treated Soil
Heavy fraction	84.14	81.95
Light fraction	6.27	6.12
Clay fraction	9.59	9.43
Total Recovery	100.00	97.50

4.3.2 Carbon and Nitrogen Measurements

The light fraction had the highest total carbon content and the heavy fraction had the lowest for both the natural and treated soil (Table 4.2). The carbon contents in Table 4.2 have been expressed in units of mmol C g^{-1} soil so that the total, native and residual substrate carbon contents are directly comparable. The high total carbon content, low particle density and high C:N

Table 4.2: Total, native and substrate carbon contents, total nitrogen contents and C:N ratios of the whole soils and the fractions isolated from the control and treated soil

		Total Carbon		Native Carbon		Substrate Carbon		Total Nitrogen		C:N
Soil	Fraction	Content [a]	Recovery [b]	Content	Recovery	Content	Recovery	Content	Recovery	Ratio [c]
Control	Whole soil	2.28		2.28		N/A[d]		0.094		21.03
	Heavy fraction	0.32	11.8	0.32	11.8	N/A	N/A	0.017	15.4	16.30
	Light fraction	24.15	66.1	24.14	66.1	N/A	N/A	0.824	55.1	25.16
	Clay fraction	5.36	22.5	5.35	22.5	N/A	N/A	0.346	35.5	13.26
	Total Recovery		100.3		100.3		N/A		106.0	
Treated	Whole soil	2.37		2.24		0.12		0.101		20.26
	Heavy fraction	0.22	7.6	0.21	7.7	0.01	6.1	0.014	11.6	13.42
	Light fraction	23.25	60.0	22.99	62.5	0.28	12.8	0.784	47.6	25.48
	Clay fraction	7.03	27.9	6.38	26.7	0.65	49.6	0.409	38.3	14.83
	Total Recovery		95.5		97.0		68.5		97.6	

[a] Contents of carbon and nitrogen are expressed in mmol of C (or N) g^{-1} fraction.

[b] Recovery values are expressed as a percentage of that present in the whole soil and were determined by multiplying the carbon or nitrogen contents by the mass balance data (Table 4.1) and dividing by the carbon or nitrogen content of the whole soil.

[c] The C:N ratios were calculated using the carbon and nitrogen contents of the samples expressed as a percentage by weight.

[d] N/A= not applicable. No ^{13}C substrate was added to the control soil.

ratio of the light fraction suggested that it contained significant quantities of unhumified organic debris of plant origin. Relative to the light fraction, the C:N ratios of the clay and heavy fractions were lower indicating that the organic carbon associated with these fractions was more humified than that of the light fraction. Reductions in the C:N ratio with decreasing particle size and increasing particle density have also been observed by Shiel (1986) and Oades et al. (1987), respectively.

Incubating ^{13}C-glucose in the soil induced a small but significant increase in the total carbon content of the whole soil. The increase was attributed to the increase observed in the total carbon content of the clay fraction since that of the light and heavy fractions decreased. Based on differences in the content of native and substrate carbon of the fractions isolated from the control and treated soils (Table 4.2), the accumulation of carbon in the clay fraction resulted from two processes:

1) a preferential accumulation of substrate carbon in the clay fraction (approximately 73 percent of the residual substrate carbon recovered was contained in the clay fraction), and
2) a movement of native carbon out of the heavy and light fractions into the clay fraction.

A preferential accumulation of substrate carbon in finer particle size fractions has been previously observed by Christensen and Sørensen (1985) and Williams et al. (1987). Using four soils with 6, 12, 23 and 46 % clay and incubated for 5-6 years with ^{14}C labelled straw, hemicellulose or glucose, Christensen and Sørensen (1985) noted that 66-84, 4-19 and <2 % of the residual substrate carbon was contained in the clay, silt and sand fractions, respectively. However, no indication of a movement of native carbon from coarser to finer fractions was presented.

Total recovery values for the total and native carbon and for the total nitrogen were essentially quantitative for the control and treated soils (Table

4.2). However, a substantial portion of the substrate carbon, approximately 30%, remained unaccounted for in the treated soil. A similar inability to quantitatively recover the residual substrate carbon from a soil incubated with ^{14}C-labelled wheat straw after using an ultrasonic probe to disperse samples was noted by Oades (unpublished data). Since the rate of mineralisation of the substrate carbon from the treated soil after 34 days incubation was minimal (Chapter 3), it was assumed that the residual substrate carbon was stabilised against microbial attack and that little if any further mineralisation would occur during the fractionation procedure. These assumptions proved to be invalid. The low recovery of residual substrate carbon was suspected to result from the exposure and subsequent mineralisation of substrate carbon which was protected from the soil microbial biomass and its enzymes by its position in the soil matrix prior to dispersing the soil. After dispersing the soil the less humified residual substrate carbon would be more susceptible to microbial and enzyme attack than the native organic carbon and would therefore be preferentially mineralised. In subsequent studies, where the amount of residual substrate carbon in various particle size fractions is to be determined, it is recommended that microbial and enzyme inhibitors (eg. sodium azide or mercuric chloride) be added to all suspensions to minimize the potential mineralisation of substrate carbon during the fractionation process.

4.3.3 Solid State CP/MAS ^{13}C NMR Spectroscopy

The inability to quantitatively recover all of the residual ^{13}C after fractionating the soil should not affect the validity of the CP/MAS ^{13}C NMR analyses. A further decomposition and mineralisation of the residual substrate ^{13}C during the fractionation process would be equivalent to reincubating the samples and allowing decomposition to continue. The residual substrate ^{13}C should still reflect the chemical nature of the organic materials synthesised by the soil microbial biomass utilising substrate carbon.

Quantification of the CP/MAS ^{13}C NMR Data

The relationship between T_{CH} and $T_1\rho H$ for each type of carbon in the samples was assessed by plotting the natural logarithm of the measured signal intensity as a function of the contact time used (Figure 4.1). Maximum signal intensities were reached at contact times of ≤0.5 ms indicating that all pertinent T_{CH} values were short relative to $T_1\rho H$ values. The magnitude of the $T_1\rho H$ values was determined by calculating the reciprocal of the slope of the linear regression model fitted to the signal intensity data collected for contact times ≥1.0 ms. The statistics associated with each regression analysis and the calculated $T_1\rho H$ values are presented in Table 4.3.

Variations in the magnitude of the $T_1\rho H$ values calculated for each type of carbon were noted within the samples. This was especially evident in the data collected for the clay fraction of the control soil where the $T_1\rho H$ values ranged from 2.0 ms for the O-alkyl carbon to 6.2 ms for the phenolic carbon. The practical implication of this is that after a 1.0 ms contact time a significantly greater proportion of the O-alkyl carbon (40%) than the phenolic carbon (15%) would not be observed due to $T_1\rho H$ effects. If the signal intensities were not corrected for the amount of decay which occurred during the contact time, the proportion of the total carbon contained in O-alkyl and phenolic structures would be under and over estimated, respectively. Therefore, to assess the proportion of each type of carbon in the samples accurately, the theoretical signal intensities, I_o, which would have existed if no relaxation occurred during the contact time were calculated using equation [4.1] (Wilson 1987). In equation [4.1], I_t is the acquired signal intensity observed after a contact time t.

$$I_o = \left(\frac{I_t}{\exp -(t/T_1\rho H)} \right) \qquad [4.1]$$

Oades et al. (1987) have observed that the clay fraction isolated from

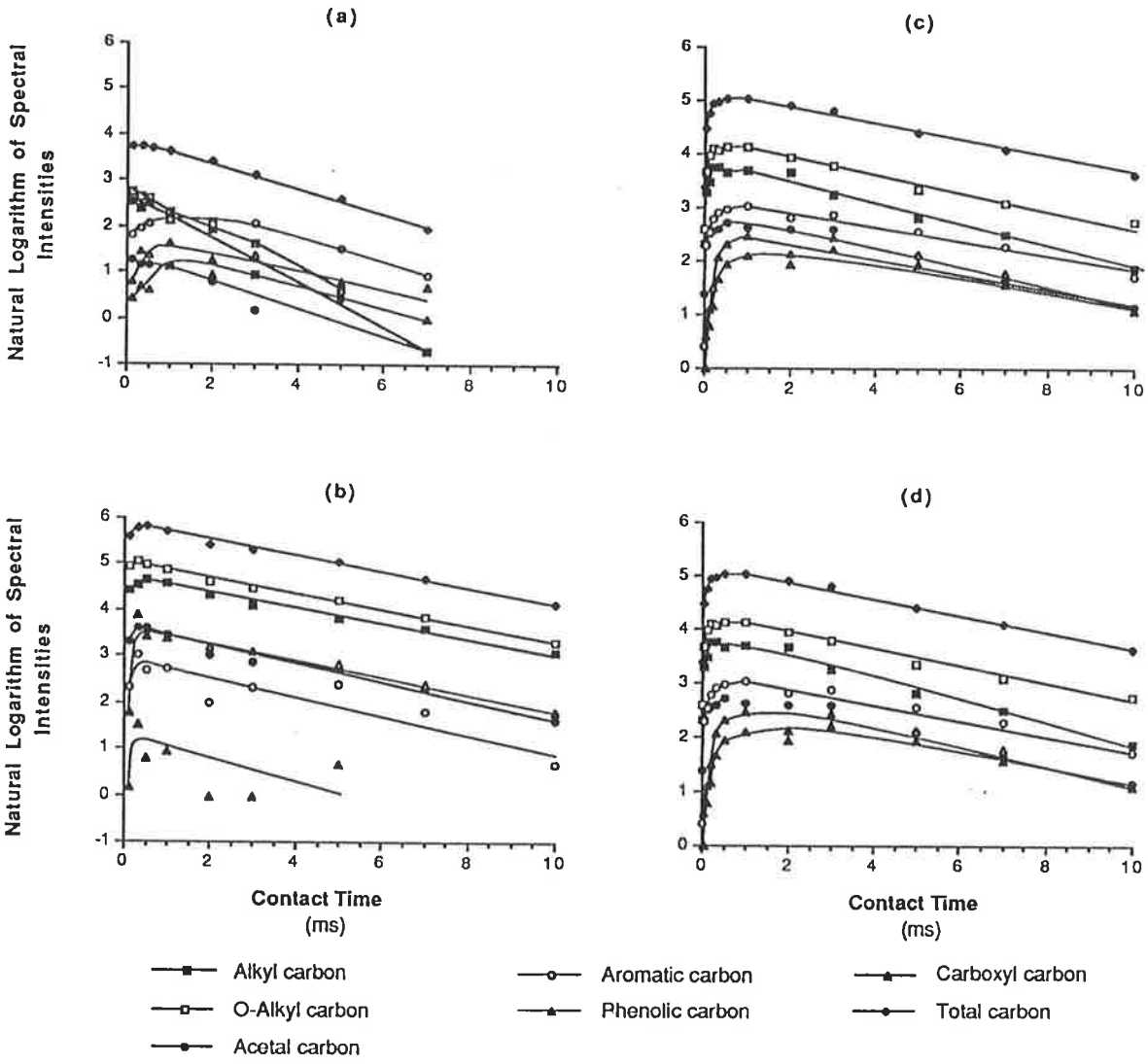

Figure 4.1: Relationship between signal intensity and contact time for each type of carbon in the clay fraction of the control soil (a), the clay fraction of the treated soil (b), the light fraction of the control soil (c) and the light fraction of the treated soil (d).

Table 4.3: Linear regression results and $T_1\rho H$ values calculated for the total signal intensity and that associated with each type of carbon analysed

Soil	Fraction	Type of Carbon	Regression Results Slope	R^2	Probability	$T_1\rho H$ (ms)
Control	Clay	Alkyl	0.46	0.96	0.004	2.16
		O-alkyl	0.51	0.98	0.009	1.96
		Acetal	0.26	0.83	0.030	3.86
		Aromatic	0.21	0.93	0.008	4.83
		Phenolic	0.16	0.83	0.032	6.17
		Carboxyl	0.16	0.80	0.040	6.06
		Total Signal	0.28	1.00	0.000	3.55
Treated	Clay	Alkyl	0.16	0.99	0.000	6.41
		O-alkyl	0.17	0.99	0.000	5.95
		Acetal	0.17	0.90	0.004	5.84
		Aromatic	0.18	0.75	0.026	5.52
		Phenolic	0.24	0.77	0.125	4.12
		Carboxyl	0.17	0.99	0.000	5.78
		Total Signal	0.17	0.99	0.000	5.99
Control	Light	Alkyl	0.21	0.99	0.000	4.76
		O-alkyl	0.15	0.98	0.000	6.49
		Acetal	0.18	0.97	0.000	5.65
		Aromatic	0.14	0.97	0.000	7.09
		Phenolic	0.11	0.84	0.011	9.01
		Carboxyl	0.14	0.87	0.007	7.09
		Total Signal	0.16	1.00	0.000	6.14
Treated	Light	Alkyl	0.20	0.92	0.002	4.88
		O-alkyl	0.18	0.96	0.000	5.56
		Acetal	0.16	0.94	0.001	6.14
		Aromatic	0.14	0.91	0.003	7.09
		Phenolic	0.13	0.72	0.319	7.46
		Carboxyl	0.16	0.90	0.004	6.32
		Total Signal	0.16	0.97	0.000	6.30

a Red-brown earth was dominated by alkyl carbon. If the $T_1\rho H$ values of the carbon associated with that clay fraction were similar to those observed in the present study, the dominance of alkyl carbon would be much greater than Oades et al. suggested. One complicating factor of applying the $T_1\rho H$ results obtained in this study to that of Oades et al. (1987) is the greater iron content of

the Red-brown earth which, because of iron's paramagnetic behaviour, would undoubtedly influence $T_1\rho H$ values.

Differences in the $T_1\rho H$ values of each type of carbon were also observed between samples. The $T_1\rho H$ values of the alkyl and O-alkyl carbon in the clay fraction of the control soil were much shorter than those of the treated soil. The ^{13}C present in the treated soil was dominated by the newly synthesised materials released by the microbial biomass as it utilised the substrate ^{13}C. Relative to the ^{13}C present in the clay fraction of the treated soil, that present in the control soil was much older, more humified and presumably more strongly adsorbed onto clay particle surfaces. It is suspected that at least part of the difference in the relaxation behaviour of the ^{13}C in the two clay fractions resulted from differences in the extent and strength of adsorption of these materials onto clay surfaces.

The ability of the CP/MAS ^{13}C NMR analysis to view quantitatively all of the ^{13}C contained in the samples was assessed by plotting the theoretical total signal intensity, I_o, as a function of the amount of ^{13}C contained in the samples analysed (Figure 4.2). The amount of ^{13}C, expressed in units of

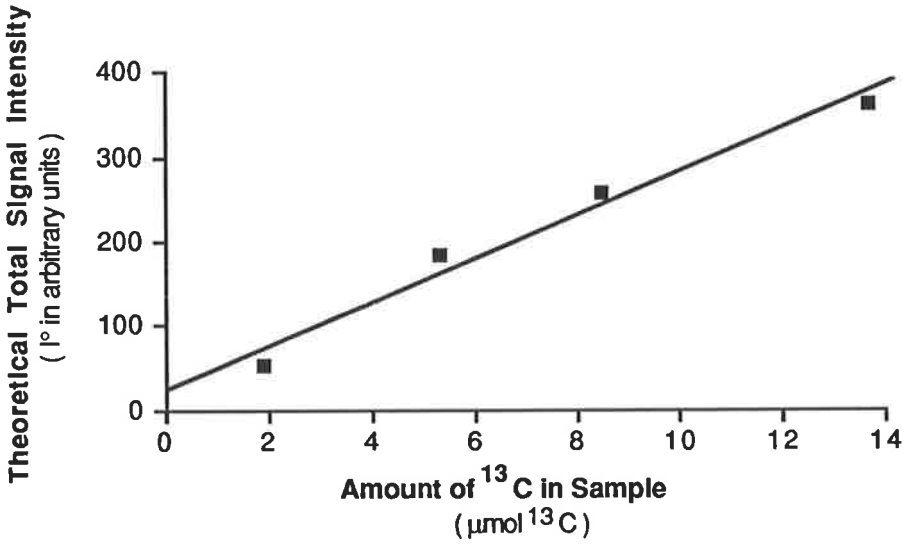

Figure 4.2: Plot of the calculated total theoretical signal intensity as a function of the amount of ^{13}C present on each sample analysed.

μmol, contained in each sample was calculated by multiplying the ^{13}C content of the samples, calculated as described in Chapter 3, by the weight of sample analysed. Statistical analysis of the data presented in Figure 4.2 indicated that a significant ($p=0.0131$) linear ($R^2=0.974$) relationship existed between the calculated theoretical total signal intensity and the amount of ^{13}C present in the samples. Therefore, after correcting for $T_1\rho H$ effects, the ^{13}C in all the samples was viewed with the same efficiency and the calculated theoretical signal intensities are quantitatively comparable both within and between individual samples.

Conventional CP/MAS ^{13}C NMR Spectra

The conventional CP/MAS ^{13}C NMR spectra acquired for the clay and light fractions of both soils appear in Figure 4.3. Relative to the spectra obtained for the unfractionated treated soil (Chapter 3), the use of particle size and density fractionations to concentrate carbon into specific fractions significantly improved the resolution of the acquired spectra and reduced the time required to obtain spectra of equivalent signal to noise ratio by a factor of 7 (15,000 transients were collected as opposed to 100,000). Although no spectra for the unfractionated control soil were presented in Chapter 3, the same observations apply.

In the clay fractions the peaks observed at approximately 30, 72, 103, 130, 155, and 175 ppm originated from alkyl, O-alkyl, acetal, aromatic, phenolic and carboxyl carbon, respectively. The low signal intensity obtained for the clay fraction of the control soil precluded any further spectral interpretation.

For the clay fraction of the treated soil the alkyl signal was centred at 30 ppm. Resonances in the vicinity of 30 ppm can originate from a range of alkyl structures (Duncan 1987). The presence of shoulders located on the high field side of the alkyl peak at approximately 23 and 15 ppm suggested

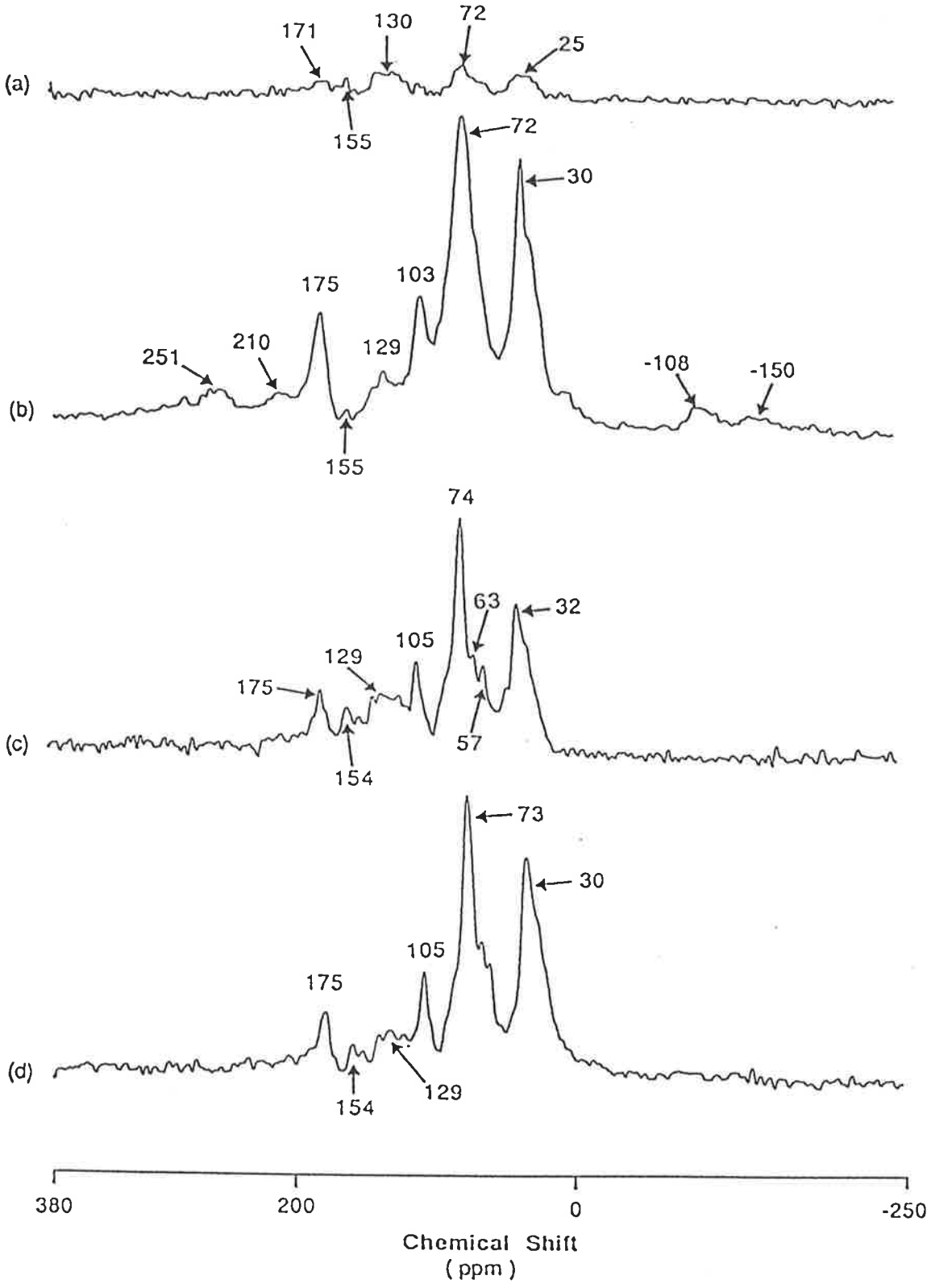

Figure 4.3: Conventional CP/MAS ^{13}C NMR spectra acquired for the clay fraction of the control soil (a), the clay fraction of the treated soil (b), the light fraction of the control soil (c) and the light fraction of the treated soil (d) using a 1.0 ms contact time and a 0.5 s recycle delay. Line broadening was 1 Hz and 15,000 transients were collected.

that the alkyl carbon was composed of several different structures. Oades et al. (1987) concluded that in a 20-53 μm particle size fraction of a Red-brown earth a portion of the alkyl material contained in a peak centred at 30 ppm had a polymethylene $-(CH_2)_n-$ structure. VanderHart (1976) showed that the chemical shift values of the carbon contained in a polymethylene structure (n-eicosane, $CH_3-CH_2-(CH_2)_{16}-CH_2-CH_3$) were 17, 27, and 35 ppm for the terminal CH_3 carbon, the CH_2 carbon adjacent to the terminal CH_3 carbon and the $-(CH_2)_n-$ carbon, respectively. The close agreement between the chemical shift values obtained by VanderHart and those observed for the alkyl peak and its shoulders in this study, indicated that the presence of $-(CH_2)_n-CH_2-CH_3$ structures in the alkyl carbon was likely.

The O-alkyl peak was centred at 72 ppm and therefore arose mainly from oxygenated carbon contained in the R_2CH-OH structures (Duncan, 1987) of carbohydrate materials. The presence of a peak for dioxygenated carbon at 103 ppm indicated that at least a portion of the carbohydrate carbon was linked together to form polysaccharides. The dipolar dephasing behaviour of this carbon (to be discussed later) indicated that the majority of it was protonated and originated from acetal structures. The presence of acetal carbon suggested that a portion of the carbohydrate carbon was probably cellulose (Oades et al. 1987).

The small peaks located at -150 and 210 ppm and -108 and 251 ppm were the high and low field spinning side bands resulting from the alkyl and O-alkyl peaks, respectively. These spinning side bands arise from homonuclear dipolar interactions between adjacent ^{13}C nuclei (Raleigh et al. 1987) and are not due to an incomplete removal of chemical shift anisotropy as a result of insufficient sample spinning speeds.

In addition to all of the peaks observed for the clay fractions, peaks at 57 and 63 ppm were observed in the spectra of the light fractions. The resonances of methoxyl carbon and the carbon adjacent to the amine group in

proteins appear in this region. Naito et al. (1981,1983) demonstrated that the resonances associated with the carbon adjacent to the amine group in alanine and serine have chemical shift values of 51 and 58, respectively. The dipolar dephasing behaviour of the carbon associated with these peaks (to be discussed later) indicated that it was dominated by CH_2 and/or CH carbon and therefore probably arose from carbon adjacent to amine groups rather than methoxyl carbon. The shoulder on the high field side of the O-alkyl peak in the spectrum of the clay fraction of the treated soil suggested that similar peaks were also present, but were not completely resolved from the carbohydrate peak.

The distribution of ^{13}C within the different types of carbon, expressed as a percentage of the total carbon detected in the conventional CP/MAS ^{13}C NMR spectra, are presented in Figure 4.4. All the data presented in Figure 4.4 was corrected for $T_1\rho H$ effects. In all the fractions analysed alkyl and O-alkyl carbon were dominant, but signals were observed for each type of carbon.

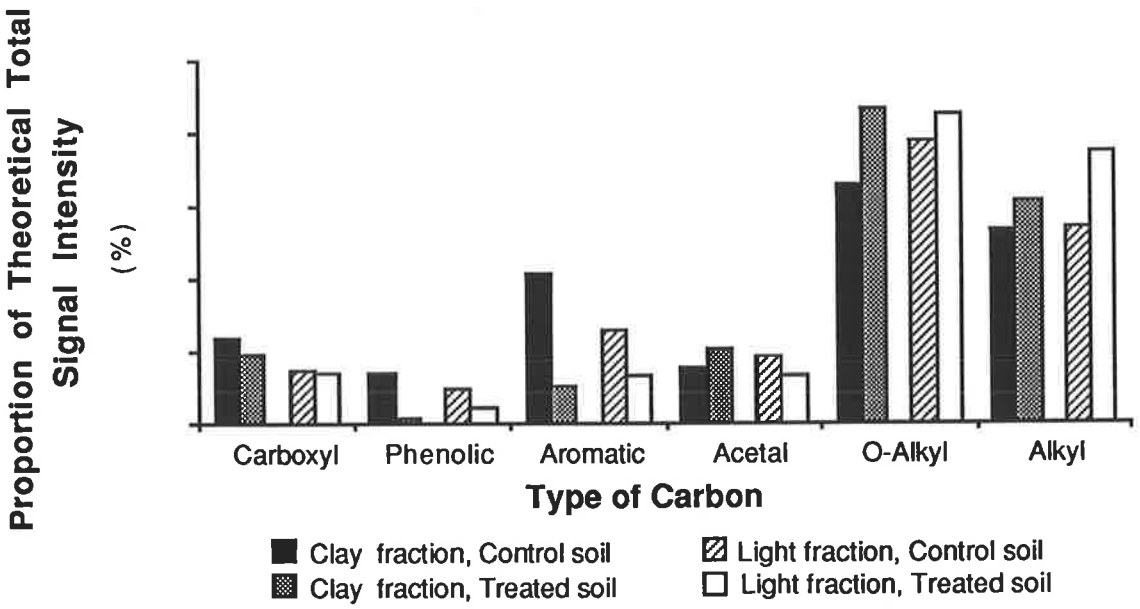

Figure 4.4: Proportion of the total ^{13}C observed in each spectral region for the clay and light factions of the control and treated soils.

The clay fraction of the control soil contained an appreciable content of aromatic carbon, much higher than that observed previously for a clay fraction separated from a red-brown earth by Oades et al. (1988).

The content of each type of ^{13}C expressed in units of µmol ^{13}C g^{-1} soil was calculated using equation [4.2] to quantitatively compare the distribution of the types of ^{13}C in the samples and to determine in which fractions the residual substrate ^{13}C resided. Equation [4.2] was developed using the

$$^{13}C \text{ Content} = \left(\frac{I_o - 22.9}{25.7} \right) \left(\frac{1}{W} \right) \qquad [4.2]$$

relationship observed between the calculated theoretical signal intensities, I_o values, and the amount of ^{13}C in the samples (Figure 4.2) and the weight of sample used in the analysis, W. The values of 22.9 and 25.7 correspond to the intercept and slope derived from the linear regression analysis of the data presented in Figure 4.2. The resulting ^{13}C contents are shown in Figure 4.5.

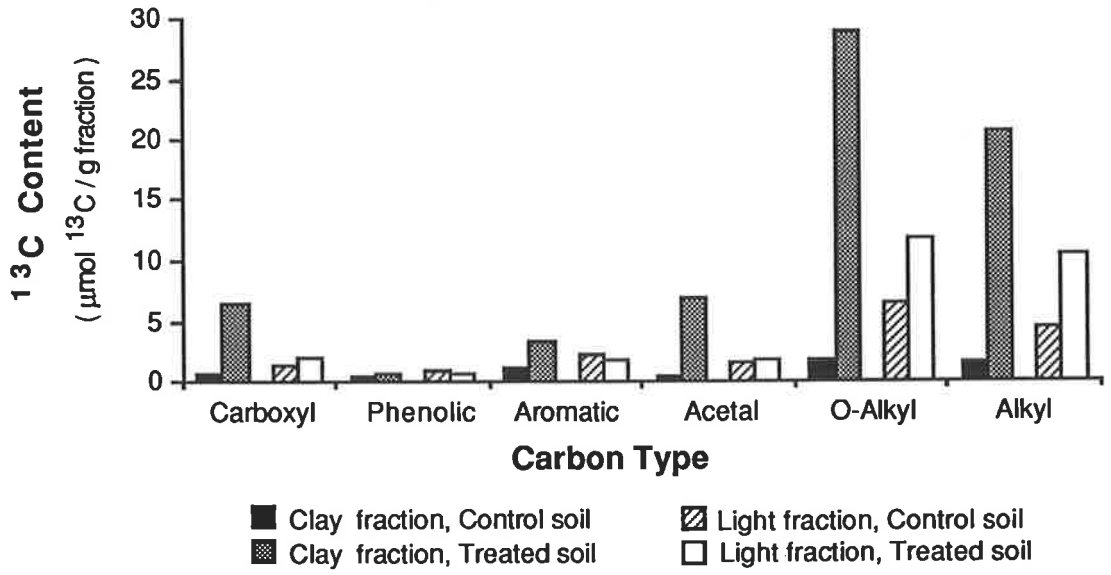

Figure 4.5: ^{13}C contents of each type of carbon in the clay and light fractions of the control and treated soils.

The ^{13}C contents of the fractions isolated from the control soil reflect the distribution of native soil organic carbon, while that of the treated soil reflects the distribution of both the native soil organic carbon and the residual substrate carbon. Since the mineralisation of native soil organic carbon from the control and treated soils during the incubation was not different (Chapter 3), the difference between the ^{13}C contents of each type of carbon in the control and treated samples represents the chemical composition of the residual substrate ^{13}C. In the clay fraction the residual substrate carbon was found in alkyl, O-alkyl, acetal, aromatic and carboxyl structures (Figure 4.5). The residual substrate carbon in the light fraction only accumulated in alkyl and O-alkyl structures.

The conventional CP/MAS ^{13}C NMR spectra acquired for the heavy fractions isolated from the control and treated soils are shown in Figure 4.6. Since no $T_1\rho H$ values were determined for the carbon contained in these fractions and the number of transients used to collect the two spectra were different (see Figure 4.6), no quantitative comparisons can be made. However, the better spectral quality and resolution in the spectrum collected for the treated soil even though far fewer transients were collected suggested that the residual substrate ^{13}C associated with the heavy fraction accumulated in alkyl, O-alkyl and carboxyl carbon structures.

Irrespective of the soil fraction, the residual substrate ^{13}C accumulated in alkyl and O-alkyl carbon structures. An accumulation of aromatic and carboxyl carbon structures and carboxyl carbon structures was also noted in the clay and heavy fractions, respectively. No appreciable accumulation of phenolic carbon was observed in any fraction, suggesting that little if any was synthesised by the soil microbial biomass utilising the ^{13}C originating from the added substrate. These results confirm those collected in the previous chapter but also suggest that some aromatic carbon was synthesised by the soil microbial biomass utilising the substrate ^{13}C. The aromatic carbon was only

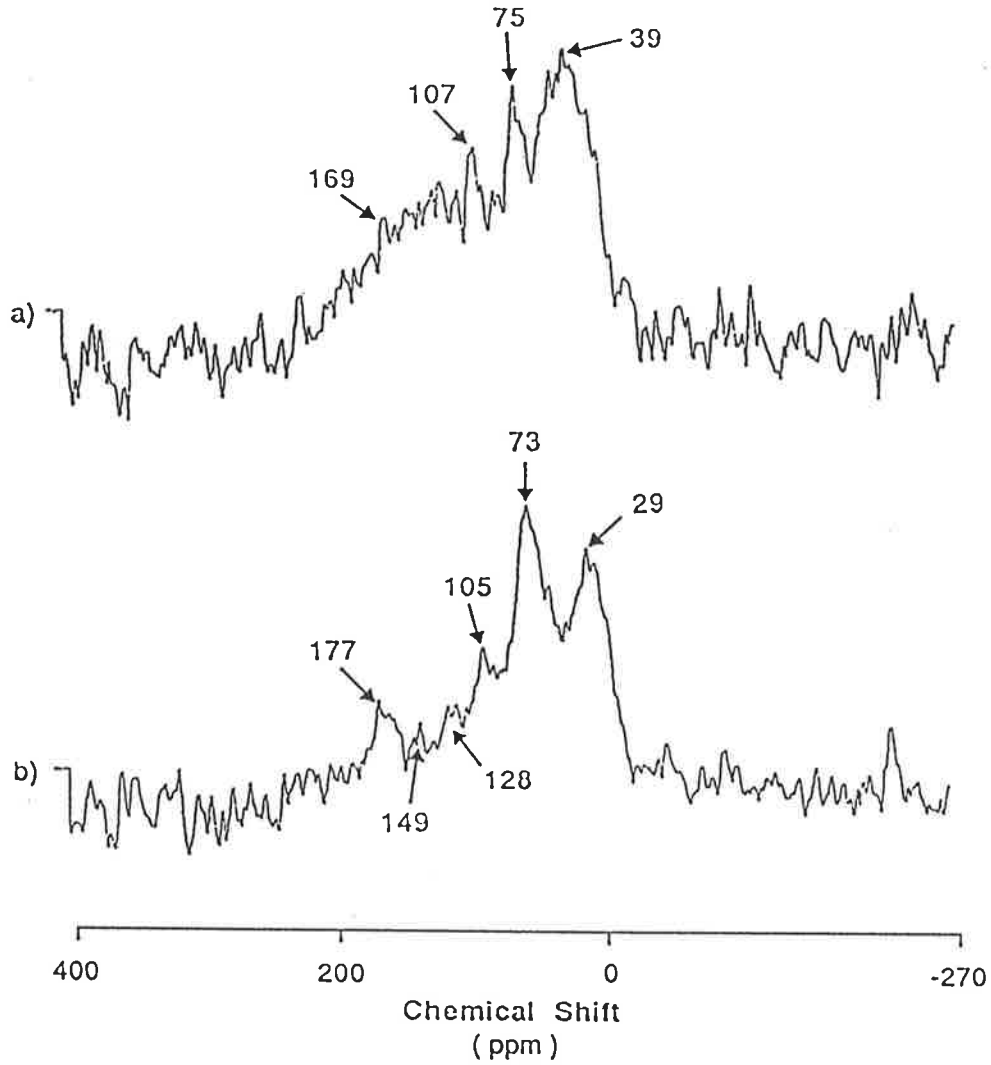

Figure 4.6: Conventional CP/MAS ^{13}C NMR spectra acquired for the heavy fraction isolated from the control (a) and treated (b) soils using a 1.0 ms contact time and a 1 s recycle delay time. Line broadening was 1 Hz and 140,000 and 90,000 transients were collected for the heavy fraction isolated from the control and treated soils, respectively.

observed in the clay fraction and was suspected to result from the synthesis of aromatic amino acids.

Dipolar Dephased CP/MAS ^{13}C NMR Spectra

As already noted in dipolar dephased CP/MAS ^{13}C NMR experiments, signals originating form CH_2 and CH carbon decay at a faster rate than those originating from nonprotonated and CH_3 carbon and any mobile material

during the dipolar dephasing time, t_1. Almost all the signal arising from CH_2 and CH carbon is lost if t_1 values ≥40 μs are used (Opella and Frey 1979). The dipolar dephased spectra acquired using t_1 values of 1 μs and 40 μs have been compared to give an indication of the proportion of each signal derived from CH_2 and CH carbon (Figure 4.7). Very little signal was observed for the clay fraction of the control soil even when t_1=1 μs, presumably because of its lower ^{13}C content. For the remaining fractions significant decreases in the signal intensities of the alkyl, O-alkyl, and acetal carbon were observed, indicating the presence of large amounts of CH_2 and CH carbon.The signal intensity of the aromatic, phenolic, and carboxyl carbon remained relatively constant. For the phenolic and carboxyl carbon this was consistent with the fact that these forms of carbon are nonprotonated. The lack of a large loss of signal for the aromatic carbon indicated that a proportion of the aromatic carbon in the fractions was also nonprotonated.

The dipolar dephasing behaviour of the peaks located at 57 and 63 ppm in the light fractions separated from the control and treated soils can be used to aid in the structural identification of the carbon from which they originated. As mentioned previously, two types of carbon structures resonate in this region: 1) carbon adjacent to the amine group in amino acids and 2) methoxyl carbon. The large decrease induced by increasing t_1 from 1 to 40 μs indicated that the carbon in question was contained in predominantly CH_2 and CH carbon structures and therefore originated from proteinaceous materials. However, since small peaks were still observed at 57 and 63 ppm after a 60 μs dipolar dephasing time, it is possible that methoxyl carbon was also present. The dipolar dephasing behaviour of the peaks located at 57 and 63 ppm therefore suggested that the carbon from which these peaks originated was dominated by amino carbon but also contained some methoxyl carbon. Given the proposed origin of the carbon contained in the light fractions (ie. partially humified plant debris), a greater contribution of methoxyl carbon may have

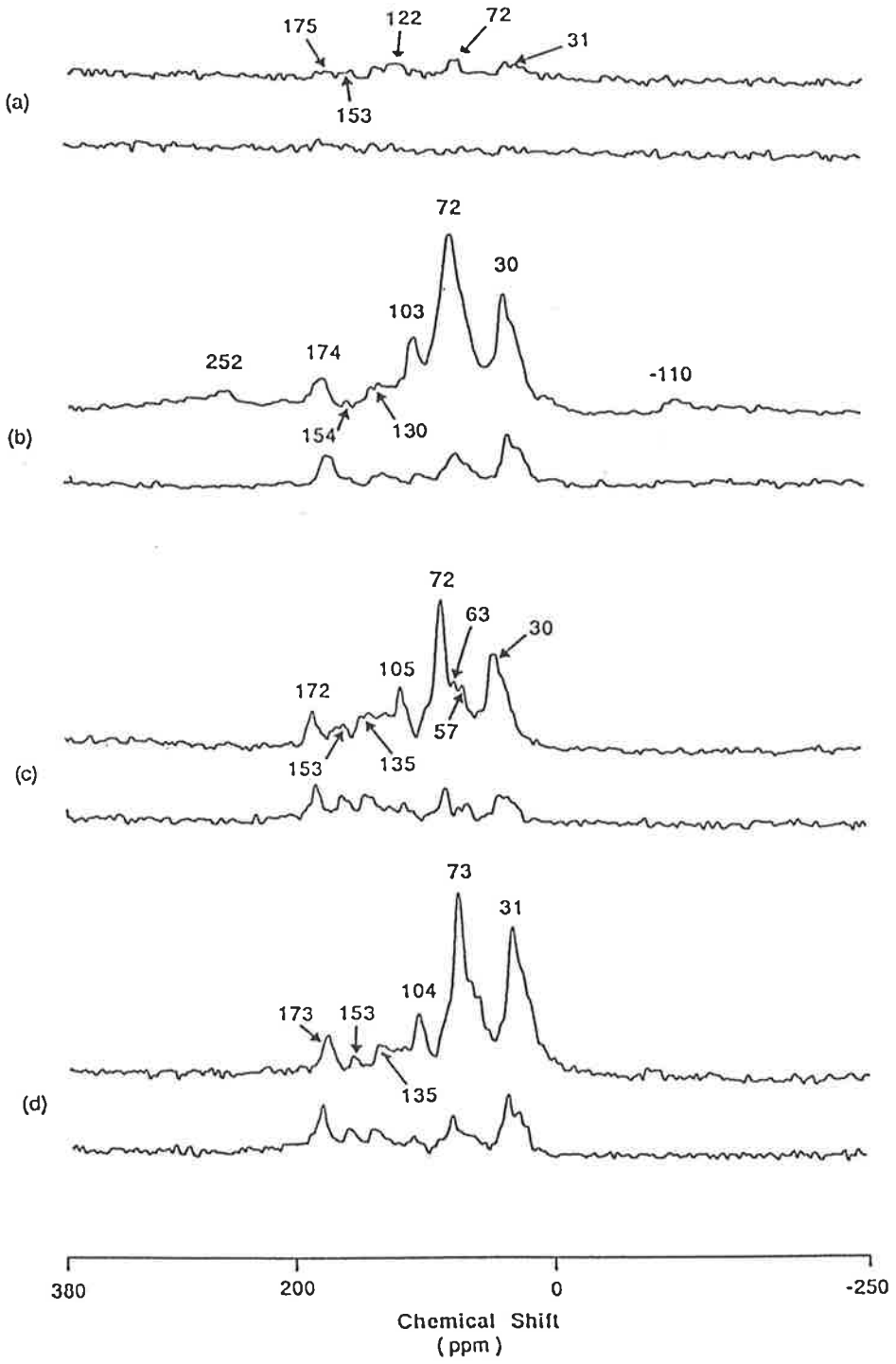

Figure 4.7: Dipolar dephased CP/MAS ^{13}C NMR spectra acquired for the clay fraction of the control soil (a), the clay fraction of the treated soil (b), the light fraction of the control soil (c) and the light fraction of the treated soil (d). The upper and lower spectra in each pair are those collected using a dipolar dephasing time of 1 and 40 μs, respectively. A contact time of 1.0 ms was used. Line broadening was 1 Hz and 10,000 transients were collected.

been expected due to the presence of lignin residues, but Kirk (1984) indicated that the removal of methoxyl carbon is one of the first steps in lignin decomposition.

Murphy et al. (1982a,b), Alemany et al. (1983) and Wilson et al. (1984) have shown that for a given type of carbon the decay of signal intensity during t_1 follows equation [4.3], where I is the signal intensity acquired using a dipolar dephasing time of t_1, I^o is the theoretical signal intensity which would have existed if $t_1=0$ and T_2' is the time constant for signal decay.

$$I = I^o \exp -(t_1/T_2') \qquad [4.3]$$

For aromatic signals containing two components which decay at different rates the observed decay of signal intensity with increasing t_1 can be described using equation [4.4] (Wilson 1987). In equation [4.4] the subscripts A and B are used to denote the parameters associated with the faster relaxing CH carbon and the slower relaxing nonprotonated carbon, respectively.

$$I = I_A^o \exp -(t_1/T_{2A}') + I_B^o \exp -(t_1/T_{2B}') \qquad [4.4]$$

Since Opella and Frey (1979) showed that the signal intensity of CH_2 and CH carbon decays to zero by using a t_1 value of 40 μs, at t_1 values >40 μs the contribution of CH_2 and CH carbon to the measured signal intensity becomes negligible and equation [4.4] simplifies to

$$I = I_B^o \exp -(t_1/T_{2B}') \qquad [4.5]$$

From a plot of ln I versus t_1, for t_1>40 μs, the values of I_B^o and T_{2B}' can be estimated from the intercept and reciprocal slope of the fitted linear regression line, respectively. By substituting the values of I_B^o and T_{2B}' into

equation [4.4] and rearranging it to give equation [4.6], the values of I_A^o and T_{2A}' can be calculated from the intercept and reciprocal slope of the linear regression line fitted to a plot of ln $[I - I_B^o \exp -(t_1/T_{2B}')]$ versus t_1 for $t_1 \leq 40$ μs, respectively.

$$[I - I_B^o \exp -(t_1/T_{2B}')] = I_A^o \exp -(t_1/T_{2A}') \qquad [4.6$$

T_{2A}' and T_{2B}' values were calculated for the carbon contained in each spectral region using the signal intensities acquired for each t_1 value and equations [3] - [6] (Table 4.4). In addition to t_1 values of 1 μs and 40 μs , spectra were also collected for t_1 values of 10, 20, 30, 60, 100, 120, 140, 160 , 180, and 200 μs. The values calculated for the rates of signal decay, T_{2A}' and T_{2B}', were well within the ranges indicated by Wilson (1987) for defined organic structures.

The aromatic I_A^o and I_B^o values indicated that approximately 25-40% of the aromatic carbon relaxed quickly. This implied that in the samples analysed only two of the six carbon atoms contained in the aromatic structures were protonated. Since little if any of the aromatic carbon was synthesised from the substrate carbon, these structures were contained in the soil's native organic fraction which may help account for their high degree of substitution.

The calculation of the proportion of CH_2 and CH carbon in the alkyl signal is complicated by the fact that molecular motion can significantly increase T_2' values and thereby reduce the rate of signal decay observed in dipolar dephased experiments. The influence of molecular motion on the rate of signal decay is exemplified by the behaviour of CH_3 carbon. Although highly protonated, CH_3 carbon has a long T_2' because its rapid rotational motion reduces the ^{13}C-^1H dipolar interaction which induces signal decay during t_1. Therefore, the proportion of the alkyl signal which relaxes slowly could arise from CH_2 and CH carbon exhibiting molecular motion as well as

nonprotonated and CH_3 carbon. In the clay and light fraction of the treated soil 20 and 34 % of the alkyl carbon had long T_2' values, respectively.

Table 4.4: Rates of signal decay, T_2' during the dipolar dephasing time, t_1, and the proportion of CH_2 and CH carbon contained in each type of carbon in the clay fraction of the treated soil and the light fraction of the control and treated soils.

Soil	Fraction	Type of Carbon	T_{2A}' [1] (μs)	T_{2B}' [1] (μs)	Proportion of CH_2 [2] and CH carbon (%)
Control	Light	Alkyl	30		100
		O-Alkyl	26		100
		Acetal	30		100
		Aromatic	37	107	34
		Phenolic		98	0
		Carboxyl		100	0
Treated	Clay	Alkyl	24	130	80
		O-Alkyl	33		100
		Acetal	31		100
		Aromatic	24	78	
		Phenolic	(no signal observed)		
		Carboxyl		109	0
Treated	Light	Alkyl	24	76	66
		O-Alkyl	32		100
		Acetal	31		100
		Aromatic	53	104	41
		Phenolic		95	0
		Carboxyl		92	0

All R^2 values and probabilities associated with the regression analyses used to calculate the T_{2A}' and T_{2B}' values were ≥0.89 and <0.05, respectively.

[1] The absence of a value for T2A' or T2B' indicates that no fast or slow relaxing carbon was observed in the acquired spectra, respectively.

[2] The proportion of the total signal derived from CH_2 and CH carbon for the alkyl and aromatic carbon was calculated using the I_A^o and I_B^o values obtained from the plots used to values in the formula $[I_A^o/(I_A^o+I_B^o)]$. For the remaining types of carbon, CH_2 and CH carbon either accounted for all or none of the signal intensity as described in the text.

4.4 Conclusions

Fractionating a soil incubated for 34 days with uniformly labelled ^{13}C-glucose on the basis of particle size and density has allowed the distribution and chemical nature of the residual ^{13}C within the isolated fractions to be determined. Spectral quality was improved by the fractionation procedure and the time required to obtain the spectra was decreased by a factor of seven. The residual ^{13}C accumulated predominantly in the clay fraction (≤2 μm diameter particles) of the soil in the form of alkyl, O-alkyl, acetal, and carboxyl carbon. However, significant amounts of alkyl and O-alkyl materials derived from the substrate ^{13}C were also found in the light fraction (>2 μm diameter particles with a density >2.0 Mg m^{-3}). Utilisation of glucose by the soil microbial biomass therefore resulted in the synthesis of alkyl, O-alkyl, acetal and carboxyl but only small amounts of aromatic carbon. All of the O-alkyl and acetal and 80% and 66% of the alkyl carbon associated with the clay and light fractions of the treated soil, respectively, was contained in CH_2 and CH carbon structures.

Chapter 5

Solid State CP/MAS ^{13}C NMR Analysis of Bacterial and Fungal Cultures Isolated from a Soil Incubated with Glucose

5.1 Introduction

In the previous two chapters solid state CP/MAS ^{13}C NMR spectroscopy was used to monitor changes in the chemical structure of carbon originating from uniformly labelled ^{13}C-glucose as it was decomposed by the microbial biomass in the Meadows fine sandy loam, an Alfisol. It was shown that utilisation of the substrate ^{13}C by the microbial biomass led to a significant accumulation of alkyl, O-alkyl and carboxyl carbon but only small amounts of aromatic carbon in the clay fraction (≤2 μm diameter particles) and the light fraction (>2 μm diameter particles with a density ≤2.00 Mg m-3) of the soil. Since all of the ^{13}C-glucose added to the soil undoubtedly passed through the microbial biomass, it was suspected that the chemical composition of the residual substrate ^{13}C after 34 days incubation should reflect the nature of the materials synthesised by the microbial biomass which utilised the ^{13}C-glucose. The objectives of this study were:

1) to perform conventional and dipolar dephased solid state CP/MAS ^{13}C NMR experiments on soil bacteria and fungi isolated from a sample of the Meadows soil incubated with glucose, and
2) to compare the chemical composition of the soil bacteria and fungi with that of the clay and light fractions separated from the Meadows soil.

Once again the objectives of this study do not address the initial objective of the thesis directly; however, the results will complement the information presented in the Chapters 3 and 4 and allow a better understanding of decomposition processes operating in soils to be obtained.

5.2 Materials and Methods

5.2.1 Soil

Bacteria and fungi were isolated from a sample of the Meadows fine sandy loam amended with glucose and incubated for 48 hours at 20°C and a soil water matric potential of -35 kPa. The soil came from the same bulk sample as that used in Chapters 3 and 4 to follow the decomposition and incorporation of uniformly labelled ^{13}C-glucose into the soil's organic fraction. The procedures involved in sample collection and some relevant chemical and physical data pertaining to the soil used have been presented in Chapter 3. The bacteria and fungi were isolated from soil incubated with glucose so that the composition of the microbial biomass in the soil would be similar to that which utilised the ^{13}C-glucose in the previous studies. The glucose was added to the soil at a rate of 37 mg glucose g^{-1} soil in the solution used to wet the soil.

5.2.2 Isolation and Preparation of the Bacterial and Fungal Cultures

Using the incubated soil, 10^{-4} and 10^{-1} soil dilutions were prepared by adding an appropriate weight of soil to a volume of filter sterilised distilled water. All filter sterilisations were performed using 0.22 μm Millipore filters. The bacterial and fungal cultures were prepared by adding 3.0 cm^3 of the 10^{-4} and 10^{-1} soil dilutions to 1.5 dm^3 of a filter sterilised nutrient solution, respectively. Different nutrient solutions were used to culture the bacteria and fungi. The composition of the nutrient solutions, per 2 dm^3, was as follows:

1) Bacterial nutrient solution - 6.00 g TSB (Tryptic Soy Broth) and 0.235 g cycloheximide
2) Fungal nutrient solution - 25 g glucose, 1.320 g $NaNO_3$, 0.660 g KH_2PO_4, 0.330 g KCl, 0.330 g $MgSO_4 \cdot 7H_2O$, 0.330 g yeast extract, 0.200 g streptomycin and 0.010 g tetracycline hydrochloride.

Cycloheximide was added to the bacterial nutrient solution to prevent the growth of any fungi. Similarly, streptomycin and tetracycline hydrochloride were added to the fungal nutrient solution to prevent the growth of any bacteria.

After addition of the appropriate soil dilution to the nutrient solutions, each mixture was shaken and divided into four sterilised 1 dm^3 Erlenmeyer flasks. The opening in each flask was plugged with a ball of glass wool and all flasks were placed in an incubator at 20°C. After 5 days the cultures were removed from the incubator. The fungi were collected by filtration through a piece of Nylex 5TI nylon screen with 5µm openings, washed with deionised water, frozen in liquid nitrogen and freeze dried. The bacteria were removed from the nutrient solution by centrifugation at 10,000 g for 20 minutes, washed with deionised water, frozen in liquid nitrogen and freeze dried.

5.2.3 Solid State CP/MAS ^{13}C NMR Spectroscopy

Conventional and dipolar dephased solid state CP/MAS ^{13}C NMR spectra were acquired for the bacterial and fungal cultures using the spectrometer and pulse sequences described in Chapter 3. The conditions required to obtain quantitative results from CP/MAS ^{13}C NMR experiments were discussed in Chapter 4 and can be summarised as follows:

1) T_{CH} values must be much shorter than $T_1\rho H$ values,
2) $T_1\rho H$ values for each type of carbon must be determined, and
3) the recycle delay must be long enough to allow for complete signal decay.

For the bacterial and fungal cultures the relationship between T_{CH} and $T_1\rho H$ and the actual $T_1\rho H$ values associated with each type of carbon were determined by measuring the signal intensities acquired by varying the contact time in the conventional CP/MAS ^{13}C NMR pulse sequence between 0.05 and 10.0 ms. The importance of the length of the recycle delay time was

determined by measuring the signal intensity acquired using the conventional pulse sequence with a 1.0 ms contact time and recycle delay times of 0.5 to 20 s.

In dipolar dephased CP/MAS ^{13}C NMR experiments (as discussed in Chapters 3 and 4) a delay period is inserted into the pulse sequence during which signals arising from CH_2 and CH carbon in rigid chemical structures decay faster than that of nonprotonated and CH_3 carbon and CH_2 and CH carbon in structures experiencing molecular motion. The rate at which signals decay is denoted by T_2' and when utilised in conjunction with chemical shift values can provide information on the chemical structure and molecular motion of the carbon in a sample. The T_2' value of each type of carbon in the bacterial and fungal materials was determined by acquiring spectra using dipolar dephasing times ranging from 1 μs to 200 μs.

The total signal intensity and the proportion contributed by each type of carbon in the spectra were determined by integration of spectral regions. Resolution in the acquired spectra was sufficient to delineate seven spectral regions. The regions identified and their chemical shift limits were as follows: alkyl (10-45 ppm), N-alkyl (45-65 ppm), O-alkyl (65-90 ppm), acetal (90-110 ppm), aromatic (110-140 ppm), phenolic (140-160 ppm) and carboxyl (160-200 ppm). The labels assigned to each region are considered to be only indicative of the dominant type of carbon present.

5.3 Results and Discussion

5.3.1 Solid State CP/MAS ^{13}C NMR Relaxation Parameters

The duration of the recycle delay period used between pulses was observed to influence the magnitude of the total signal intensity acquired for the bacterial and fungal materials (Figure 5.1). For recycle delay times <3.0 s a decrease in signal intensity was observed as the recycle delay time decreased for both the bacterial and fungal materials indicating that relaxation

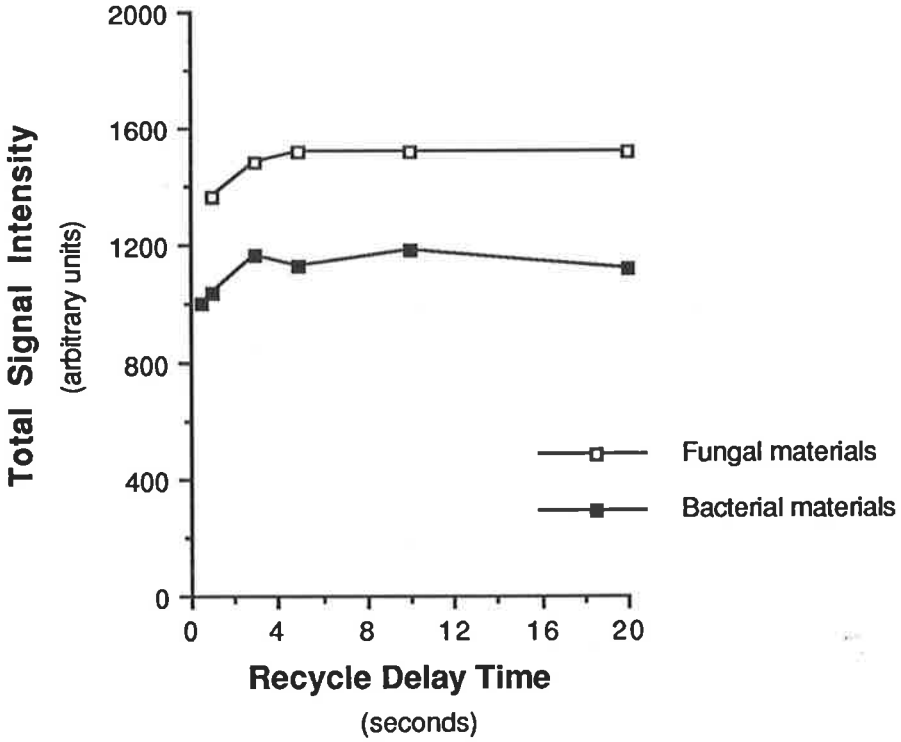

Figure 5.1: Influence of increasing the duration of the recycle delay period used to acquire conventional CP/MAS ^{13}C NMR spectra for the bacterial and fungal materials. The spectra were acquired using a contact time of 1.0 ms. The spectral line broadening used was 20 Hz and 5000 transients were collected for each spectrum.

between pulses was incomplete. Under such circumstances not all of the carbon in a sample would be observed. Therefore a recycle delay time of 3.0 s was used in the analysis of all samples.

The relationship between T_{CH} and $T_1\rho H$ for the total carbon and each type of carbon in the bacterial and fungal materials was assessed by plotting the natural logarithm of the acquired signal intensities as a function of the contact time used (Figure 5.2). Maximum signal intensities were reached at contact times of ≤0.5 ms indicating that all pertinent T_{CH} values were short relative to $T_1\rho H$ values. The magnitude of the $T_1\rho H$ values was determined by calculating the reciprocal of the slope of the linear regression model fitted to the signal intensity data (Figure 5.2) collected for contact times ≥1.0 ms. The

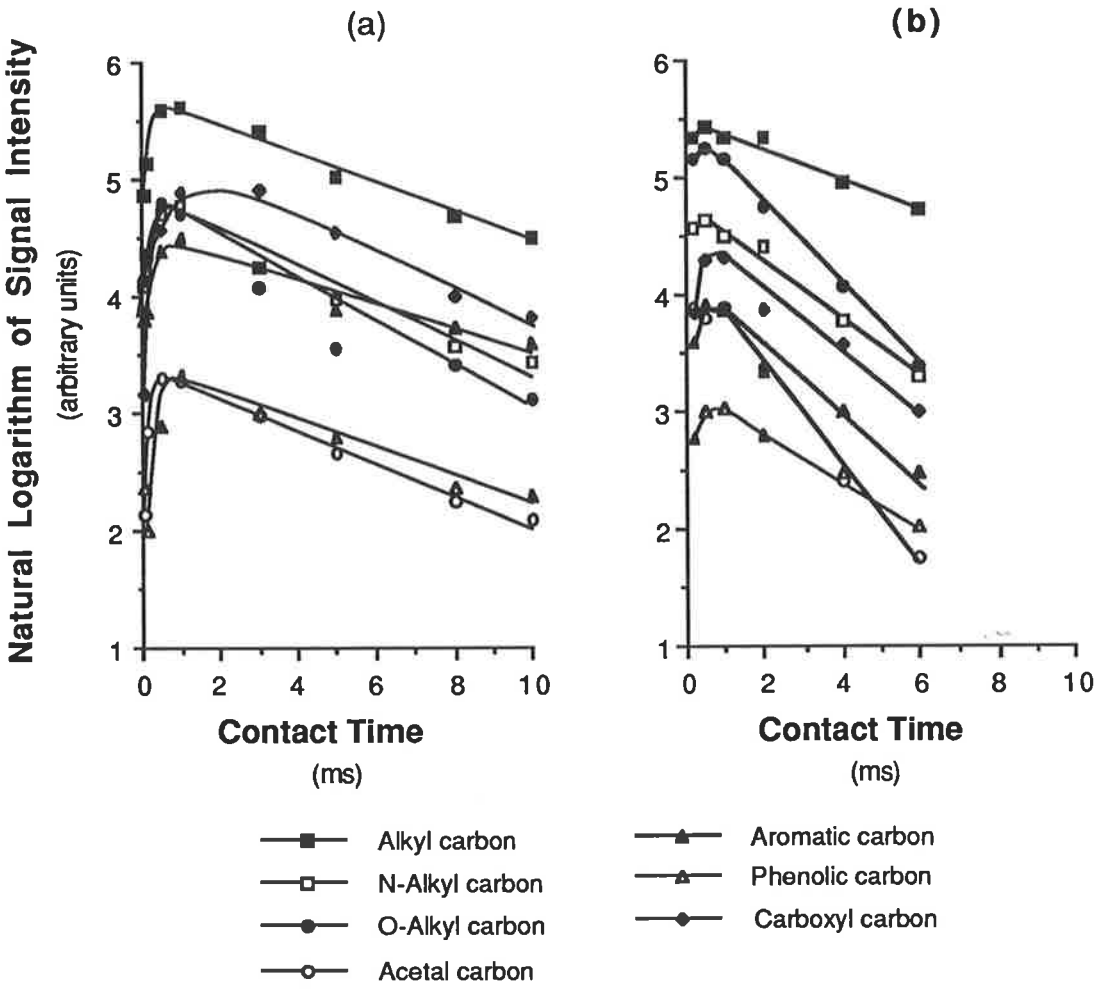

Figure 5.2: Relationship between signal intensity and contact time for each type of carbon in the bacterial (a) and fungal (b) materials. The line broadening used to acquire the spectra was 20 Hz and 5000 transients were collected for each spectrum.

calculated $T_1\rho H$ values and the statistics associated with each regression analysis are presented in Table 5.1.

The $T_1\rho H$ values calculated for the bacterial and fungal materials ranged from 6.2-9.8 ms and from 2.3-7.4 ms, respectively. The variation in the $T_1\rho H$ values of the different types of carbon in each sample indicated that the amount of signal decay which occurred during the contact time varied with the type of carbon. An accurate determination of the distribution of the different types of carbon in the samples was therefore not possible using the signal

Table 5.1: $T_1\rho H$ values calculated for the total carbon and each type of carbon in the bacterial and fungal materials and the statistics associated with each regression analysis.

Sample	Type of Carbon	Regression Results			$T_1\rho H$ (ms)
		Slope	R^2	Probability	
Bacteria	Alkyl	0.13	0.99	0.001	7.7
	N-alkyl	0.15	0.96	0.003	6.8
	O-alkyl	0.16	0.90	0.013	6.2
	Acetal	0.14	0.99	0.001	7.3
	Aromatic	0.10	0.94	0.006	9.8
	Phenolic	0.12	0.98	0.001	8.6
	Carboxyl	0.13	0.95	0.004	7.4
	Total Signal	0.13	0.99	0.001	7.5
Fungi	Alkyl	0.13	0.97	0.016	7.4
	N-alkyl	0.25	0.99	0.007	4.0
	O-alkyl	0.35	1.00	0.001	2.8
	Acetal	0.43	0.99	0.004	2.3
	Aromatic	0.26	0.96	0.018	3.9
	Phenolic	0.19	0.99	0.003	5.2
	Carboxyl	0.25	0.97	0.013	4.0
	Total Signal	0.23	1.00	0.001	4.4

intensity data as collected. To determine the proportion of each type of carbon contained in the soil, the theoretical signal intensities, I_o, which would have been observed if no signal decay occurred during the contact time were calculated using equation [5.1]. In equation [5.1] I_t is the signal intensity acquired using a contact time, t, of 1.0 ms.

$$I_o = \left(\frac{I_t}{\exp -(t / T_1\rho H)} \right) \qquad [5.1]$$

With the exception of the alkyl carbon, the $T_1\rho H$ values of each type of carbon in the bacterial materials were longer than the corresponding values in the fungal materials. It was suspected that the differences in the relaxation

behaviour of the different types of carbon in the bacterial and fungal materials may be related, at least partially, to differences in the chemical structure of the materials synthesised by the two types of microorganisms.

5.3.2 Solid State CP/MAS ^{13}C NMR Spectra

The conventional and dipolar dephased CP/MAS ^{13}C NMR spectra acquired for the bacterial and fungal materials appear in Figures 5.3 and 5.4, respectively. The results obtained from the dipolar dephasing experiments will be discussed first since an accurate interpretation of the conventional spectra required a knowledge of the dipolar dephasing behaviour of each type of carbon in the spectra.

Dipolar Dephasing Experiments

In dipolar dephasing experiments signals arising from CH_2 and CH carbon in rigid chemical structures decay more rapidly than the other forms of carbon during the dipolar dephasing time, t_1, and are not observed using t_1 values ≥40 μs (Opella and Frey 1979). In this study dipolar dephased spectra were collected using t_1 values of 1, 10, 20, 30, 40, 60, 100, 120, 140, 160 , 180, and 200 μs. The rates of signal decay, T_{2A}' and T_{2B}', and the proportion of the signal intensity attributable to the fast and slow relaxing components, I_A^o and I_B^o, respectively, were calculated (Table 5.2) from the acquired spectra using the procedure presented in Chapter 4.

For the bacterial and fungal materials no N-alkyl, O-alkyl and acetal carbon signals were observed using t_1 values >40 μs. As a result only T_{2A}' and I_A^o values could be calculated for these types of carbon and the proportion of each type of carbon contributed by CH_2 and CH carbon was calculated to be 100 percent indicating that these signals were dominated by CH_2 and CH carbon. For the carboxyl and phenolic carbon only T_{2B}' and I_B^o values were calculated since their chemical structure precludes the existence

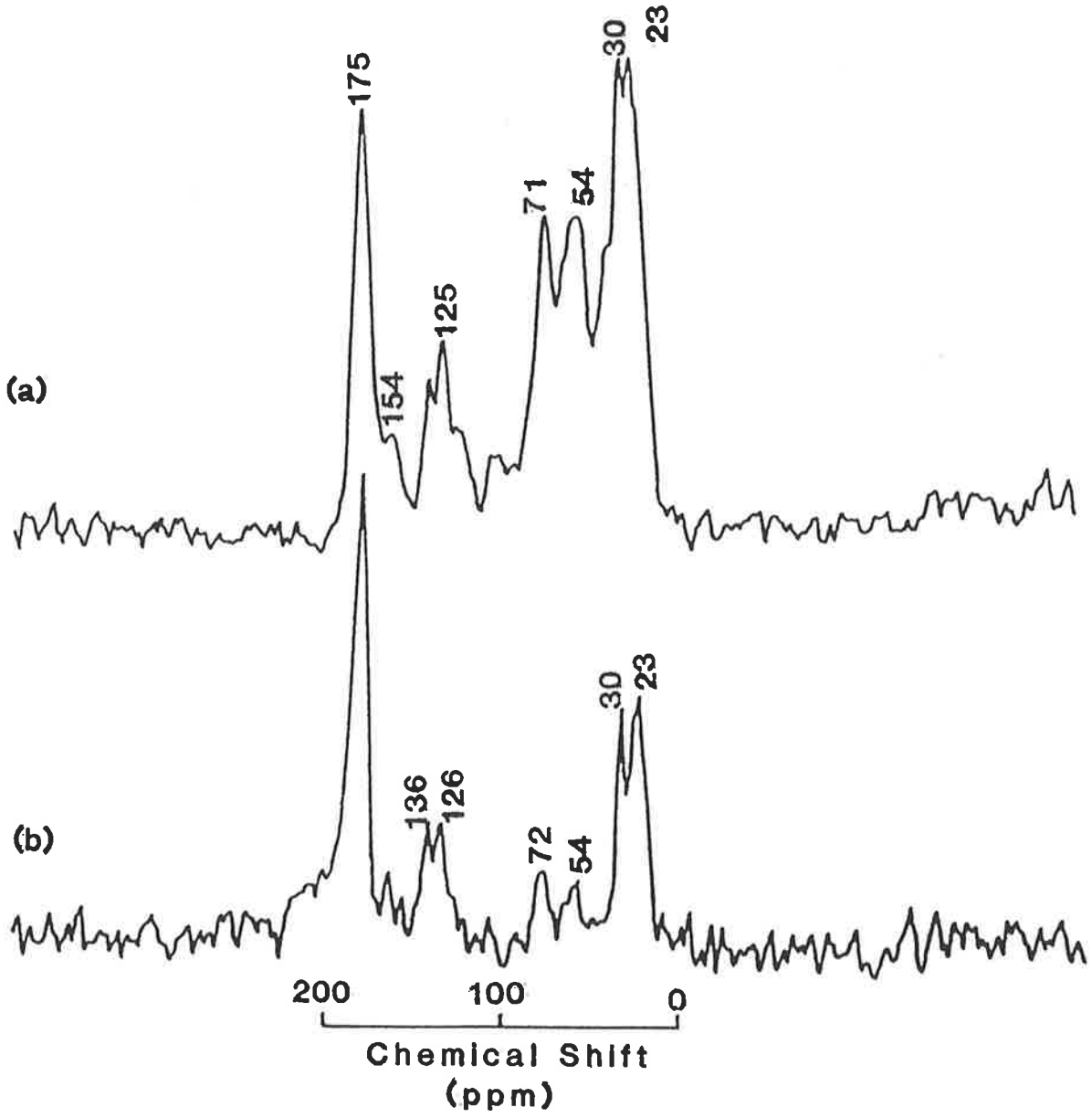

Figure 5.3: The conventional (a) and dipolar dephased (b) CP/MAS ^{13}C NMR spectra acquired for the bacterial materials using a 1.0 ms contact time and a 3.0 s recycle delay time. The dipolar dephasing time used was 40 μs. Line broadening was 20 Hz and 5000 transients were collected.

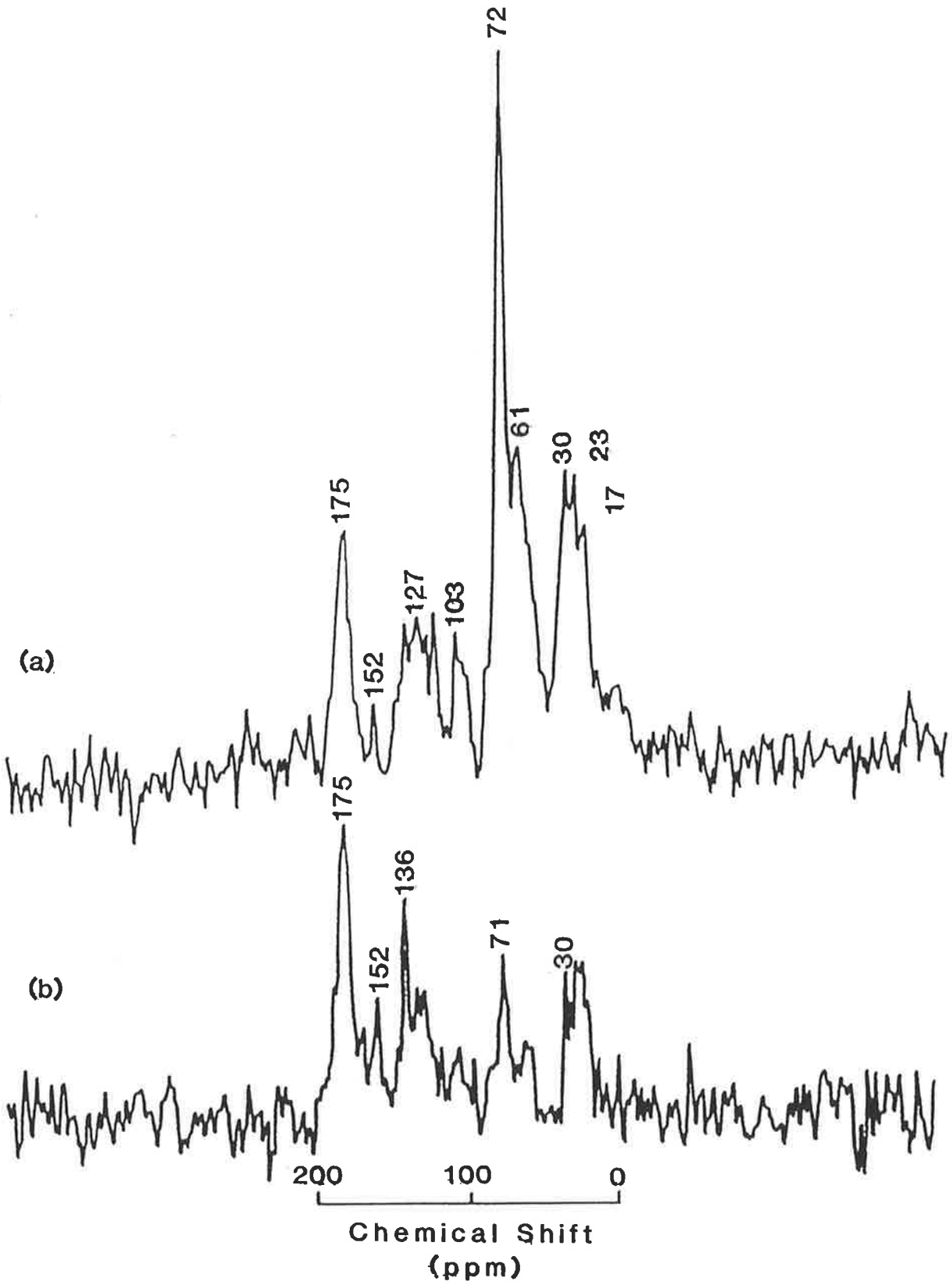

Figure 5.4: The conventional (a) and dipolar dephased (b) CP/MAS ^{13}C NMR spectra acquired for the fungal materials using a 1.0 ms contact time and a 3.0 s recycle delay time. The dipolar dephasing time used was 40 μs. Line broadening was 20 Hz and 5000 transients were collected.

of CH_2 and CH carbon. The I_A^o and I_B^o values calculated for the aromatic signals indicated that that the chemical structure of the aromatic materials synthesised by the bacteria and fungi differed since 66% of the aromatic carbon in the bacterial materials was protonated as apposed to only 31% in the fungal materials. For the alkyl carbon, the I_A^o and I_B^o values indicated that 66 and 13% was contained in CH_2 and CH structures in the bacterial and fungal materials, respectively. The exceptionally low value obtained for the

Table 5.2: Rates of signal decay, T_2', during the dipolar dephasing time, t_1, and the proportion of CH_2 and CH carbon contained in each type of carbon in the bacterial and fungal materials.

Sample	Type of Carbon	T_{2A}' [1] (μs)	T_{2B}' [1] (μs)	Proportion of CH_2 [2] and CH carbon (%)
Bacteria	Alkyl	21	111	66
	N-Alkyl	20		100
	O-Alkyl	21		100
	Acetal	25		100
	Aromatic	24	178	66
	Phenolic		154	0
	Carboxyl		115	0
Fungi	Alkyl	40	83	13
	N-Alkyl	53		100
	O-Alkyl	51		100
	Acetal	49		100
	Aromatic	51	130	31
	Phenolic		55	0
	Carboxyl		108	0

All R^2 values and probabilities associated with the regression analyses used to calculate the T_{2A}' and T_{2B}' values were ≥ 0.89 and <0.05, respectively.

[1] The absence of a value for T_{2A}' or T_{2B}' indicates that no fast or slow relaxing carbon was observed in the acquired spectra, respectively.

[2] The proportion of the total signal derived from CH_2 and CH carbon for the alkyl and aromatic carbon was calculated using the I_A^o and I_B^o values obtained from the plots used to values in the formula $[I_A^o/(I_A^o+I_B^o)]$. For the remaining types of carbon, CH_2 and CH carbon either accounted for all or none of the signal intensity as described in the text.

fungal materials was suspected to result from molecular motion since the alkyl peak in the dipolar dephased spectra was centred around 30 ppm which is indicative of CH_2 carbon. In dipolar dephasing experiments molecular motion reduces the rate of signal decay by weakening the ^{1}H-^{13}C dipolar interactions such that CH_2 and CH carbon contained in structures experiencing molecular motion would still be observed after t_1 values >40 μs. The influence of molecular motion on dipolar dephasing behaviour of protonated carbon is exemplified by that of CH_3 carbon. Although highly protonated, signals originating from CH_3 carbon decay more slowly than CH_2 and CH during t_1 due to its rapid rotational motion.

Conventional Experiments

The peaks observed in the vicinity of 30, 60, 72, 105, 129, 155 and 175 ppm have been ascribed to alkyl, N-alkyl, O-alkyl, acetal, aromatic, phenolic and carboxyl carbon, respectively. With the exception of the N-alkyl carbon, the chemical shift values of each type of carbon in the bacterial and fungal materials were similar.

The alkyl carbon resonances extended from 10 to 45 ppm and in the bacterial materials consisted of at least two components as indicated by the peaks at 30 and 23 ppm. In the fungal materials at least one additional type of alkyl carbon was also present as indicated by the peak at 17 ppm. Many different types of alkyl structures resonate within the 10-45 ppm range of chemical shift (Duncan 1987). However, the observed chemical shift values were similar to those reported by VanderHart (1976) for the terminal CH_3 carbon, the CH_2 carbon adjacent to the terminal CH_3 carbon and the $-(CH_2)_n-$ carbon in the alkane n-eicosane, CH_3-CH_2-$(CH_2)_{16}$-CH_2-CH_3. The presence of $-(CH_2)_n$-CH_2-CH_3 structures in the alkyl carbon was therefore likely. The synthesis of the fatty acid and phospholipid structures associated with microbial cell membranes could account for the presence of these structures.

The different dipolar dephasing behaviour of the alkyl carbon in the bacterial and fungal materials indicated that the chemical structure of the alkyl carbon synthesised by soil bacteria and fungi differed.

The N-alkyl region of the spectra acquired for the bacterial and fungal materials was dominated by peaks at 54 and 61 ppm, respectively. In addition to N-alkyl carbon methoxyl carbon also resonates in this region of chemical shift (Duncan 1987). However, the dipolar dephasing behaviour of the N-alkyl carbon indicated that it was dominated by either CH_2 or CH carbon structures and not the CH_3 carbon in methoxyl structures. Since the carbon adjacent to the amine group in amino acids has a CH_2 or CH structure it was suspected that these peaks were derived from proteinaceous materials. The small difference in the chemical shift values of the N-alkyl resonances indicated that the nature of the dominant proteins synthesised by the bacterial and fungal cultures was different. In the CP/MAS ^{13}C NMR spectra of the soil fractions presented in Chapter 4 two resonances with chemical shift values of 57 and 63 were observed in the N-alkyl region. If these resonances were derived from bacterial and fungal proteins, as is suggested when the chemical shift values are compared with those obtained in this study, the resonances in the N-alkyl region may be used to determine the relative contribution of bacterial and fungal carbon to the organic materials in soils and soil fractions. However, the CP/MAS ^{13}C NMR spectra of many more soil microorganisms would have to be collected to establish the validity of this statement.

The O-alkyl resonance in both cultures was centred at 72 ppm indicating a similarity in the chemical structure of the O-alkyl materials synthesised by the bacteria and fungi. This resonance was suspected to arise mainly from oxygenated carbon contained in the R_2CH-OH structures of carbohydrates (Duncan 1987). The presence of dioxygenated carbon indicated by the resonances at approximately 103 ppm suggested that at least a portion of the carbohydrate carbon was linked together into polysaccharides.

The dipolar dephasing behaviour of the dioxygenated carbon indicated that the majority of it was protonated and originated from acetal as opposed to ketal structures. The combination of O-alkyl and acetal carbon suggested the presence of cellulosic materials (Oades et al. 1987).

The resonances associated with the aromatic, phenolic and carboxyl carbon appeared at similar chemical shift values for the bacterial and fungal materials suggesting that the chemical structure of these types of carbon in the two cultures were not different. However, the dipolar dephasing behaviour of the aromatic carbon detected in the two cultures was different (Table 5.2) and indicated that the aromatic carbon synthesised by the soil fungi was either less protonated than that of the soil bacteria or it was contained in structures experiencing a higher degree of molecular motion. The resonances at 152 and 136 ppm became clearly visible in the dipolar dephased spectrum of the fungi. The former resonance is from C-1 carbon in phenolic materials but the origin of the latter is unknown, although substituted heterocyclic carbon is a possibility.

The conventional CP/MAS ^{13}C NMR spectra acquired for whole algal cells (Zelibor et al. 1988) and for bacterial spores (Lundin and Sacks 1988) are presented in Figure 5.5 for comparative purposes. The spectra acquired by Zelibor et al. (1988) for the algal cells were virtually identical to that obtained for the fungal materials in this study indicating a similarly in the chemical composition of the organic materials synthesised by both organisms. The spectra acquired for the bacterial spores by Lundin and Sacks (1988) closely resembled that acquired for the bacterial culture in this study. The chemical nature of the organic materials synthesised by the soil microorganisms therefore appears to depend on the type of soil organism involved in the decomposition of organic substrates in the soil.

The proportion of each type of carbon in the bacterial and fungal materials expressed as a percentage of the total carbon detected in the

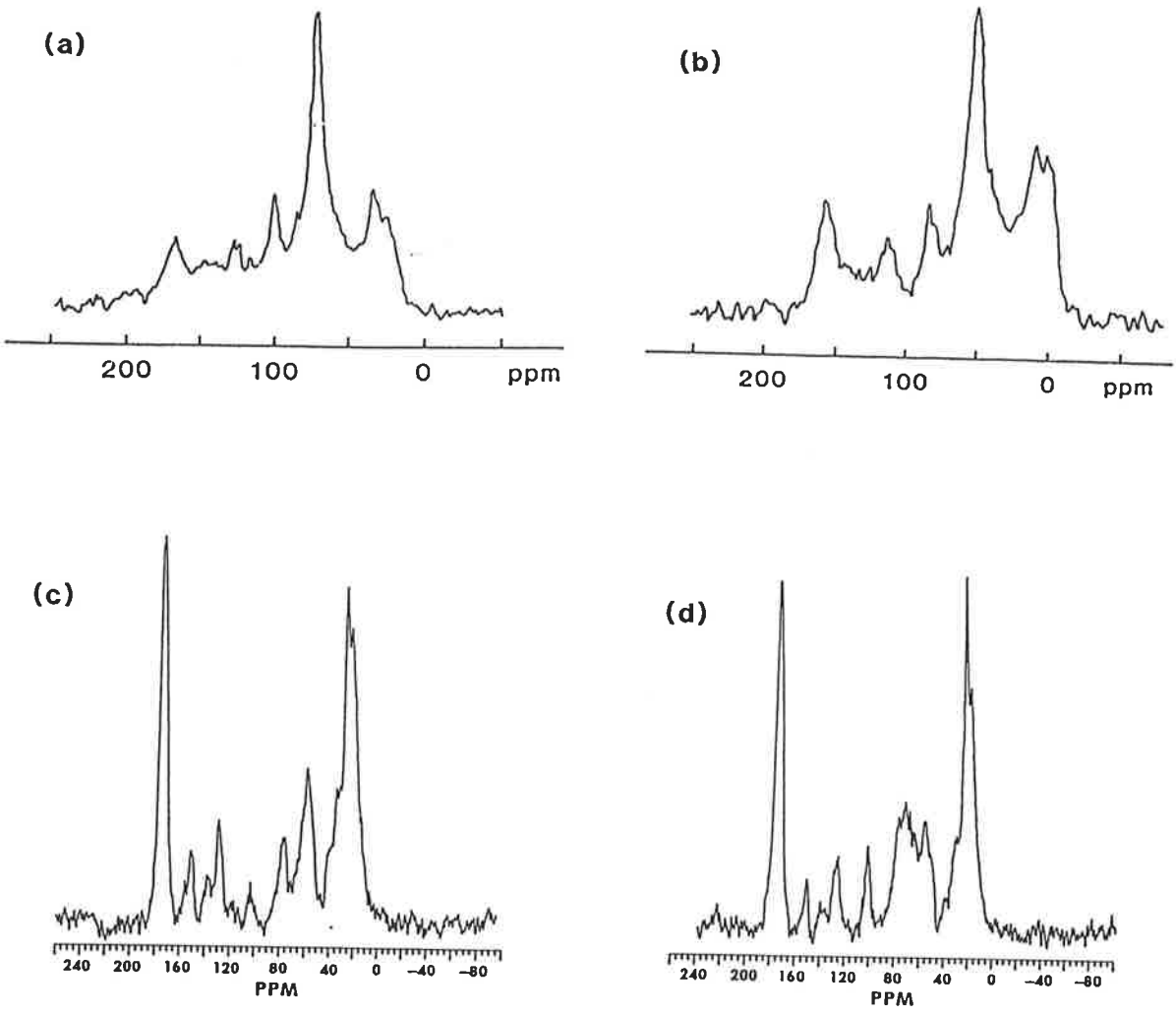

Figure 5.5: Conventional CP/MAS ^{13}C NMR spectra acquired for (a) whole cells of a mixed algal culture (*Chlorella* sp., *Chlamydomonas* sp., *Closterium* sp. and *Scenedesmus* sp.) (Zelibor et al. 1988), (b) whole cells of the filamentous algae *Zygnema* sp. (Zelibor et al. 1988) and bacterial spores isolated from (c) *Bacillus coagulans* and (d) *Bacillus macerans* (Lundin and Sacks 1988).

conventional spectra is presented in Figure 5.6a. All values presented in Figure 5.6a have been corrected for any signal decay which occurred during the contact time used in the pulse sequence (ie. due to $T_1\rho H$ effects). The major differences in the chemical composition of the bacterial and fungal materials were that the bacterial materials contained more alkyl and carboxyl carbon but less O-alkyl and acetal carbon than the fungal materials. The larger proportion of O-alkyl and acetal carbon in the fungal materials suggested that the fungi contained more polysaccharide materials than the bacteria. Although the chemical shift values of the N-alkyl carbon synthesised by the two cultures were different, the proportion of N-alkyl carbon synthesised was not. Aromatic and phenolic carbon were synthesised by both cultures with the proportion synthesised by the bacteria being greater than that synthesised by the fungi.

In Chapter 4 conventional CP/MAS ^{13}C NMR spectra were acquired for the clay (≤2 μm diameter particles) and light (>2 μm diameter particles with a density of ≤2.00 Mg m^{-3}) fractions separated from the Meadows fine sandy loam incubated for 34 days with uniformly labelled ^{13}C-glucose. Carbon balance measurements allowed the proportion of each type of carbon in both the residual substrate ^{13}C (Figure 5.6b) and the native organic carbon (Figure 5.6c) in the fractions to be calculated.

The composition of the residual substrate ^{13}C was suspected to reflect the chemical structure of the organic materials synthesised by the soil microorganisms utilising the added ^{13}C-glucose carbon. When compared to the data collected for the bacterial and fungal materials, the composition of the residual substrate ^{13}C in the clay fraction more closely resembled that of the fungal than bacterial carbon. This observation, when considered in conjunction with the fact that 73% of the recovered residual substrate ^{13}C was contained in the clay fraction, indicated that the glucose added to the Meadows soil was utilised predominantly by the fungi. The dominance of

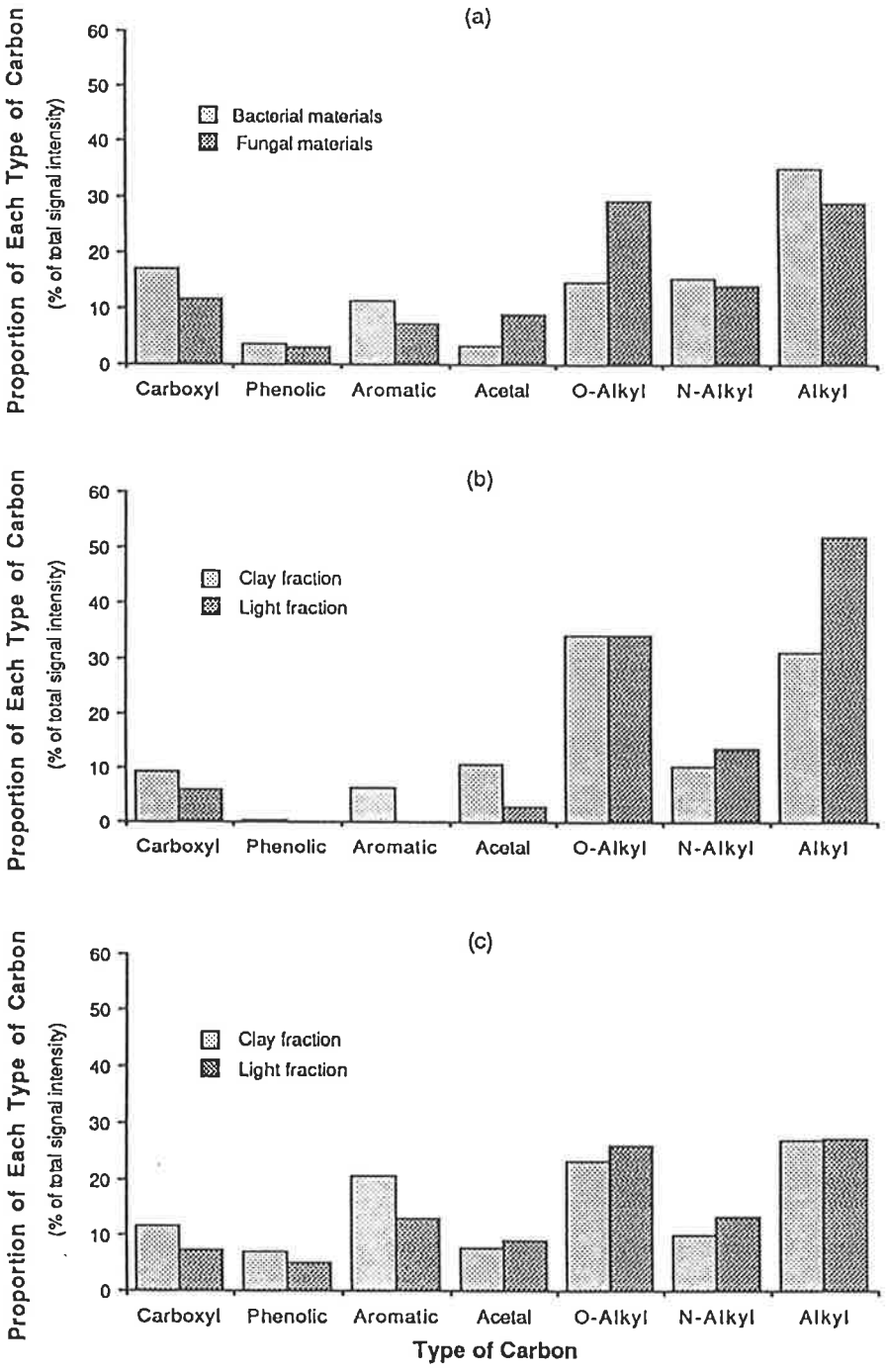

Figure 5.6: The chemical composition of the organic carbon synthesised by the bacteria and fungi isolated from the Meadows soil incubated with glucose (a) and the residual substrate ^{13}C (b) and the native soil organic carbon (c) contained in the clay and light fractions isolated from the Meadows soil incubated with uniformly labelled ^{13}C-glucose for 34 days.

fungi in this soil was also evident from the similarity in the composition of the native organic carbon in the clay and light fractions to that of the fungi. The similar nature of soil organic carbon and the organic materials synthesised by fungi has also been suggested by Gonzalez-Vila et al. (1978) and Lüdeman et al. (1982) from comparisons between the ^{13}C NMR spectra of fungal melanins and the humic acids extracted from soil.

The chemical composition of the residual substrate carbon contained in the light fraction was quite different from that of either the bacterial or fungal carbon. The absence of any aromatic carbon appears questionable as aromatic carbon was synthesised by both the soil bacteria and fungi.

Conclusions

The conventional CP/MAS ^{13}C NMR spectra acquired for the soil bacteria and fungi isolated from the Meadows fine sandy loam indicated that both microorganisms synthesised organic materials containing alkyl, O-alkyl, N-alkyl, acetal, aromatic, phenolic, and carboxyl carbon. To quantitatively determine the distribution of each type of carbon in the bacterial and fungal materials a recycle delay time of 3.0 s was required and the acquired signal intensities had to be corrected for the signal decay which occurred during the contact time ($T_1\rho H$ effects). The bacterial materials contained more alkyl, aromatic, and carboxyl carbon but less O-alkyl and acetal carbon than the fungal materials. The proportion of N-alkyl and phenolic carbon synthesised by both cultures was similar.

Comparison of the composition of the bacterial and fungal carbon to that of the native and residual substrate carbon contained in the clay and light fraction of a sample of the Meadows soil incubated with ^{13}C-glucose indicated that the soil microbial biomass was dominated by fungi.

Chapter 6

Influence of Gypsum Addition on the Mineralisation of Carbon Originating from Uniformly Labelled ^{14}C-glucose in 9 South Australian Red-brown Earths

6.1 Introduction

In Chapter 3, contrary to previous evidence presented by Muneer and Oades (1989a,b), the addition of gypsum to a soil amended with an organic substrate had only a small influence on the amount of substrate carbon mineralised to carbon dioxide. The studies reviewed in Chapter 1 combined with the results of Chapter 2 suggested that the decreased mineralisation of substrate carbon noted by Muneer and Oades (1989a,b) resulted from the formation of calcium-organic complexes, presumably with carboxylic functional groups located on organic materials synthesised by the soil microbial biomass. If the decreased mineralisation of the substrate carbon noted by Muneer and Oades (1989a,b) resulted solely from the formation of calcium-organic complexes, a similar observation would be expected in subsequent experiments irrespective of soil type. The decreased ability of gypsum to stabilise glucose carbon against microbial attack in the Meadows fine sandy loam relative to that observed by Muneer and Oades (1989a,b) for the Urrbrae fine sandy loam suggested that soil properties are also important to the biological stabilisation of organic carbon in calcium amended soils.

When the properties of the Meadows soil were compared to those of the Urrbrae soil, the most outstanding difference between the two soils was the content and mineralogy of the clay fractions. The clay content of the Urrbrae fine sandy loam was 16% and contained approximately 60% illite and 40% kaolinite. The clay content of the Meadows fine sandy loam was 9% and was dominated by coarse grained kaolinite. The Urrbrae soil therefore had a higher content of clay minerals with more reactive surfaces. Based on these

observations it was postulated that the decreased mineralisation of substrate carbon in the presence of gypsum resulted from the formation of clay-Ca^{2+}-organic complexes in which the Ca^{2+} operate as a bridge binding the organic materials to the clay surfaces. Such a mechanism would also account for the ability of calcium additions to decrease the amount of water soluble organic carbon (Sokoloff 1938).

The ability of Ca^{2+} to bind organic materials to mineral surfaces in soils was proposed in the early literature (Peterson 1947). The mechanism(s) involved are assumed to be similar to those proposed by Edwards and Bremner (1967) for the formation of clay-polyvalent cation-organic matter complexes.

The objectives of this study were to determine the influence of adding gypsum to 9 Red-brown earths having clay contents ranging from 3 to 29% and amended with uniformly labelled ^{14}C-glucose on:

1) the mineralisation of glucose ^{14}C and native soil organic carbon, and
2) the amount of water soluble residual glucose ^{14}C and native soil organic carbon present in the soils after 8 weeks incubation.

6.2 Materials and Methods

6.2.1 Soils

Nine soils were selected from a series of 40 Red-brown earths collected by Wegener et al. (1989) from the lower- to mid-north region of South Australia. All samples were collected from the surface 0.10 m of the soil, oven dried at 30°C and passed through a 2 mm sieve. Selection of the nine soils used in this study was based on soil pH values and clay contents. It was essential that all the soils selected had a slightly acidic pH values so that accurate measurements of the amount of glucose ^{14}C mineralised during the incubation period could be obtained. Under these pH conditions, the collection of erroneous results due to the precipitation of carbonate species in

the soil matrix would be unlikely. Once the soils with a slightly acidic pH were identified, the required nine soils were selected such that the range of soil clay contents was maximised. The location and some relevant physical and chemical properties of the soils used in this study are presented in Table 6.1.

6.2.2 Sample Preparation and Incubation

For each of the nine soils, 2 bulk samples containing 200 g (oven dry equivalent weight) of air dried soil were prepared. Gypsum (AR grade $CaSO_4$ $2H_2O$) was added to one of the bulk samples from each soil at a rate of 0.5% by weight (5 t/ha assuming a bulk density of 1.0 Mg m^{-3} and a 0.10 m depth of incorporation). The bulk samples receiving no gypsum will be referred to as the control samples and those receiving gypsum as the treated samples.

The control and treated bulk samples were amended with 11.0 g of a ^{14}C-glucose solution prepared by adding 37 mBq of uniformly labelled glucose and 200 g of natural glucose to 240 cm^3 of deionised water. To each 200 g bulk sample, 11.00 g of the glucose solution was added which corresponded to an addition of 25 mg glucose carbon g^{-1} soil (1% glucose carbon by weight) and 4.62 kBq ^{14}C g^{-1} soil. An amount of deionised water, in addition to that added in the ^{14}C-glucose solution, was added to each bulk sample to bring their soil water matric potentials to -35 kPa. The wetted bulk samples were thoroughly mixed and left for 16 hours at 4°C to equilibrate.

The gravimetric water content of each soil at -35kPa matric potential and the initial osmotic pressure of the soil solution in the glucose amended samples are presented in Table 6.2. Although the osmotic pressure of the soil solutions was so high at the beginning of the incubation that the activity of soil microorganisms would be limited, this problem was expected to exist only during the early stages of the incubation before most of the glucose was

Table 6.1: The location and some relevant physical and chemical properties of the soils used in this study.

Soil Number	Location (by hundred)	Soil pH [a] Water	Soil pH [a] 0.01M $CaCl_2$	Clay Content [b] (% by weight)	Surface Area [c] (m^2 g^{-1} soil)	Organic Carbon [d] (% by weight)	Total Nitrogen [e] (% by weight)
1	Caltowie	6.4	5.7	7	17	0.88	0.08
2	Gilbert	5.9	5.0	10	13	0.94	0.10
3	Dalkey	6.6	6.0	12	25	1.02	0.09
4	Grace	6.1	5.2	14	15	1.05	0.11
5	Grace	6.4	5.6	16	25	1.16	0.11
6	Kapunda	6.3	5.5	19	22	1.24	0.13
7	Gilbert	6.2	5.3	22	24	2.50	0.24
8	Gilbert	6.5	6.0	27	43	1.83	0.18
9	Gilbert	6.4	5.6	29	23	1.89	0.20

[a] Determined using a 1:5 soil to solution ratio. Measurements were made on the clear supernatant after soil particles had settled from solution.

[b] Determined by Wegener et al. (1989) using a plummet balance technique described by Heanes (1981).

[c] Determined as the surface area available to nitrogen.

[d] Determined using a LECO CR-12 carbon analyser

[e] Determined by Wegener et al. (1989) using a micro Kjeldahl digestion procedure (Heanes 1981).

Table 6.2: The gravimetric water content of the soils at -35 kPa matric potential and the calculated osmotic pressures of the soil solutions in the glucose amended soils.

Soil	Gravimetric Water Content [a] (%)	Osmotic Pressure [b] (kPa)
1	13.0	2826
2	14.6	2421
3	13.3	2887
4	14.2	2593
5	15.0	2543
6	17.7	2097
7	21.9	1712
8	23.0	1692
9	22.2	1662

[a] Determined using a pressure plate apparatus.

[b] Calculated using the equation:

$$\Pi v = -RT \ln x_A$$

in which Π is the osmotic pressure, v is the volume occupied by one mole of solvent, R is the gas constant, T is the temperature in °K and x_A is the mole fraction of the solvent. In using this equation it was assumed that the glucose added to the samples was uniformly distributed through out the soil solution and that the soil solution behaved ideally.

utilised. By allowing the incubation to proceed for eight weeks any initial limitations should be overcome.

Five replicate samples were prepared for incubation from each bulk sample by adding the equivalent of approximately 30.0 g on an oven dry weight basis of the bulk soil to sterilised 75 cm^3 plastic containers. Each incubation sample was sealed in a 1 dm^3 glass food jar containing 5.0 cm^3 of deionised water and incubated for 57 days at room temperature (20-30°C). The deionised water was added to the jars to minimise evaporative drying of the soil surface during the incubation period. The jars were opened daily for

15 minutes to reduce the potential of the samples becoming anaerobic. Once the samples were removed from the incubation they were oven dried at 50°C and homogenised by grinding with a mortar and pestle.

6.2.3 Analyses

Carbon Contents of the Bulk and Incubated Samples

None of the soils used in this study contained significant amounts of inorganic carbon. Therefore all references made to the carbon contents of the soils or samples refer to organic carbon contents.

The total carbon content, expressed as a percentage by weight of soil, was determined for the unincubated bulk samples and the incubated samples using a LECO CR-12 carbon analyser. The weight percentage of total carbon, %C, was converted into a total carbon content, C_T, expressed in mmol C g^{-1} soil, using equation [6.1] in which MW_C is the molecular weight of carbon.

$$C_T = \left(\frac{\%\,C}{MW_C}\right) 10 \qquad [6.1]$$

The ^{14}C content of the unincubated bulk samples and the incubated samples was determined by the method of Amato (1983). The carbon in the samples was converted to carbon dioxide by heating in a digestion mixture (Dalal 1979) and trapped in 5 cm^3 of 2 M NaOH. The amount of ^{14}C trapped in the 2 M NaOH was determined by liquid scintillation counting using a Beckman Model LS 3801 Scintillation Counter. All samples were removed from light for at least 36 hours to minimise any problems due to chemilumescence. Background interference was assessed by the inclusion of blanks in each set of samples analysed and the counting efficiency was established by adding LKB internal standards to the counting mixture. The measured radioactivity was corrected for both background interference and counting efficiency. The content of ^{14}C in the samples was calculated in units

of dpm g^{-1} soil. Using the ratio, R, of the contents of natural and labelled glucose carbon (expressed in mmol C cm^{-3} and dpm ^{14}C cm^{-3}, respectively) in the glucose solution used to amend the soils, the content of substrate carbon, C_S, expressed in mmol C g^{-1} soil was calculated from the measured ^{14}C content of the samples (equation [6.2]).

$$C_S = (^{14}C \text{ content}) \, R \qquad [6.2]$$

The content of native carbon, C_N, expressed in units of mmol C g^{-1} soil in the samples was calculated as the difference between the total carbon and substrate carbon contents (equation [6.3]).

$$C_N = C_T - C_S \qquad [6.3]$$

The influence of gypsum addition on the mineralisation of substrate ^{14}C and native soil carbon was determined by expressing the residual carbon contents of the incubated samples as a percentage of their corresponding initial contents obtained from the unincubated bulk samples.

Water Soluble Carbon

The amount of total water soluble carbon and substrate ^{14}C released from the incubated samples in a 1:5 soil to deionised water extract was determined. The equivalent of 5.00 g on an oven dry weight basis of the incubated samples was added to 25 ml of deionised water and shaken on an end-over-end shaker for 10 min at 13.5 rpm. The suspensions were centrifuged immediately after shaking and the clear supernatant collected. Particulate organic materials floating on and in the supernatant were removed by filtration. The total carbon content of the supernatant was determined by measuring the dissolved organic carbon (DOC) contents using the procedure

outlined by Hine and Bursil (1985). The amount of water soluble ^{14}C was determined by liquid scintillation counting. Using a similar series of calculations to those outlined above, the proportion of the native and substrate carbon in the incubated samples which was water soluble was calculated.

The electrical conductivity and concentrations of Ca^{2+} and Na^{+} in the supernatant collected from 1:5 extracts were also determined. Electrical conductivity measurements were made using a Radiometer CDM 2e conductivity meter and the concentrations of Ca^{2+} and Na^{+} were measured by atomic absorption spectroscopy and flame ionisation photometry, respectively.

6.2.4 Experimental Design

The influence of gypsum on each of the parameters measured in this study was assessed using an unpaired two tailed t-test to compare the means of the control and treated samples from each soil. Least significant difference, LSD, values at a 5% level of significance were calculated for each comparison. In the presentation of the results (Figures 6.1-6.3), the LSD values are shown by the error bars associated with the treated sample data and the probability of accepting the hypothesis that no difference existed between the treatment means is given above the data used in each t-test. For the electrical conductivity and calcium concentration data presented in Figure 6.3, no probability values were placed on the data since all the t-tests were significant at a level ≤ 0.01.

6.3 Results and Discussion

A detailed presentation of the data collected in this study is given on the computer disk located on the back cover of the thesis.

6.3.1 Influence of Gypsum Addition on the Mineralisation of Substrate and Native Carbon.

The residual substrate carbon contents in the control and treated

samples of the nine soils varied between 19% and 42% of that initially present before incubation (Figure 6.1a). The large loss of substrate carbon from the soils during the incubation period indicated that the microbial population was able to overcome the initial osmotic pressure of the soil solution and utilise the added substrate carbon.

Although no statistical comparisons were made between the different soils, as the clay content of the samples increased (ie. in progressing from soil 1 to 9) the residual substrate carbon content tended to decrease. The observation of a decrease in residual substrate carbon with increasing clay content was contrary to what was expected based on results presented by Van Veen et al. (1985) and Oades (1988). By incubating ^{14}C-glucose in a sandy loam and clay soil having 12% and 42% clay, respectively, Van Veen et al. (1985) noted that although the clay soil contained more biomass early in the incubation, the mineralisation of glucose ^{14}C was reduced relative to the sandy loam. Oades (1988) indicated that the loss of organic carbon from soils receiving no organic inputs for 8 years decreased as the amount of clay increased. The decline in residual substrate carbon with increasing clay content was suspected to result from a greater inhibition of microbial activity in the low clay content soils with a higher osmotic pressure early in the incubation.

The influence of gypsum addition on the amount of substrate carbon mineralised from the nine soils, as indicated by their residual substrate carbon contents, was variable (Figure 6.1a). For soils 1, 3, 4 and 9 the content of residual substrate carbon was increased significantly by 5.5, 25.0, 6.2 and 3.2 percent, respectively. As indicated by the probability values shown in Figure 6.1a, the differences in the remaining soils were not significant.

The lack of any relationship between clay content and the reduction in mineralisation of substrate carbon induced by gypsum in the soils used in this

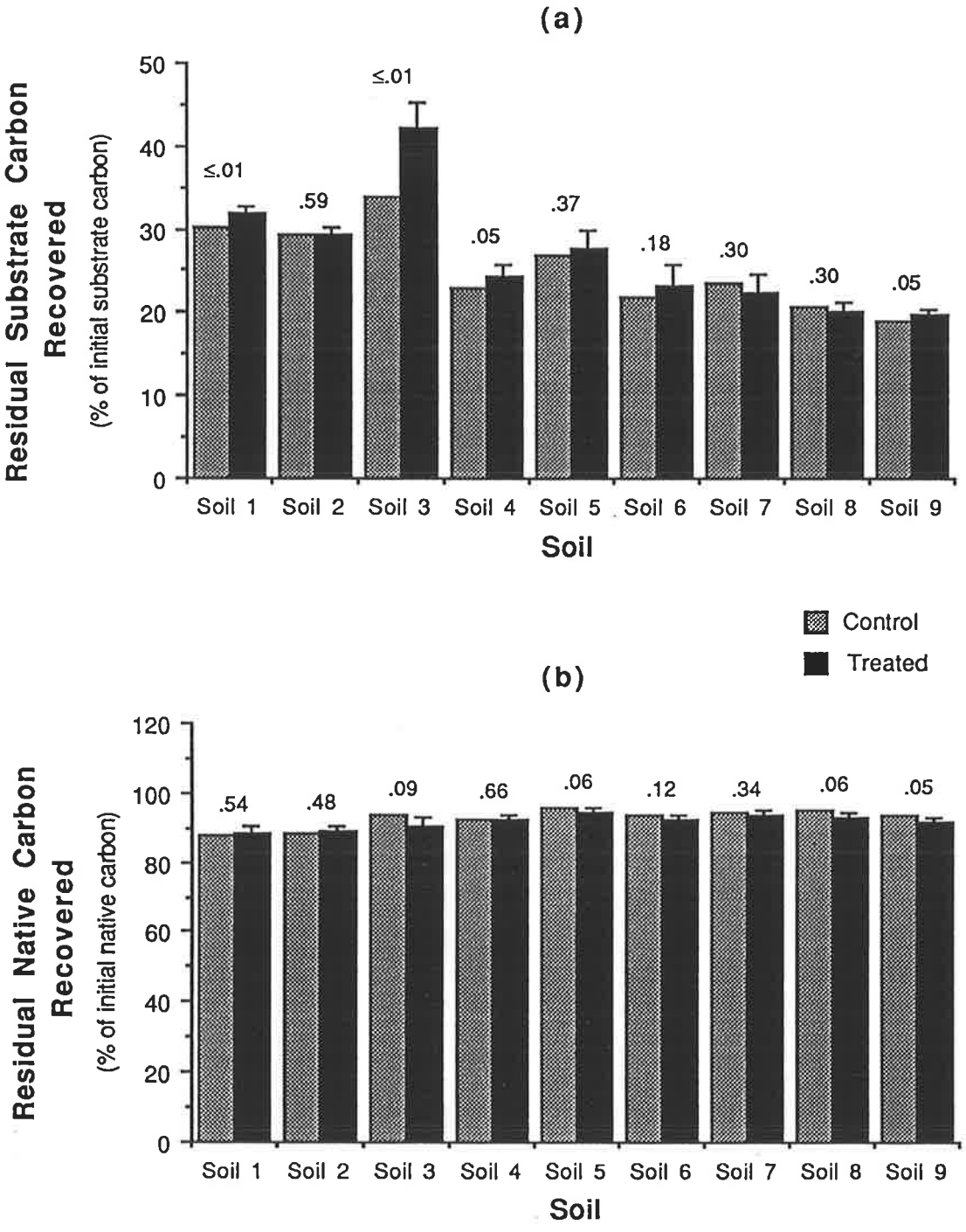

Figure 6.1: The residual substrate carbon (a) and residual native carbon (b) recovered from the control and treated samples of each soil after 57 days incubation.

study indicates that soil properties other than clay content may be involved in the stabilisation of organic carbon in soils by calcium. Since the formation of clay-Ca^{2+}-organic complexes requires the presence of exposed clay surfaces, it is possible that the microaggregation of clay particles in a soil may limit the available surface area. The surface area of the nine soils used in this study was determined by a nitrogen absorption method (Table 6.1). As with clay content, the influence of gypsum addition on the mineralisation of organic substrates in the soils was not related to the surface area of the soils.

The presence of gypsum did not significantly alter the mineralisation of native organic carbon in the nine soils (Figure 6.1b). However, the amount of native carbon mineralised was decreased by approximately 30 percent as the clay content of the soils increased from 7 to 29 percent by weight, supporting the possible role of clay in the stabilisation of native soil organic materials.

The different behaviour of the residual substrate ^{14}C and residual native carbon with increasing clay content may indicate that the mineralisation of substrate carbon occurred independently of soil properties. It is suggested that as a result of the high osmotic pressure of the soil solution a portion of the carbon assimilated by the microbial biomass did not come into contact with soil particles during the incubation. This suggestion is supported by the appearance of a mat of fungal hyphae over the surface of the samples during incubation. Since the substrate ^{14}C utilised by the fungi to synthesise these structures would not come into contact with the soil or calcium, it is possible that no influence of gypsum or soil properties would be observed on the mineralisation of the substrate carbon. In subsequent studies it is suggested that either insoluble organic compounds such as cellulose be used as the substrate or that glucose be added in small quantities sequentially. The inclusion of regular wetting and drying cycles with a thorough mixing of the samples after each drying cycle is also encouraged to ensure that a good contact is made between the residual substrate carbon and the soil.

6.3.2 Influence of Gypsum Addition on the Amount of Water Soluble Substrate and Native Carbon

The amount of water soluble substrate carbon extracted from the control and treated samples varied between 10 and 17 percent (Figure 6.2a). The addition of gypsum to the soils decreased the proportion of water soluble substrate carbon; however, the decreases were small and those associated with soils 4, 7 and 8 were not statistically significant. The decreased amount of water soluble residual substrate carbon in the gypsum amended soils suggested that a portion of the residual substrate carbon was rendered insoluble in the presence of Ca^{2+} . Whether the decreased solubility resulted from an influence of gypsum on the entrapment of substrate carbon within packets of coagulated soil clays or from the formation of insoluble Ca^{2+}-organic complexes could not be differentiated, but the lack of an increase in the effect of gypsum with increasing clay content supports the notion of the formation of insoluble Ca^{2+}-organic complexes.

The proportion of water soluble native organic carbon was small for all soils and varied between 0.4 and 3.2 percent (Figure 6.2b). The influence of gypsum on the proportion of water soluble native carbon was highly variable and inconsistent.

6.3.3 Influence of Gypsum on the Electrical Conductivity and Concentrations of Ca^{2+} and Na^{+} in the 1:5 Extracts

The influence of gypsum on the electrical conductivity of the nine soils used in this study is presented in Figure 6.3a. In all the soils gypsum addition resulted in a substantial and highly significant increase in the electrical conductivity of the 1:5 soil extracts. The concentration of Ca^{2+} and Na^{+} in the soil extracts were also measured to ensure that in the gypsum amended soils the dominant cation in solution was Ca^{2+}. The results shown in Figures 6.3b and 6.3c clearly demonstrate the dominance of Ca^{2+} in the gypsum amended soils.

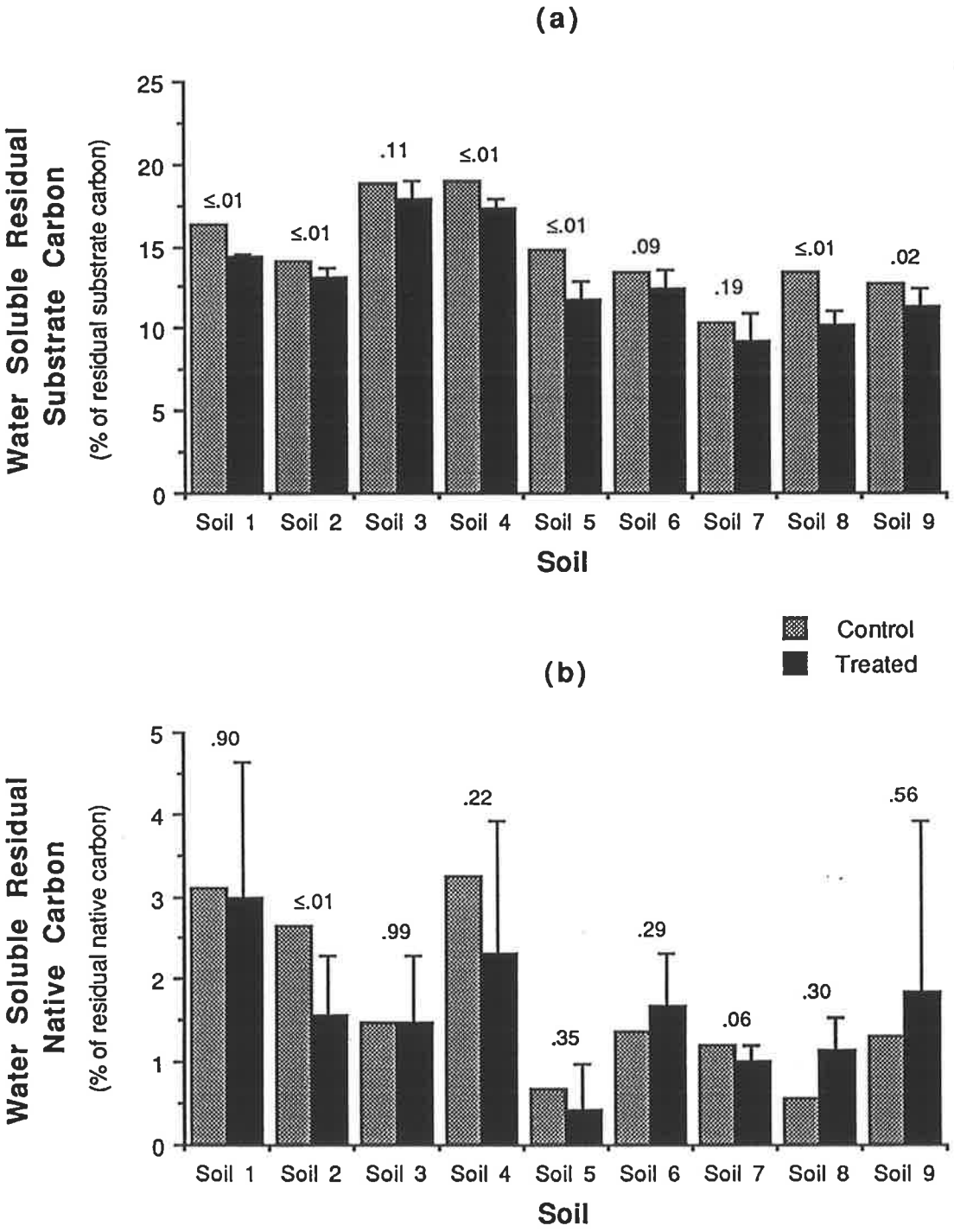

Figure 6.2: The proportion of the residual substrate carbon (a) and native soil carbon (b) soluble in the 1:5 soil to water extracts obtained from the control and treated samples of each soil.

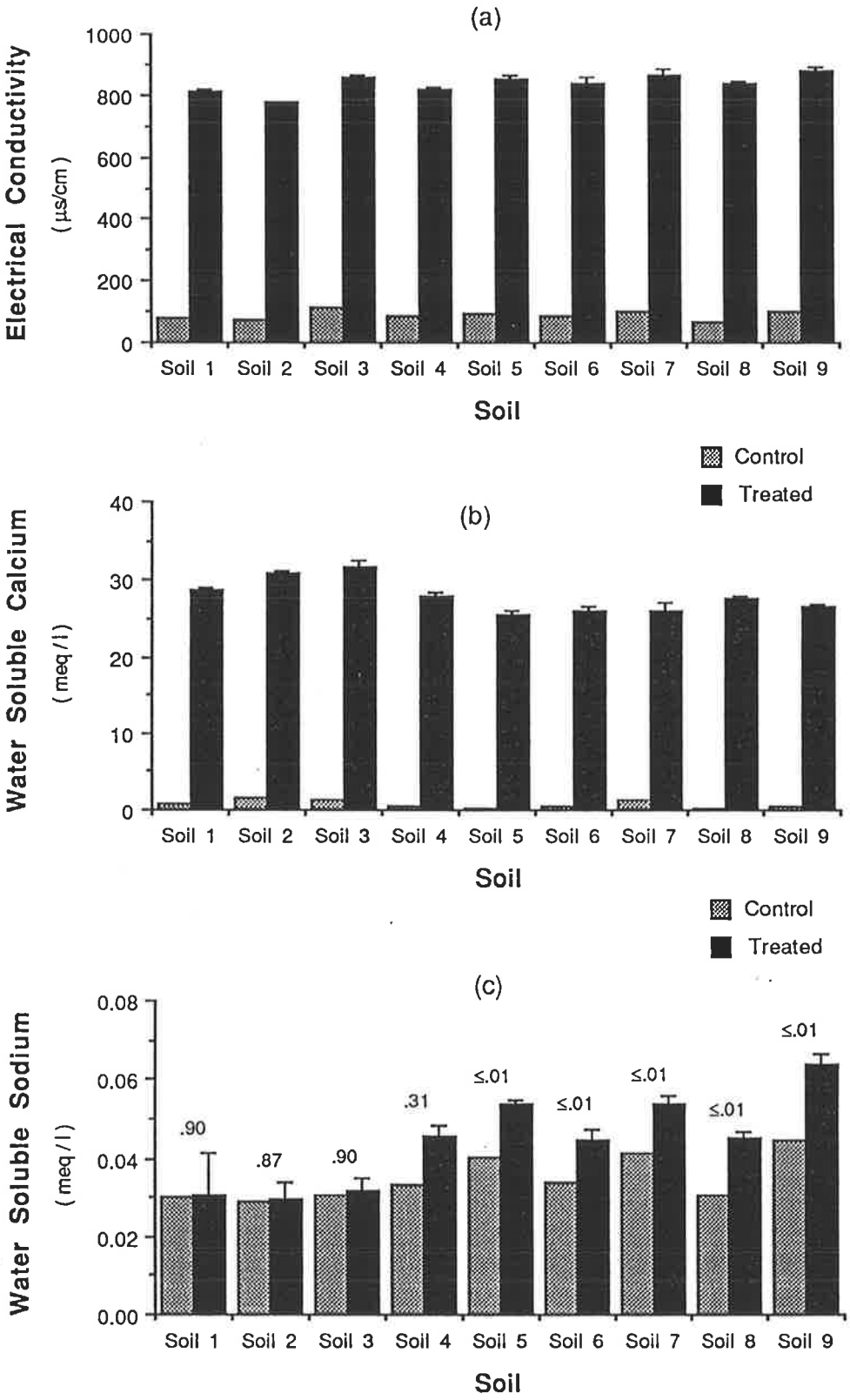

Figure 6.3: The electrical conductivity (a), water soluble Ca^{2+} concentration (b) and water soluble Na^{+} concentration (c) of the 1:5 soil to deionised water extracts obtained from the control and treated samples each soil.

extracts obtained from the control and treated samples each soil.

6.4 Conclusions

Gypsum was added to nine soils in this study to increase the concentration of Ca^{2+} in the soil solution in an attempt to encourage the formation of clay-Ca^{2+}-organic complexes and stabilise substrate carbon against microbial attack. A significant biological stabilisation of the substrate carbon was noted in four of the nine soils, with that noted for soil three being much larger than was observed previously by Muneer and Oades (1989a). The results demonstrated that the influence of calcium on the biological stability of substrate carbon in the soils varied and was not a function of either the clay contents or surface areas of the soils.

The significance of adding calcium to some soils to preserve organic carbon was clearly demonstrated by the magnitude of the biological stabilisation noted for soil three. However, further work is required to determine why an effect was not observed in all the soils tested before general recommendations can be made to the agricultural community.

Chapter 7

Summary and Conclusions

In this final section of the thesis the acquired results will be summarised and their implications discussed. The results pertaining to the influence of Ca^{2+} on the stabilisation of organic materials in soils and those pertaining to the solid state CP/MAS ^{13}C NMR spectroscopy experiments will be addressed separately since the addition of Ca^{2+} was not observed to influence the results of the CP/MAS ^{13}C NMR analyses.

7.1 Influence of Ca^{2+} on the Biological Stability of Organic Materials in Soil

The addition of calcium, as gypsum or agricultural lime, to the Urrbrae fine sandy loam was shown to decrease the extent of mineralisation of glucose carbon in a laboratory experiment (Muneer and Oades 1989a) and wheat straw carbon in a field experiment (Muneer and Oades 1989b). When these observations were considered collectively with the results reviewed in Chapter 1, an influence of Ca^{2+} on the biological stability of soil organic materials in soils became apparent. The stabilisation appeared to involve the formation of Ca^{2+}-organic complexes, presumably by an interaction of Ca^{2+} with the anionic carboxylate carbon contained in the materials synthesised by soil microorganisms. In addition to the formation of Ca^{2+}-organic complexes, biological stabilisation of organic materials induced by the addition of gypsum to soil may have been at least partially due to an inhibition of microbial activity in response to an increased electrolyte concentration.

The influence of electrolyte concentration on microbial activity was investigated in Chapter 2 by monitoring the rate of glucose decomposition in soil amended with increasing amounts of calcium chloride. At Ca^{2+} concentrations ≤ 0.05 M neither the rate nor the extent of glucose utilisation by

soil microorganisms was altered. The maximum electrolyte concentration expected in gypsum amended soils would be approximately 0.03 M (the solubility of gypsum). It was therefore unlikely that the decreased substrate mineralisation induced by the addition of gypsum resulted from an inhibition of microbial activity induced by either an osmotic effect or a direct influence of the Ca^{2+} released from gypsum on microbial activity.

In Chapter 3 a laboratory incubation experiment was completed in which uniformly labelled ^{13}C-glucose was incubated in the Meadows fine sandy loam in the presence and absence of gypsum for up to 34 days. The Meadows soil, which had different chemical and physical properties to those of the Urrbrae soil used by Muneerand Oades (1989),was used to assess the influence of soil type on the ability of gypsum to decrease the amount of carbon mineralised from an organic substrate. Adding gypsum to the Meadows soil decreased the amount of substrate ^{13}C mineralised but the magnitude of the decrease was small, much less than that previously observed. The ability of calcium, as gypsum, to biologically stabilise organic materials in soils therefore appeared to vary with soil type.

The indication that the ability of Ca^{2+} to decrease the proportion of substrate carbon mineralised varied with soil type was tested using nine Red-brown earths collected from the lower- to mid-north region of South Australia (Chapter 6). The clay content and surface areas of the nine soils ranged from 7 - 29% and 17 - 43 $m^2\ g^{-1}$ soil, respectively. All soils were wetted to a matric potential of -35 kPa and incubated with uniformly labelled ^{14}C-glucose in the presence and absence of gypsum. A significant influence of gypsum addition on the biological stabilisation of the glucose ^{14}C was noted in four of the nine soils and no relationship was observed between the magnitude of the decreased mineralisation and the clay content or surface area of the soils.

Although the mechanism for the decreased mineralisation of organic carbon from gypsum amended soils has not been identified in the research

presented in this study, the results have indicated that in certain soils the addition of calcium, as gypsum, can reduce the amount of carbon mineralised from an organic substrate.

Further work is required to determine why an effect was not observed in all the soils tested before general recommendations can be made to the agricultural community.

7.2 The Use of ^{13}C-labelled Organic Materials in Conjunction with Solid State CP/MAS ^{13}C NMR Spectroscopy to Study Carbon Dynamics in Soil.

CP/MAS ^{13}C NMR spectroscopy was used in this study to monitor the changes associated with the chemical structure of the glucose carbon as it was utilised by soil microorganisms and incorporated into the soil organic fraction. Both conventional and dipolar dephasing experiments were performed on the soil incubated with the labelled ^{13}C-glucose. Utilisation of the glucose by soil microorganisms resulted in the synthesis of alkyl, O-alkyl and carboxyl carbon. No significant resonances were observed in the aromatic region of the acquired spectra indicating that little if any aromatic carbon was synthesised by the microbial biomass utilising the glucose.

The largest changes in the chemical composition of the substrate ^{13}C occurred during the first 10 days of the incubation, which corresponded to the time when the largest change in the half-life of the substrate carbon was observed. Subsequent to day 10 as the mineralisation of the substrate continued, albeit at a much reduced rate, no significant changes in the chemical structure of the substrate carbon were noted. Whether the increased half-life of the substrate ^{13}C was a function of its changes in chemical structure or was due to its adsorption on soil particles remains questionable.

Since the ^{13}C content of each sample analysed by CP/MAS ^{13}C NMR was known the opportunity existed to assess the quantitativeness of the CP/MAS ^{13}C NMR analysis. The absence of a linear relationship between the

total signal intensity acquired and the amount of ^{13}C in the analysed samples indicated that the proportion of the total ^{13}C detected in the samples changed as the incubation progressed. Correcting the CP/MAS ^{13}C NMR results for $T_1\rho H$ effects during the contact time did not improve the relationship. It was postulated that the discrepancy between the acquired signal intensity and the ^{13}C content of the samples resulted from a portion of the ^{13}C being rendered NMR invisible, probably due to an association with paramagnetics.

The soil incubated for 34 days with and without the uniformly labelled ^{13}C-glucose was fractionated on the basis of particle size and density to determine the distribution of residual substrate ^{13}C and its chemical structure within the isolated fractions. The residual ^{13}C accumulated in the clay fraction (≤2 μm diameter particles) in the form of alkyl, O-alkyl and carboxyl carbon predominantly. There was some indication that aromatic carbon was also present in the clay fraction. Significant amounts of alkyl and O-alkyl carbon were also observed in the light (>2 μm diameter particles with a density ≤2.00 Mg m^{-3}) and heavy (>2 μm diameter particles with a density >2.00 Mg m^{-3}) fractions. The quality of the acquired solid state CP/MAS ^{13}C NMR spectra were significantly improved by the fractionation procedure and the acquisition time required was reduced by a factor of 7.

The solid state CP/MAS ^{13}C NMR spectra acquired for the soil incubated with the ^{13}C-glucose reflected the chemical nature of the organic materials synthesised by soil microorganisms from the added glucose. CP/MAS ^{13}C NMR spectra were therefore also acquired for a bacterial and a fungal culture isolated from the Meadows soil. The chemical composition of the organic materials synthesised by the isolated bacteria and fungi differed. The bacterial materials contained more alkyl, aromatic and carboxyl carbon and less carbohydrate and acetal carbon. Both cultures contained equivalent amounts of N-alkyl carbon, but the chemical shift values of the dominant peak differed. The chemical composition of the residual substrate carbon in the soil

resembled that of the fungal materials more closely than that of the bacterial materials. Therefore, glucose decomposition in the Meadows soil appeared to be dominated by the fungal biomass.

The results of this work demonstrate the potential of utilising ^{13}C-labelled organic substrates in conjunction with solid state CP/MAS ^{13}C NMR spectroscopy to gain an increased understanding of the dynamics of organic materials in soils.

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