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dissertation entitled

Mineralization, Immobilization, and ^{15}N-NMR Spectroscopy of Organic Nitrogen in Whole Soil and Particle-Size Fractions

presented by

Ralph John DiCosty

has been accepted towards fulfillment
of the requirements for

Ph.D degree in Crop and Soil Sciences

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Date 8/27/99

MINERALIZATION, IMMOBILAZATION AND ^{15}N-NMR SPECTROSCOPY OF ORGANIC NITROGEN IN WHOLE SOIL AND PARTICLE-SIZE FRACTIONS

By

Ralph John DiCosty

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Crop and Soil Sciences

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ABSTRACT

MINERALIZATION, IMMOBILAZATION AND ^{15}N-NMR SPECTROSCOPY OF ORGANIC NITROGEN IN WHOLE SOIL AND PARTICLE-SIZE FRACTIONS

By

Ralph John DiCosty

Knowledge of the mechanisms controlling nitrogen (N) turnover in soils is necessary to maximize agricultural production while minimizing environmental pollution. Important mechanisms include adsorption, aggregate protection, and humification. Humification has been linked to increases in heterocyclic N by some authors, but this is disputed by others who consider that soil N is largely proteinaceous. The objectives of this study were to (1) test the quantitativeness of ^{15}N-cross-polarization magic-angle spinning nuclear magnetic resonance (CPMAS-NMR), (2) identify organic N functional groups during clover decomposition, and (3) evaluate mechanisms of N stabilization. The CPMAS-NMR spectrum of a prepared, complex, soil-organic mixture was quantitative for both heterocyclic and noncyclic N. However, peak overlap was an important but not fatal weakness of this technique. A sandy loam soil was incubated for 14 months in the laboratory with ^{15}N-clover (*Trifolium pratense* L.) with periodic samples taken for ultrasonic particle-size fractionation followed by ^{15}N-CPMAS-NMR and a shaken slurry N mineralization test. In the incubation, clover-N was quickly transferred from macroorganic matter in coarse fractions to clays, and thereafter underwent slow decomposition. One-third or less of clover-N was mineralizable in shaken slurry tests of size fractions, and therefore the remainder was stabilized via

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adsorption or humification, and possibly aggregate protection. The composition of clover-N per NMR was invariably 90% amide, 5-10% guanidinium N of arginine, and 5% amino. Any humification must have involved incorporation of protein into humus without change of functional group. Discrepancies in N composition between this and other studies may be due to the shortness of the incubation in this study or the questionable assumption in other studies that all proteinaceous soil N is hydrolyzable. The mechanisms of N stabilization observed here were relevant to the field as judged by comparison of this study to field studies.

I dedicate this dissertation to Jesus Christ, my Lord and Savior.

I thank Him for life, love, and peace.

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ACKNOWLEDGMENTS

I thank my wife Utami for her love for me, for working late into the night in the laboratory with me, for helping me with slide presentations and formatting, and for her patience. I thank my parents Gene DiCosty and June Robinson for their continual encouragement and belief in me. I thank Anna for enduring long hours spent working on my thesis.

Dr. Eldor Paul supported me generously, encouraged me to be a deeper thinker, helped me to appreciate the breadth of soil science, and always had time for me. Dr. Sharon Anderson allowed me to take the risk of this research project, thus facilitating my learning from both success and failure. She supported me generously, always took time for me, and helped me to think more deeply. Dr. Boyd Ellis encouraged and helped me both personally and professionally. Dr. David Weliky taught me to be an NMR spectroscopist, allowing the use of his equipment and working with me late into the night. Dr. Nathaniel Ostrom shared his laboratory and helpful advice with me.

Kermit Johnson troubleshot almost every imaginable NMR problem for me, and Dr. Long Le provided my initial NMR training plus continued support. Dr. Karl Bishop freely shared helpful ideas for my NMR work. Dr. Dave Harris helped me overcome the challenges of CO2 measurement, elemental analysis, and mass spectrometry. Bryan Pape and Jason Franti worked hard and skillfully in many of my laboratory experiments. Dr. Tom Willson, Dr. Sherri Morris, and Ann-Marie Ezanno freely shared ideas, data, and expertise with me. Mike Rutter and Dr. Oliver Schabenberger gave me crucial help in

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the area of statistics. Keith and Karyn Sanders gave me important help and friendship in the laboratory.

Eugene Jones has been a faithful and encouraging friend for many years. Eric Thomas supported and encouraged me many times. Steve Fitton, Basim Aljazi, Scott Stryd, Jack Brown, John VanderSloot, Evan Vanderwey, Dr. and Mrs. Mark and Sherre Whalon, and Dr. Bill Metcalfe supported me with encouragement and helpful advice. Thanks to the seemingly countless friends who prayed for and encouraged me at University Reformed Church.

I am grateful for the financial support of the US Department of Agriculture through a Water Sciences Fellowship and a grant within the National Research Initiative Competitive Grants Program.

This material is based upon work supported by the Cooperative State Research Service, U.S. Department of Agriculture, under Agreement No. 94-37107-0387. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author and do not necessarily reflect the view of the U.S. Department of Agriculture.

TABLE OF CONTENTS

Append

Append

Appendi

Appendix

Appendix

Appendix

Appendix

LIST OF TABLES

LIST OF FIGURES

Figure 18

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INTRODUCTION

An understanding of the mineralization (and immobilization) of organic nitrogen (N) in soils is necessary to maximize agricultural production while minimizing environmental degradation. Therefore, soil scientists have devoted considerable effort to understanding the controls on N mineralization. These controls include four (non-independent) categories: (1) carbon mineralization, (2) physical location of organic N within the soil matrix, (3) chemical structure of organic N, and (4) environmental factors (e.g. temperature, moisture, pH). The first three controls are relevant to the present study and are described below.

Control of N Mineralization in Soils

Carbon Mineralization

Carbon mineralization is closely linked to N mineralization. Decomposition of agricultural crop residues with C:N ratios greater than 25 is typically accompanied by net microbial uptake of inorganic N (Paul and Clark, 1996), whereas decomposition of residues with C:N < 25 results in net microbial production of inorganic N. Under aerobic conditions, net C mineralization is expected to exceed net N mineralization; mineralized C escapes as CO_2 but mineralized N can again be immobilized. For example, McGill (1971, p. 77) incubated a soil with ^{14}C-acetate + ^{15}N-ammonium sulfate and found that the half-life of amino acid N was 2700 d, whereas Sorenson and Paul (1971) found that the half-life of amino acid C in the same soil incubation was 1600 d.

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Physical Location of Organic N within the Soil Matrix

The physical location of organic N within the soil matrix is undoubtedly an important factor in N mineralization. Soil scientists have established that the physical fractionation of soil yields biologically and chemically distinct pools of organic matter (e.g. Catroux and Schnitzer, 1987). Although many fractionation schemes have been published, the four most important pools of soil organic matter (SOM or OM) appear to be (1) soluble, (2) macroorganic matter (MOM), (3) microbial biomass, and (4) sorbed. (These pools of SOM have some overlap with one another.) See Christensen (1996) for details.

The soluble fraction likely represents free or weakly bound compounds that readily support microbial growth, such as sugars or low molecular weight polypeptides. The MOM fraction probably consists mainly of large plant fragments in the early stages of decomposition. As defined here, this material is isolated by flotation in water or recovered on a sieve with other sand-sized material during particle-size separation (Christensen, 1992). Generally, the MOM fraction is short-lived but it may contain resistant components (Christensen, 1992) such as lignin or charcoal.

The third pool of SOM, microbial biomass, accounts for only 1-3 % of total soil C and at most 5 % of total soil N (Smith and Paul, 1990). Nonetheless, the microbial biomass is important as a transformer and short-term reservoir of soil nutrients. Direct microscopy and chloroform fumigation are two important methods for estimating the microbial biomass (see Paul and Clark, 1996). In the chloroform method, living microbial cells are lysed by chloroform fumigation; the biomass of the lysed cells is then estimated by observing the CO_2 or NH_4 released upon re-inoculation of the fumigated soil or by immediately extracting the fumigated soil and analyzing for elements released from the

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The sorbed fraction is important in the medium- to long-term stabilization of soil organic matter, in contrast to the relatively short-lived soluble, MOM, and microbial biomass fractions. This fraction is primarily associated with silt and clay and is responsible for the relatively high levels of SOM found in fine-textured soils. The OM associated with the fine silt and coarse clay appears to be more resistant than that associated with fine clay. In regard to the long-term stability of these fractions, Anderson and Paul (1984) determined the radiocarbon age of C in particle-size fractions from three soils in the Northern Great Plains (North America); the ^{14}C ages of fine silt and coarse clay were consistently greater than those of fine clay. In two of the three soils, the ^{14}C ages of fine silt and coarse clay were 200 yr or greater. Tiessen and Stewart (1983) found that conversion of grassland to agricultural land led to a net loss of soil organic C and N after several decades; the conversion was also associated with an increase in the proportion of C and N bound to fine silt and coarse clay.

Relatively few studies have focused on the short- or medium-term stabilization of soil organic N in clay and silt fractions. Ladd et al. (1977a,b) amended soils with $^{15}NO_3$ in conjunction with either wheat straw or glucose; after 160 d of incubation in the laboratory they considered that organic ^{15}N in the fine clay was being transferred to silt and coarse clay, and that silt was important in the long-term stabilization of OM. Paul and McGill (1977), in association with Myers, amended field soils with $^{14}C^{15}N$-straw or ($^{15}NO_3$ plus straw) and followed the labeled material for 2 - 4 yr. Generally, the proportion of ^{15}N found in the > 0.2 μm fraction increased, whereas the proportions of ^{15}N found in the 0.04 to 0.2 μm and < 0.04 μm fractions decreased. In a field study of the decomposition of

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$^{13}C^{15}N$-wheat straw during 574 d, Aita et al. (1997) found that substantial amounts of ^{15}N (and ^{13}C) were retained in the fraction < 50 μm, with negligible net decomposition in said fraction. Balabane and Balesdent (1995) supplied ^{15}N to maize fields in the form of either $^{15}NH_4{}^{15}NO_3$ or maize residues in a four-year study, and found limited net decomposition of ^{15}N associated with particles < 50 μm. With ^{15}N supplied in inorganic form, kinetic analysis revealed that there were two pools of ^{15}N within the < 50 μm fraction; one pool was considered to undergo zero decomposition and the other had a decay rate constant of 1.8 yr^{-1}. Where ^{15}N was supplied as maize residue, the ^{15}N in the < 50 μm fraction was modeled as a (one-pool) accumulation function without decay.

Possible mechanisms for the stabilization of soil organic N in the clay and silt fractions include (1) adsorption per se, (2) *aggregate protection*[1] (i.e. entrapment of OM in pores inaccessible to microbes), and (3) chemical structure. The first two mechanisms are discussed below, and the third mechanism is the topic of the next section. In regard to adsorption, Hassink (1996,1997) and Hassink and Whitmore (1997) have compiled considerable evidence that clay and silt behave as saturable adsorptive surfaces with respect to organic matter. In a study of soils from five continents, Hassink (1997) demonstrated that the sorption capacity, or *protective capacity*, for soil C was as follows:

protective capacity (g C kg^{-1}) =

4.09 + 0.37 x (% particles < 20 μm) (1)

[1] *Aggregate protection* has also been referred to as *physical protection*. The former term is used in this study to avoid ambiguity.

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Evidently, the sorption of organic C by silt and clay is non-specific, as Equation 1 was applicable to soils with different mineralogy. Hassink and Whitmore (1997) developed a similar equation based on the proportion of particles < 2 μm:

protective capacity (g C kg^{-1}) =

21.1 + 0.375 x (% particles < 2 μm)

These authors integrated the protective capacity into a simple mechanistic adsorption-desorption model. The model assumed that desorption must precede decomposition of adsorbed OM, and indicated that adsorption was relatively quick whereas desorption was slow. The assumption that desorption must precede decomposition is probably an oversimplification, but reflects the fact that clays inhibit decomposition (Stevenson, 1994).

The aggregate protection of soil organic N in pores inaccessible to microbes is explained by the macroaggregate-microaggregate concept of Edwards and Bremner (1967). Based on dispersion studies, these researchers proposed that soil is composed of weakly bound macroaggregates (> 250 μm) and tightly bound microaggregates (< 250 μm), the latter being most important in aggregate protection. The structure of the microaggregates is represented by $[(C\text{-}P\text{-}OM)_x]_y$, where C = clay mineral particle, P = polyvalent metal such as Ca, Al, or Fe, and OM = humified organo-metallic complex. The subscripts x and y are whole numbers, and reflect that microaggregates contain a hierarchy of C-P-OM units. Experimentally, Edwards and Bremner (1967) investigated the aggregate protection of N by means of an anaerobic (waterlogged) incubation (two weeks, 30 °C) of previously air-dried and ground soils. Mineralization in two soils ground to 50

μm was found to be about 2.5-fold higher than mineralization in the same soils ground to 2000 μm. If the 50 μm soil was moistened after grinding, allowed to air dry at 25 °C, and sieved to 2000 μm size, the mineralization enhancement was lost; evidently, the microaggregates had re-formed in the wetting-drying process. Craswell and Waring (1972) extended the results of Edwards and Bremner (1967), and found increases in aerobic N mineralization with grinding in fine- but not coarse-textured soils.

One might argue that the increases in aerobic N mineralization upon grinding are the result of enhanced oxygen diffusion to the interior of microaggregates rather than entrapment of OM in pores inaccessible to microbes. However, Rovira and Greacen (1957) showed that the uptake of oxygen by an undisrupted silt loam soil was the same under air or oxygen atmospheres.

Chemical Structure of Organic N

The chemical structure of soil organic N is controversial; therefore the relationship between the mineralization and chemical structure of N has not been adequately characterized. Broadly speaking, soil organic N has been studied by one of three methods: (1) chemical extraction, (2) pyrolysis mass spectrometry, and (3) ^{15}N-nuclear magnetic resonance spectroscopy. One widely used method of chemical extraction is acid hydrolysis; soils are typically treated with hot 3 to 6 M HCl for 12 to 24 h (Stevenson, 1994). The fractions identified are summarized in Table 1. The nature of the N in the NH_3, hydrolyzable unknown, and acid-insoluble fractions is not well known; Stevenson (1994) and Schnitzer (1985) consider that about one-half of soil N remains unidentifiable by soil hydrolysis techniques. Despite these problems, the hydrolysis fractions have been

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Table 1

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observed to exhibit some differences in mineralizability. For example, amino acid-N generally decreases (as a proportion of total N) upon cultivation (Stevenson, 1994) and is therefore a labile N form.

Table 1. Fractions of soil organic N identified by acid hydrolysis.†

fraction	explanation	% of soil N
amino acid N	Determined in hydrolysate by ninhydrin methods.	30-45
"NH_3"-N	Ammonia recovered upon steam distillation of MgO-treated hydrolysate, sources include amino acids destroyed by hydrolysis and clay-fixed NH_4.	20-35
amino sugar N	Determined by either steam distillation of hydrolysate at pH 11.2 in the presence of phosphate-borate buffer (with correction for NH_3-N) or direct colorimetric analysis of hydrolysate.	5-10
hydrolyzable unknown N (HUN)	Hydrolyzable N minus (amino acid N + amino sugar N + NH_3-N).	10-20
acid-insoluble N	-	20-35

† Adapted from Stevenson (1994).

The chemical fractionation of SOM into *humin*, *humic acid*, and *fulvic acid*, is a milder fractionation than acid hydrolysis and has been useful in studies of proteinaceous soil N. A typical separation might involve treatment of soil with 0.1 to 0.5 M NaOH, followed by separation of liquid and solid phases by centrifugation, and adjustment of the

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liquid pH to 2 with 2 M HCl (Schnitzer, 1982). The humin is contained in the residue of the NaOH extraction, the humic acid is precipitate of the acidified liquid, and the fulvic acid remains in solution. Simonart et al. (1967) applied phenol extraction and electrophoresis to humic acid to isolate a "humoprotein" complex and protein. They considered that the protein was hydrogen-bonded to the humic acid. Haworth (1971) summarized numerous studies on humic acid and hypothesized that protein was attached to an aromatic humic "core" via both covalent and hydrogen bonds. Biederbeck and Paul (1973) extracted humic acid with phenol, and purified the phenolic extract with polyvinylpyrrolidone (PVP, a phenol-complexing agent) to isolate relatively pure peptidic materials. The phenol and PVP were considered to have disrupted hydrogen bonds between peptides and an aromatic humic acid fraction. These studies demonstrated that protein is an important N-containing constituent of humic acid.

Soil scientists have applied cross-polarization magic-angle spinning ^{15}N-nuclear magnetic resonance spectroscopy in the solid state (CPMAS-NMR) and pyrolysis-mass spectrometry (PyMS) to gain further information about the chemical structure of soil organic N. The proponents of each technique claim to be able to assign all but a small fraction of soil N to specific functional groups, but the conclusions of different investigators are often contradictory. Based on PyMS studies of whole soils and hydrolysis fractions, and on detailed analysis of hydrolysis fractions (e.g. gel chromatography, gas chromatography, mass spectrometry), Schulten and Schnitzer (1998) state that soil N is distributed as follows: proteins + peptides + amino acids, 40 %; amino sugars, 5 - 6 %; heterocyclic N, 35 %; and NH_3, 19 %. (NH_3 is an operationally defined pool; see Table 1.) In a PyMS study of agricultural soils, Schulten and Hempfling (1992)

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found a negative correlation between heterocyclic N and total soil N. According to these authors, the low-N soils had a decreased capacity to immobilize N (as a result of management practices) and thereby became enriched with relatively resistant heterocyclic N.

In contrast to the aforementioned PyMS studies, NMR researchers have found 80 % or more of soil N in amide form (peptide bonds in proteins), with the remainder in the form of amino acids, amino sugars, or the amino groups of nucleic acid bases (Knicker et al., 1993, 1997; Clinton et al., 1995; Hopkins et al., 1997). Heterocyclic N was identified in fulvic acid by Zhou and Wen (1992), but comprised only 9% of the spectral intensity. Given that Schulten and Hempfling (1992) proposed that heterocyclic N is an indicator of agricultural management practices (see above), it is important to reconcile the controversy concerning the chemical nature of soil organic N.

Both PyMS and CPMAS-NMR have potential problems in quantitation of soil organic N. In the case of PyMS, incomplete sample volatilization or thermal rearrangement may confound the analysis (Schulten et al., 1995). In CPMAS-NMR, quantitation may fail if differential relaxation effects during the contact and delay times are not considered. These effects have been assessed in plant material, compost, and soluble extracts (Knicker and Lüdemann, 1995; Knicker et al., 1997), but have not been thoroughly investigated in soil. (The nature of these effects is described in the Material and Methods). Another potential problem of CPMAS-NMR is that rigid aromatic structures may have excessively long T_{1H} values; the delay times required to detect the N in such structures may be too lengthy for a practical experiment. In a CPMAS-NMR study of ^{13}C, Wilson et al. (1984) showed that T_{1H} values exceeded 50 s for rigid, planar,

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aromatic molecules such as naphthalene, anthracene, and phenanthrene! Similar N-containing structures, such as those proposed by Flaig et al. (1975) or those identified by PyMS (Schulten and Schnitzer, 1998), might also exhibit long T_{1H} values.

Additional problems may confound quantitation of soil N by CPMAS NMR. Most NMR studies of soil N require addition of ^{15}N; incubation time of the ^{15}N with soil may be insufficient for formation of heterocyclic N (Schulten and Schnitzer, 1998). Also, NMR may be currently unable to detect the numerous heterocyclic N structures thought to be present in soil (Schulten and Schnitzer, 1998). The structures may be obscured by the high frequency shoulder of the amide-N peak. If numerous heterocyclic N structures exist in soil and have a wide range of chemical shifts, the structures may give rise to numerous small signals that are undetectable in a typical CPMAS-NMR experiment.

Despite the limitations of CPMAS-NMR, its noninvasiveness makes it attractive for studies of soil organic N. The technique has not been applied to soil particle-size fractions, which are known to contain biologically and chemically distinct pools of OM. Each fraction may contain fewer functional groups than whole soil, and thus better resolved NMR spectra seem possible. Further, the results of Bedrock et al. (1998) suggest that ^{15}N-CPMAS-NMR can serve to identify soil microbial populations. These authors cultured soil bacteria and fungi axenically and found that the organisms had distinct ^{15}N-CPMAS-NMR signatures. The bacterial cells exhibited a small peak at 115-140 ppm (referenced to NH_4^+ = 0 ppm), which was attributed to aromatic N of purine bases. The fungal cells had a smaller peak in said region, but showed a unique peak at 10 - 25 ppm, which was attributed to N-acetyl-glucosamine. Bedrock et al. (1998) also incubated unlabeled straw in mesh bags in a field soil fertilized with $^{15}NH_4NO_3$. After 12

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months of decomposition, the degraded straw had a fungal NMR signature. Bedrock et al. (1998) supported their finding with other microbiological data, which indicated that bacteria comprised less than 5% of the microbial biomass.

In contrast to nitrogen, the relationship between the chemical structure of soil organic C and C mineralization is reasonably well characterized. This topic merits discussion because C and N mineralization are related (see above). Based upon the results of ^{13}C-NMR studies, Baldock et al. (1992) proposed a simple three-stage model, which relates C structure to mineralization (Table 2). Overall, the model describes a progression from carbohydrate to aliphatic C, with an intermediate stage marked by the selective preservation of aromatic C of lignin. The O-alkyl, aromatic, and alkyl C tend to be concentrated in the sand (> 53 μm), silt (2-53 μm), and clay (< 2 μm) fractions, respectively.

Table 2. Oxidative model of plant decomposition in mineral soils.†

	stage of decomposition		
	early	intermediate	late
major peaks in ^{13}C-NMR spectra	O-alkyl	O-alkyl alkyl aromatic	alkyl
probable types of molecules	plant carbohydrate	microbial carbohydrate selectively preserved plant lipids? selectively preserved plant lignin	peptidoglycans of bacterial cell walls? bacterial and plant lipids? humic polymers?

† Adapted from Baldock et al. (1992).

N Dynamics in Cover Crop Systems

Knowledge of N dynamics within cover crop systems is of fundamental importance with the increasing emphasis on low-input sustainable agriculture. Cover crops are beneficial because they can reduce erosion, conserve water (if kills are timed appropriately), reduce weed biomass, and decrease fertilizer costs (Bowman et al., 1998; Fisk, 1997). Top cover crops for the Great Lakes region of the USA include red clover, hairy vetch, annual ryegrass, and rye (Bowman et al., 1998). Sarrantonio (1998) suggests that from 25-50% of the N from cover crops is available to a subsequent crop, but this estimate may be somewhat high per the ^{15}N studies discussed below.

Harris and Hesterman (1990) evaluated the uptake of alfalfa-^{15}N by first-year corn and second-year barley at two sites in Michigan. After one growing season, 46% of the ^{15}N remained in organic forms in the soil and 17 or 25% of the ^{15}N was found in the plant. The higher plant uptake occurred in a relatively coarse-textured soil and may have been related to a lack of clay protection. The alfalfa-derived ^{15}N was much less available during the second growing season, as the barley crop recovered only 1% of the initial ^{15}N.

Harris et al. (1994) extended the findings of Harris and Hesterman (1990) by studying the fate of fertilizer- versus legume-^{15}N (red clover) in conventional and low-input farming systems in Pennsylvania. Losses of ^{15}N from the two plant-soil systems were similar after two growing seasons (39% of input). Plant uptake of ^{15}N was higher in the fertilized system than in the legume system (40 versus 17% of input), with nearly all uptake in both systems occurring in the first growing season. The legume system retained more ^{15}N in the soil than the fertilizer system (47 versus 17% of input), and contained a larger microbial biomass. The high microbial biomass and soil N retention in the legume

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system suggest enhanced N-supplying capacity in long-term cover-cropped soils.

Objectives

The objectives of this study were to (1) test whether CPMAS-NMR is quantitative for both heterocyclic and noncyclic soil organic N, (2) identify the soil organic N functional groups derived from decomposing clover, and (3) evaluate the mechanisms for the short- to medium-term stabilization of soil organic N.

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MATERIALS AND METHODS

Soil Incubation Experiment

Overview

Objectives 2 and 3 (see Introduction) were addressed via a soil incubation experiment, as depicted in Figure 1 and summarized below. The Ap horizon of an agricultural soil was amended with ^{15}N-labeled red clover (*Trifolium pratense* L.) and incubated in laboratory-scale microlysimeters for 14 months. *In situ* C and N mineralization were measured several times during the incubation. On five dates, soil was ultrasonically fractionated into five size classes, and the concentrations of N, ^{15}N, and C were determined for each size fraction and whole soil. On three of the five dates, the fractions and whole soil were characterized by solid-state ^{15}N-NMR and by a shaken-slurry N mineralization test.

Soil and Plant Material

The soil used was a fine-loamy, mixed mesic Glossoboric Hapludalf (Marlette series) from East Lansing, Michigan, USA. The Ap layer of soil was excavated from 0 to 10 cm depth, air-dried, sieved to remove fragments > 2 mm in diameter, and thoroughly mixed by passing several times through a soil splitter.

The particle-size distribution of the soil was determined by the conventional pipet method. The method was based on reports by Gee and Bauder (1986), Whittig and Allardice (1986), and Dr. Sharon Anderson (pers. comm.) Carbonates were removed and

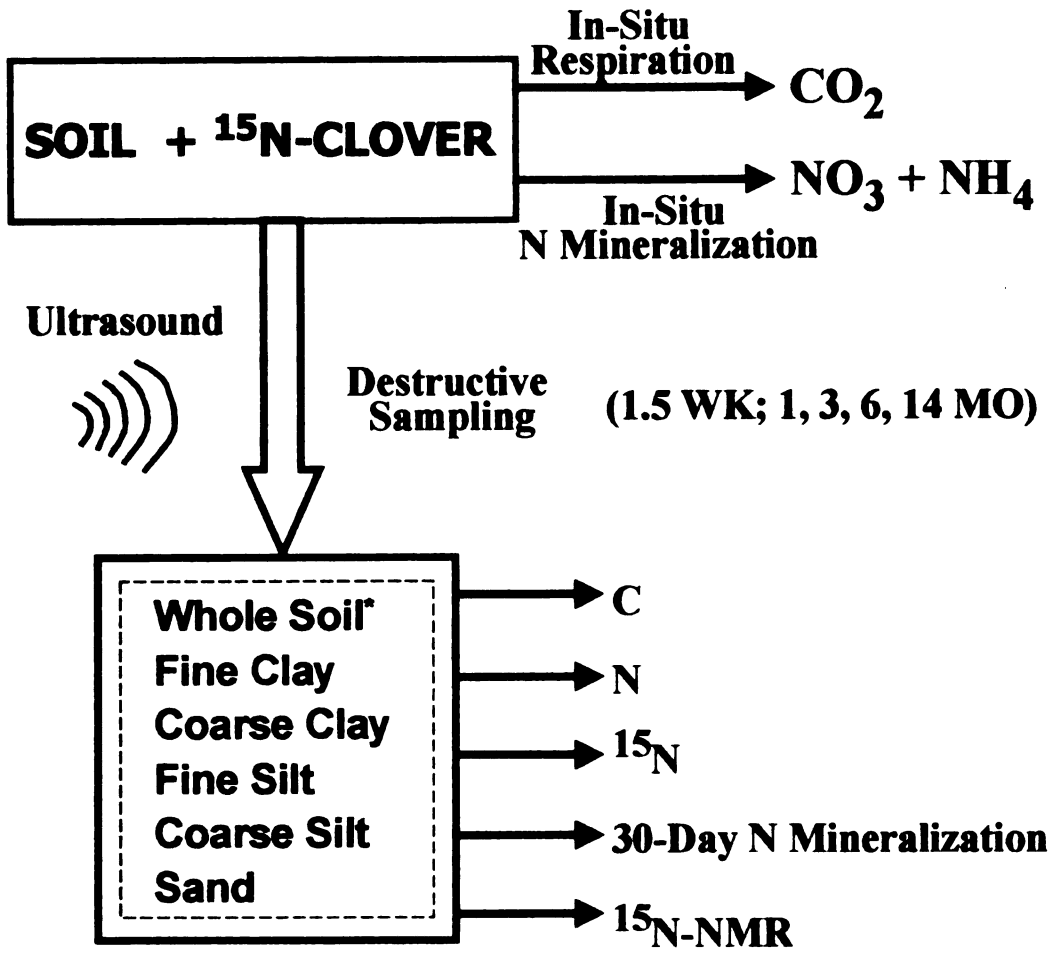

Figure 1. Overview of soil incubation experiment. * Whole soil was not sonicated.

the soil cation exchange sites were saturated with sodium by treatment with 1 M sodium acetate (adjusted to pH 5), at 70 – 80 °C. Excess salts were then removed by centrifugation with water, after which organic matter was removed by addition of 30% hydrogen peroxide at 70 °C. Next, appropriate dispersant (sodium hexametaphosphate) was added followed by either 24 h or more of soaking or about 16 h of shaking; soaking and shaking gave the same results. Sand (> 53 μm diameter) was determined by sieving, and fine silt (2 – 10 μm) and coarse clay (0.2 – 2 μm) were determined by shaking the remaining suspension and taking pipet aliquots at the appropriate settling times. Finally, fine clay (< 0.2 μm) was determined in a tube centrifuge based on an integrated version of Stokes' law. Throughout the procedure, the presence of residual salts was taken into account by appropriate measurements and corrections. The results are shown in the leftmost data column of Table 3. The soil was identified as a sandy loam per the USDA textural classification system.

Table 3. Particle-size distribution as determined by conventional pipet or ultrasound methods.

fraction	method		ultrasonic yield / conventional yield
	conventional pipet (%) †	ultrasound (38.3 kJ / 28 g soil) (%)†	
fine clay (< 0.2 μm)	6.50 (0.28)	3.81 (0.56)	0.59
coarse clay (0.2-2μm)	9.05 (0.87)	12.4 (0.7)	1.37
fine silt (2-10 μm)	9.94 (0.90)	12.0 (0.4)	1.21
coarse silt (10-53 μm)	19.7 (0.2)	15.4 (2.9)	0.78
sand (53-2000 μm)	54.8 (0.2)	56.3 (3.1)	1.03
SUM	100	100	

† Sample standard deviations in parentheses.

The chemical characteristics of the soil prior to amendment with clover were as follows (means ± sample standard deviations): pH in 1:2 soil H_2O = 7.20 ± 0.03, C_{total} (g kg^{-1}) = 13.6 ± 1.6, N_{total} (g kg^{-1}) = 1.12 ± 0.01, and molar C:N ratio = 14.1 ± 1.8.

The clover (*Trifolium pratense* L.) used as a soil amendment had been grown hydroponically using $^{15}NO_3$ as the sole N source. The clover was freeze-dried and ball-milled to a powdery texture prior to mixing it with soil. The characteristics of the clover were as follows (means ± sample standard deviations): C_{total} (g kg^{-1}) = 403 ± 4, N_{total} (g kg^{-1}) = 38.9 ± 1.0, molar C:N ratio = 12.9 ± 0.4, atom % ^{15}N = 91.8 ± 2.0.

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Incubation Conditions

Soils were incubated at room temperature (about 23 °C) in the dark in the laboratory-scale microlysimeters described by Nadelhoffer (1990). Before incubation, the soil was amended with 34.3 g of the ^{15}N-labeled red clover per kg amended soil (dry basis). The total C and N contents of the soil were approximately doubled as a result of adding the amendment, and the clover-C corresponded to about 1.4 % of the soil dry weight. Although agricultural soils typically receive much smaller organic inputs, Paul and Clark (1996) state that the decomposition rate of organic materials added to soil is independent of the quantity added if the C addition does not exceed 1.5 % of the soil dry weight. The high concentration of clover in the soil ensured that ^{15}N-NMR spectra could be obtained in a reasonable amount of time.

Into each lysimeter, 62.1 g of amended soil (dry basis) were packed at an approximate bulk density of 1.2 Mg m^{-3}. Then, water was added to -33 kPa soil water potential, with correction for moisture retained in the glass filter and glass wool components of the lysimeters. The soil water potential was maintained by adding water about twice per week; less than ten percent of the water evaporated between additions.

In Situ *N Mineralization*

In situ N mineralization was defined as the cumulative, soluble, inorganic N leached from the lysimeters. The method was modified from Nadelhoffer (1990). For leaching, soils were allowed to equilibrate with an N-free nutrient solution for 30 min, and then vacuum-extracted at 45 - 60 kPa. Potassium chloride was added to the leachates as a

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preservative[2], after which the leachates were stored at -20 °C until analysis. The N-free nutrient solution contained 1.3 mM $CaCl_2$, 0.67 mM KH_2PO_4, 0.33 mM K_2SO_4, 0.33 mM $MgSO_4$, 8.33 μM H_3BO_3, 0.67 μM $MnCl_2$, 0.67 μM $ZnSO_4$, 0.17 μM $CuSO_4$, and 0.17 μM Na_2MoO_4, with final pH = 5.1. About 5 to 8 mL of excess nutrient solution remained in each lysimeter after leaching; the excess liquid was allowed to evaporate by uncapping the lysimeters until they again reached -33 kPa water potential. At the end of the study, about 11 pore volumes of leachate had been collected from each lysimeter.

In Situ *C Mineralization*

In situ C mineralization was measured by temporarily making the lysimeters gastight, during which time respired CO_2 accumulated in the headspace (Nadelhoffer, 1990). For a single measurement of respiration in an individual lysimeter, three to four headspace gas samples were taken at specific times and immediately injected into an infrared gas analyzer for determination of CO_2 concentration. Respiration in each lysimeter was defined as the slope of a linear regression of CO_2 concentration versus time. The squared correlation coefficient of the regression (R^2) exceeded 0.95 for most measurements; measurements with $R^2 < 0.95$ were omitted from the final data.

For the respiration measurement at incubation time = 1.3 d, the first-measured lysimeters respired more than the last-measured lysimeters. This observation is consistent with general principles of the decomposition of plant material in soil (see Paul and Clark,

[2] According to Keeney and Nelson (1982), filtered 2 M KCl extracts of soil are stable under refrigeration for several months. In the present study, KCl was added to 1 or 2 M final concentration.

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1996); the rate of C loss is expected to decrease rapidly with time in the initial stages of decomposition. Therefore, the respiration at time = 1.3 d was estimated by constructing a scatter diagram of the natural logarithm of respiration versus exact time of measurement, fitting a regression line through the points, and calculating a predicted value of respiration for time = 1.3 d.

After the 14-month soil incubation, the respiration data were fitted to a two-pool model (Paul et al., in press):

$$r = C_1k_1 \exp(-k_1t) + C_2k_2 \exp(-k_2t) \qquad (2)$$

where r = respiration (amount of C per time), C_1 and C_2 are the amounts of C in the two pools, k_1 and k_2 are rate constants ($time^{-1}$), and t = time. Respiration was defined to be a positive number and thus equals –dC/dt. The sum of the parameters C_1 and C_2 was constrained to equal initial whole soil C minus resistant C. Resistant C was estimated as 45% of the native (non-clover) C. This estimate is derived from the data of Collins et al. (in press); these researchers determined that nonhydrolyzable C (after removal of visible plant residues) in cultivated topsoils of the Northern Prairie and Corn Belt regions of the USA ranged from 39 to 51% of total C. Paul et al. (1997) classify nonhydrolyzable C as resistant based on its old radiocarbon age. For cultivated topsoils in Colorado, Nebraska, and Arizona, Paul et al. (1997) found that nonhydrolyzable C pool had a mean ^{14}C age ranging from 900 to 3300 yr whereas the ^{14}C age of the total C in these soils ranged from 400 to 1300 yr.

For simplicity, no correction for microbial biosynthesis was made in the model. The fitting procedure is explained below in the section *N Mineralizability in Whole Soil and Particle-Size Fractions.*

Ultrasonic Fractionation of Soil

On five dates, the soil from two lysimeters was composited, sonicated, and separated into five organo-mineral size classes. The size classes were fine clay (0-0.2 μm diameter), coarse clay (0.2-2 μm), fine silt (2-10 μm), coarse silt (10-53 μm), and sand (53-2000 μm). An ultrasonic energy of 38.3 kJ per 28 g soil was used, per the results of a preliminary study. This energy produced a particle-size distribution similar to the conventional pipet method (Table 3); the energy was measured as described by Morra et al. (1991) and North (1976). Sonication was carried out in a 250-mL beaker that was resting in an ice bath, with a 1:5 soil:water ratio, 28 g soil (dry basis), 1.27 cm (0.5 in) ultrasonic probe diameter and 3 cm probe depth. The incubated soils required brief hand-shaking before sonication; the shaking served to break up large clods of moist soil.

After sonication, the three finest size fractions were physically separated on the basis of Stokes' law. The fine clay was isolated by centrifugation, the coarse clay by gravity sedimentation at 4 °C (the low temperature served to slow microbial transformations), and the fine silt by gravity sedimentation at room temperature. Each separation consisted of eight centrifugation or sedimentation cycles. During the separations, the concentration of clay was kept below 10 g/L; Elonen (1971) showed that clay concentrations above 10 g/L significantly increase the suspension viscosity relative to pure water and thereby confound the Stokes' law calculations. After the three finest size fractions were isolated, the coarse silt and sand were separated by wet sieving.

When the separations were completed, the three fine size fractions were concentrated by flocculation with $MgCl_2$; the supernatant was removed by siphoning, with

centrifugation as necessary. The two coarse size fractions were either flocculated as above or allowed to settle under gravity, and were concentrated by siphoning the supernatant. Salts were removed by dialysis at 4 °C (cellulose membrane with molecular weight cutoff 12,000 to 14,000, Spectra/Por, Spectrum Medical Industries), such that the amount of Mg remaining in the soil was small compared to Mg supplied by the liquid medium in subsequent shaken-slurry N mineralization tests. The separation, concentration, and dialysis were completed in about 2 weeks, with samples stored at 4 °C between steps. The separated fractions and a sample of whole soil were freeze-dried, and then stored at -70 °C.

N Mineralizability in Whole Soil and Particle-Size Fractions

N mineralizability in whole soil and particle-size fractions was measured by two complementary methods. The first method consisted of the kinetic analysis of clover-N and native-N in whole soils and particle-size fractions as a function of incubation time. The amounts of clover-N and native-N were computed from ^{15}N measurements according to the equations given in Appendix 1. As appropriate, the data were fit to one of four mathematical models.

The first model is a one-pool exponential decay:

$$N_t = N_0 \exp(-kt) \qquad (3)$$

where N_t is the amount of N at time = t, N_0 is the N amount in the fraction at the beginning of incubation, and k is a rate constant ($time^{-1}$).

The second model is a two-pool exponential decay:

$$N_t = N_1 \exp(-k_1 t) + N_2 \exp(-k_2 t) \qquad (4)$$

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where the parameters are defined analogously to Equation 3. In some cases, one of the two pools exhibited essentially zero decay and the two-pool model was simplified as follows:

$$N_t = N_1 \exp(-k_1 t) + N_2 \qquad (5)$$

The third model is applicable where a period of net N accumulation precedes a period of net N depletion:

$$N_t = -A \exp(-k_A t) + B \exp(-k_D t) \qquad (6)$$

where A and B are (positive) constants, k_A is the accumulation rate constant, and k_D is the depletion rate constant. The derivation of this model is given in Appendix 2.

The fourth model simulated N accumulation without depletion:

$$N_t = N_0 + N_1 (1 - \exp(-k_A t)) \qquad (7)$$

where N_0 is the N amount in the fraction at the beginning of incubation, N_1 is related to the size of an unspecified pool from which N is being transferred, and k_A is the accumulation rate constant.

For simplicity, no corrections for microbial biosynthesis were made in the models. Parameter estimates and standard errors in the models were generated via the Levenberg-Marquardt method within the nonlinear regression module (PROC NLIN) of the SAS software (SAS Institute, 1989). The best model for each fraction was usually chosen based on visual inspection. If visual inspection did not clearly identify one model as superior, the model with the lowest ratios of the standard errors to the parameter estimates was chosen.

The second method for measuring N mineralizability involved shaking the whole soils and particle-size fractions (obtained from three dates of the 14-month soil incubation)

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for 30 days in an N-free nutrient medium. The method was based on that of Catroux and Schnitzer (1987). N mineralization was defined as shown below:

$$\text{percent N mineralized} = \frac{N_{30} - N_0 - N_{CTRL}}{N_{TOTAL} - N_0} \times 100 \qquad (8)$$

where N_{30} = extractable inorganic N in soil sample after 30 days of shaking, N_0 = extractable inorganic N in soil sample prior to shaking, N_{CTRL} = net change in inorganic N observed for a blank (no soil) sample shaken for 30 d, and N_{total} = total N in soil sample prior to shaking. Ideally, this measurement reflects the capacity of mineral-associated organic matter to supply inorganic N in the absence of the protective effects of soil aggregation. Details of the measurements are described below:

Soils and 23 to 29 mL of N-free nutrient medium were added to 125-mL Erlenmeyer flasks. Soil amounts were calculated to give 7 μmol N per mL medium, subject to sample availability and a maximum soil:water mass ratio of 1:10. The medium was designed to support generalized heterotrophic microbes, and is described in Table 4. To each flask, 1 mL of inoculum was added per 29 mL of medium. (The inoculum was derived from a moist soil that had been stored at 2 °C.[3])

[3] The inoculum served to provide microbes to the flasks without contributing a significant amount of N, and was derived from the previously described Marlette loam soil. A few grams of this soil were brought to -33 kPa water potential and incubated 5 d at room temperature. Thereafter, the soil was maintained at 2 °C and -33 kPa water potential. For each shaken-slurry N mineralization experiment, a portion of the chilled soil was combined with water in the moist soil:water mass ratio of 1:10. The resulting slurry was hand-shaken, allowed to settle for 30 min, and 1 mL of supernatant was combined with 99 mL of the N-free nutrient medium. This inoculum suspension was continuously stirred while transferring appropriate aliquots to each flask.

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Flasks were stoppered with foam plugs and inorganic N was extracted either immediately or after 30 d of rotary shaking at 250 ± 5 rpm. About twice per week during the 30-d period, evaporated water was replaced and wall deposits were resuspended. All extraction and shaking occurred in the dark. The average temperature for extraction and shaking was about 23 °C, and the maximum deviation from the average was 3 °C. To extract the soils, solid KCl was added to the suspensions to achieve a final KCl concentration of 1 M. The slurries were then shaken at 250 ± 5 rpm for 60 min and allowed to flocculate (under the influence of the salts in the medium) at 2 °C for about 2 h. (In the case of fine clay, the slurries were also centrifuged for 10 min at *relative centrifugal force* = 26.) Finally, the supernatants were passed through cellulose ester syringe-filters with pore size 0.45 μm; the filtrates were stored at -20 °C until analysis. Preliminary tests showed the filters contained no inorganic N.

Table 4. Description of N-free nutrient medium used in 30-day shaken-slurry N mineralization tests.†

compound	conc. (mM)
K_2HPO_4	22.5
KH_2PO_4	22.0
NaCl	8.56
$MgSO_4$	2.00
$CaCl_2$	0.100
H_3BO_3	46.1×10^{-3}
$MnCl_2$	9.10×10^{-3}
$FeSO_4.7H_2O$	4.89×10^{-3}
sodium tartrate	7.38×10^{-3}
$CuCl_2.4H_2O$	0.130×10^{-3}
$ZnSO_4$	0.152×10^{-3}
$CoCl_2.6H_2O$	0.170×10^{-3}
$Na_2MoO_4.2H_2O$	0.104×10^{-3}

† This medium was modified from the M9 Minimal Medium (Sambrook et al., 1989, as cited by Zuberer, 1994). The phosphate salts provided buffering near pH 7. The trace elements were added via a stock solution described by Cote and Gherna (1994). Medium was not sterilized.

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C, N, and ^{15}N Analyses

Total C and N contents of clover, soils, and particle-size fractions were determined by dry combustion (Carlo Erba NA 1500 Series 2 N/C/S Analyzer). For whole soil samples with high levels of nitrate, both organic and inorganic N were determined. These samples were extracted three times with 0.01 M $CaCl_2$; organic N in the solid residue was determined by dry combustion and inorganic N in the combined extract was determined as described below.

Soluble inorganic N (NO_3^- + NO_2^- + NH_4^+) in the leachates, whole soil extracts, and shaken-slurry extracts was measured colorimetrically on a flow-injection analyzer (Lachat). Interference in the colorimetric reactions occurred in many of the extracts from the shaken-slurry mineralization tests, and was likely a result of colored organic compounds or unflocculated colloids. The interference was overcome by standard additions (Bader, 1980). Briefly, fixed volumes of sample were treated with fixed volumes of either blank solution or a solution containing inorganic N. The percent recovery of added inorganic N was computed, and the concentration of inorganic N in the original sample was calculated accordingly.

The ^{15}N concentrations of the clover, soils, particle-size fractions, and shaken-slurry extracts were determined by mass spectrometry at either Michigan State University (Europa Scientific 20-20 Stable Isotope Analyzer) or University of Georgia. Samples were diluted as necessary with natural abundance N in the form of atropine, soil, or aqueous ammonium sulfate to avoid introducing excessive ^{15}N into the mass spectrometers. The ^{15}N concentrations in the soils and fractions were computed as follows:

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$$[^{15}N]_{SMP} = \{A_M([N]_{STD}\, m_{STD} + [N]_{SMP}\, m_{SMP}) - A_{STD}\, [N]_{STD}\, m_{STD}\}/(100\, m_{SMP})$$

where $[^{15}N]_{SMP}$ denotes the sample ^{15}N concentration, A_M and A_{STD} denote the atom % ^{15}N in the mixture and natural abundance material, respectively, $[N]_{STD}$ and $[N]_{SMP}$ are the concentrations of N in the natural abundance material and sample, respectively, and m_{STD} and m_{SMP} are the masses of natural abundance material and sample, respectively. Concentrations were expressed on a molar basis.

In the kinetic analysis of N and C in whole soils and particle-size fractions, the amounts of clover-N, native-N, and C were expressed as percent of initial amounts in whole soil. For simplicity, the standard deviations of the initial amounts were ignored in computing the standard deviations of the percentages.

Statistical Treatment of Outliers

Dixon's gap test (Bliss, 1967) was used to identify outliers at the 10 % probability level, for respiration, elemental analysis (C and N), and shaken-slurry N mineralization. These outliers comprised a small proportion of the data, and were excluded from the final data due only to their extreme deviations. This was deemed necessary because it is difficult to completely avoid analytical errors in samples with low masses or low concentrations of analytes. According to Bliss (1967), "... the bias from rejecting a valid observation is usually far less than that caused by retaining a contaminant."

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NMR Spectroscopy[4]

NMR spectra of the solid state samples were acquired on a Varian VXR 400 MHz spectrometer with a frequency of 40.5 MHz for ^{15}N. Cross-polarization magic-angle spinning was used to enhance the signal-to-noise ratio and hasten data acquisition. About 200-300 mg of soil were packed into a silicon nitride rotor of 7 mm diameter and spun about the magic angle at 4500 Hz. Chemical shifts were referenced to $(^{15}NH_4)_2SO_4$ (= 0 ppm); chemical shift assignments are summarized in Table 5 and given in detail in Appendix 4.

[4] See Appendix 3 for a brief overview of NMR concepts.

Table 5. Su

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Table 5. Summary of ^{15}N chemical shifts.

shift region † (ppm)	nitrogen functionality
0	ammonium
5-34	amino groups in free and amino-terminal amino acids
42-76	amino groups of nucleic acids, guanidine nitrogens of arginine, indole-N of tryptophan
83-116	amide N of proteins
120-148	heterocyclic N in certain positions in nucleic acids, in histidine, or in flavin
158	heterocyclic N in pyrrole (chlorophyll)
170-211	heterocyclic N in nucleic acids, in flavin, or in pyrrole (chlorophyll)
312-320	heterocyclic N in oxidized flavin structures
354	nitrate

† Chemical shifts are relative to $^{15}NH_4NO_3$ (= 0 ppm). Based on hydrogen bonding considerations, the reference compound used in the present study, $(^{15}NH_4)_2SO_4$, is likely to have a chemical shift within 1 ppm of $^{15}NH_4NO_3$. See Appendix 4 for details.

Standard samples were run to ensure optimal performance of the spectrometer before each series of NMR experiments. The magic angle was checked and adjusted with KBr. Then, the 90° ^1H pulse time, the Hartmann-Hahn match, and decoupling power during acquisition were optimized on a sample comprised of ^{15}N-uracil, $(^{15}NH_4)_2SO_4$, and unlabeled soil. The uracil (Figure 2) served to verify that the spectrometer was able to detect heterocyclic N, and the relatively narrow resonance range of $(^{15}NH_4)_2SO_4$ allowed for a precise determination of the Hartmann-Hahn match. The 90° ^1H pulses ranged from

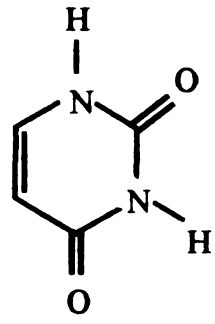

Figure 2. Chemical structure of uracil

6.5 to 10.5 μs, and decoupling power during acquisition ranged from about 65 to 80 kHz (as measured with an oscilloscope). With this decoupling power, linewidths (full width at half-height, lb = 10 Hz) were about 4 to 6 ppm for uracil and 2 ppm for ammonium sulfate. Optimal contact times were 0.2 to 0.4 ms, delay times were 0.5 s or longer to avoid probe overheating, and acquisition times ranged from 6 to 10 ms.

The uracil/ammonium sulfate/soil sample used for the above optimization process had been intimately mixed so that the molecular interactions (e.g. binding to soil particles) would be similar to those expected in a natural soil. The uracil, ammonium sulfate, and soil were combined in a sufficient volume of water for complete dissolution of both ^{15}N-labeled compounds; the resulting suspension was mechanically shaken for one hour, quickly frozen in liquid nitrogen (to avoid precipitation of relatively insoluble uracil), and lyophilized. This mixing method influenced the NMR behavior of the sample; the sample prepared as above exhibited a lower T_{1H} value than a sample mixed only by grinding with mortar and pestle. This is likely due to shorter distances between ^{15}N atoms and paramagnetic species (e.g. Fe) in the water-treated sample.

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For testing whether NMR was quantitative (Objective 1 in Introduction), an intimate mixture of ^{15}N-uracil, ^{15}N-clover, and unlabeled soil was prepared with the mixing technique described above. The peak area proportions determined by NMR were compared to the known sample composition, after correction for differential relaxation effects (as described below).

To process each NMR spectrum, the raw data (free induction decay) were multiplied by an exponential weighting function (line broadening = 100-120 Hz for soils, 40 Hz for clover), zero-filled to 16384 data points, and Fourier-transformed. The transformed spectrum was phased and baseline-corrected. The phasing and baseline correction were somewhat subjective and were performed twice. The peak area for each functional group was defined as the average peak area from the two phasing/baseline correction operations. Spinning sidebands were included in the peak area calculations.

Correction for Differential Relaxation Effects

Differential relaxation effects must be considered for accurate quantitation of NMR spectra. These effects were evaluated via two models. The first model accounts for differential rates of buildup or decay of magnetization during the contact time (Stejskal and Memory, 1994):

$$S(t_c) = So \frac{\exp\left(-t_c / T_{1\rho H}\right) - \exp\left(-t_c / T_{NH}\right)}{1 - \left(T_{NH} / T_{1\rho H}\right)} \qquad (9)$$

where $S(t_c)$ = measured signal intensity at contact time = t_c, S_0 = theoretical signal intensity, $T_{1\rho H}$ = spin-lattice relaxation time in the rotating frame, and T_{NH} is the cross-polarization time. The model excludes $T_{1\rho N}$ with the assumption that this parameter is of

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negligible effect (i.e. $T_{1\rho N} >> T_{1\rho H}$). In addition, the model assumes the molar ratio $^{15}N/^{1}H << 1$, and $T_{NH} << T_{1\rho H}$. To implement the model, NMR spectra were acquired at seven different contact times (ranging from 0.05 to 3.2 ms). Model parameters were generated with either the COMPLEX nonlinear optimization program of Box (Kuester and Mize, 1973; as modified by Dr. Thomas Manetsch) or the Marquardt method within PROC NLIN of the SAS software (SAS Institute, 1989); standard errors of the parameters were computed by SAS. For convenience, a correction factor (CF) for a particular functional group at a given contact time was defined by rearranging Equation 9:

$$CF = \frac{1 - (T_{NH} / T_{1\rho H})}{\exp(-t_c / T_{1\rho H}) - \exp(-t_c / T_{NH})} \qquad (10)$$

Thus, corrected signal intensity = measured signal intensity x CF.

The second model accounts for differential decay of magnetization during the delay time (Stejskal and Memory, 1994):

$$S(t_D) = S_0 [\, 1 - \exp(-t_D / T_{1H}) \,] \qquad (11)$$

where $S(t_D)$ = measured signal intensity at delay time = t_D, S_0 = theoretical signal intensity, and T_{1H} is the spin-lattice relaxation time of ^{1}H (in the laboratory frame). The model assumes that spin-spin relaxation is negligible. To fit the model, NMR spectra were acquired with three different delay times. Model parameters were generated with the same methods used for the contact time model (Equation 9).

RESULTS

Overall N and C Mineralization

The overall N mineralization during the 14-month incubation of the clover-amended soil is shown in Figure 3. The total N is given for the dates on which *in situ* N mineralization was measured (via leaching), and was computed as initial total N minus the cumulative leached N. Measured organic N is reported for the dates on which soils were ultrasonically fractionated[5]; the basis for the modeled organic N curve will be given later. (Gaseous losses were small as indicated by the small difference between the upper and lower curves near the end of the incubation.) There was a substantial accumulation of inorganic N in the middle of the incubation, as revealed by the large difference between total and organic N at such time. This accumulation of inorganic N is likely due to the infrequent leaching during the first half of the incubation. Such high concentrations of inorganic N would likely not be found in a field soil and may have led to some inhibition of N mineralization (e.g. no net change in measured organic N between day 34 and 95). Nevertheless, Figure 3 indicates that N mineralization after 14 months was substantial and corresponded to about 30% of the initial N.

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[5] On Day 11, no whole soil sample was taken prior to ultrasonic fractionation, and whole-soil organic N was estimated as follows:

organic N = initial whole soil N – leached inorganic N – inorganic N recovered immediately after sonication.

On Days 34, 95, and 190, organic N was measured on whole soil after removal of inorganic N with 0.01 M $CaCl_2$, as described in Materials and Methods. On Day 439, organic N was estimated as whole soil N minus inorganic N recovered immediately after sonication.

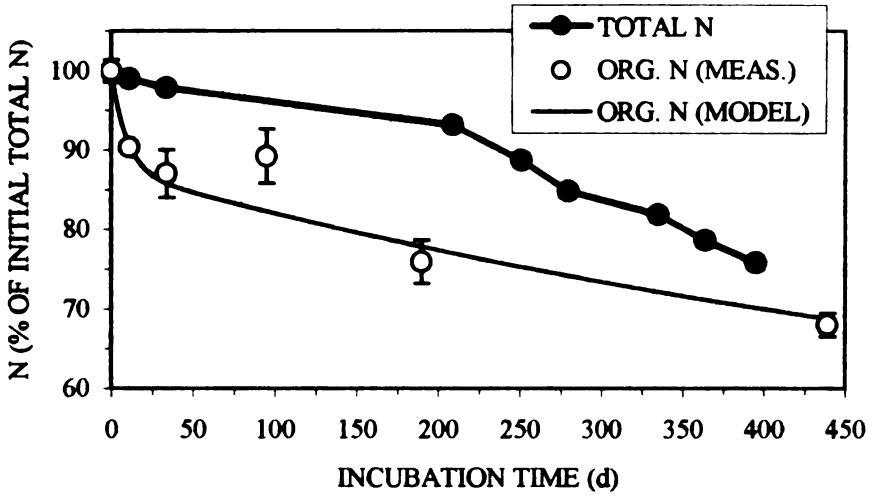

Figure 3. N remaining after mineralization during soil incubation. (Error bars represent sample standard deviations and are invisible where error < 1% of initial total N.)

The overall C mineralization is shown in Figures 4 (respiration) and 5 (whole soil C). The initial respiration rates of the clover-amended soil were extremely high, corresponding to 5 and 3 % of initial soil C per day after 1 and 5 days, respectively. These rates reflect the rapid microbial attack of labile organic constituents, such as sugars and amino acids. Respiration rates were very slow (< 0.08% of initial soil C per day) later in the incubation, probably as a result of increased proportions of slow-degrading compounds such as cellulose or hemicellulose. (See Paul and Clark (1996) for relative decomposition rates of various substrates.) The respiration data were modeled with the two-pool constrained model as described in Materials and Methods (Equation 2); the model is appropriate as indicated by the close agreement between the measured and modeled values (Figure 4) and the relatively low standard errors of the parameter estimates (Table 6). The two C pools in the respiration model do not sum to 100 because it was assumed that 45% of the native (non-clover) C was completely resistant to mineralization throughout the incubation, as explained in Materials and Methods.

The modeled respiration data are contrasted with the whole soil C data as obtained by dry combustion in Figure 5. As with the respiration data, the combustion data were reasonably modeled by assuming the existence of two C pools with non-zero decay constants and a third resistant pool (Figure 5, Table 6). The sharp difference in slope between the combustion and respiration curves during the first month of incubation is attributed to methodology. Respiration was measured only on days for which the soil was at optimum water content and thus was probably overestimated: Occasionally (eight times during the study), the soil water content substantially exceeded the optimum value as a result of the leaching procedure for measurement of *in situ* N mineralization (see

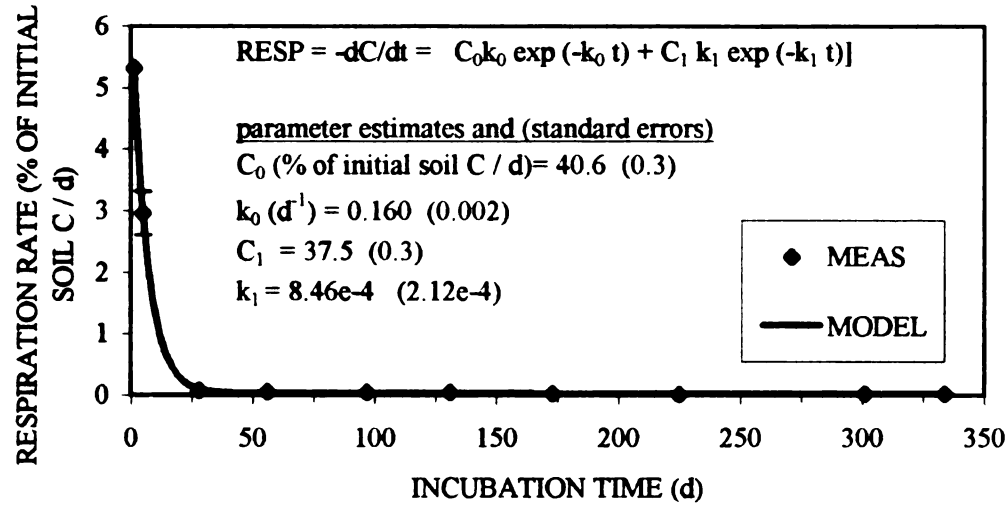

Figure 4A. Respiration rate as function of soil incubation time. About 51% of the initial soil C was derived from the clover amendment. Error bars represent sample standard deviations.

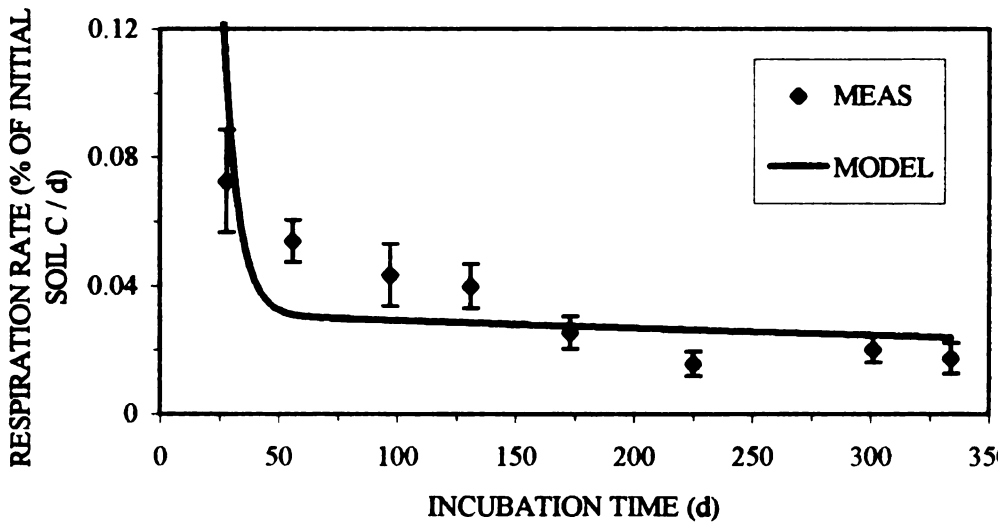

Figure 4B. Expanded view of Figure 4A.

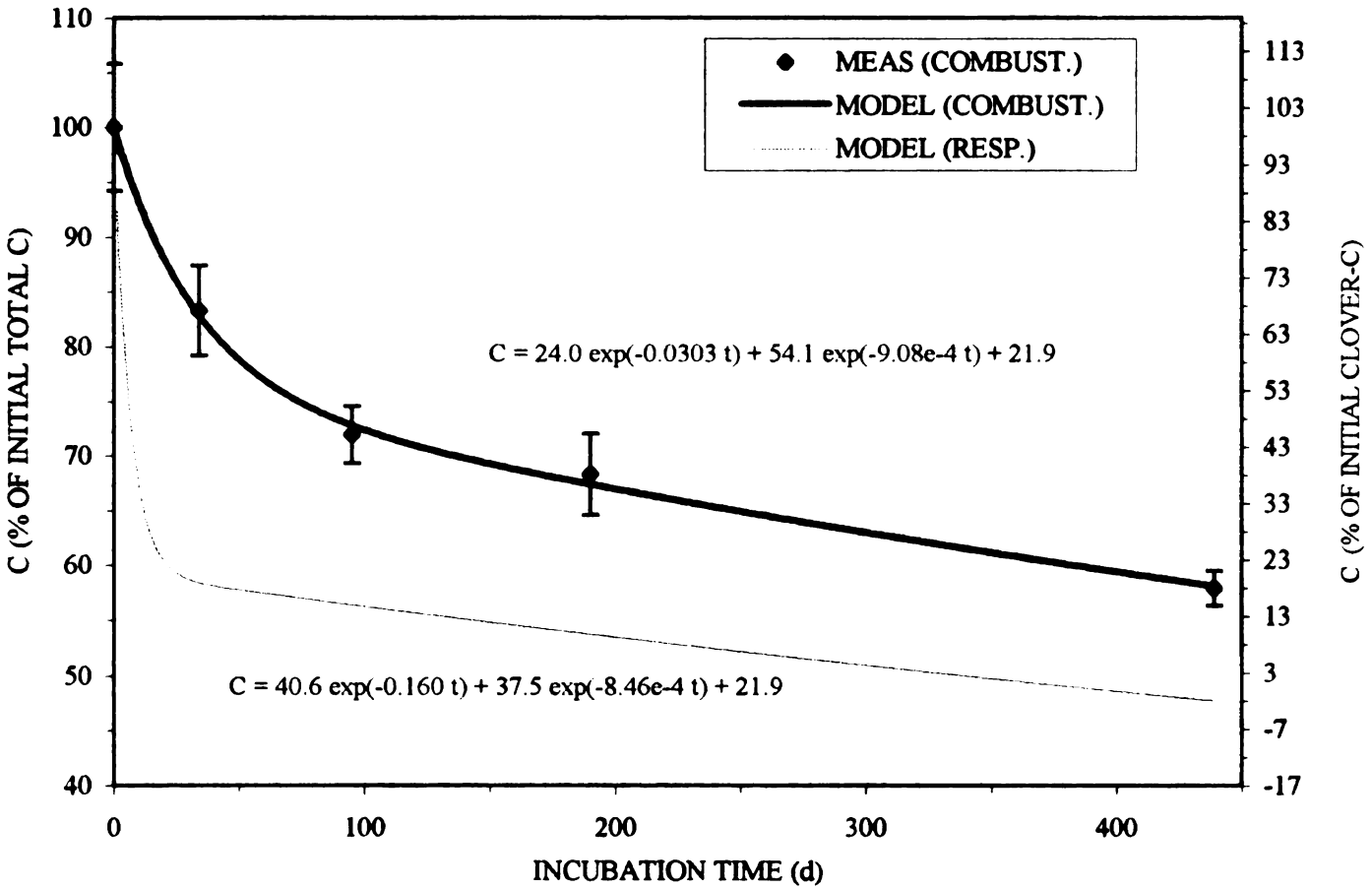

Figure 5. C remaining in whole soil as determined by dry combustion of whole soil and by respiration. The modeled respiration data are based on 10 measurement dates between Days 1 and 334.

Table 6. Summary of kinetic modeling data. †

organic matter fraction	equation number in text	equation with parameter estimates and standard errors
whole soil C (respiration)	2	respiration rate = (0.160 ± 0.002) (40.6 ± 0.3) exp[-(0.160 ± 0.002) t] + (8.46e-4 ± 2.12e-4) (37.5 ± 0.3) exp[-(8.46e-4 ± 2.12e-4) t] C = (40.6 ± 0.3) exp[-(0.160 ± 0.002) t] + (37.5 ± 0.3) exp[-(8.46e-4 ± 2.12e-4) t] + 21.9
whole soil C (combustion)	-	C = (24.0 ± 1.8) exp[-(0.0303 ± 0.0051) t] + (54.1 ± 1.8) exp [-(9.08e-4 ± 1.16e-4) t] + 21.9
whole soil clover-N	4	N = (24.7 ± 5.1) exp[-(0.106 ± 0.071) t] + (75.3 ± 5.1) exp[-(1.51e-3 ± 0.35e-3) t]
fine clay clover-N	6	N = -(6.67 ± 8.70) exp[-(0.0410 ± 0.1143) t] + (10.4 ± 5.0) exp[-(1.05e-3 ± 1.79e-3) t]
coarse clay clover-N	6	N = -(36.7 ± 11.9) exp[-(0.0661 ± 0.0335) t] + (44.6 ± 3.1) exp[-(1.30e-3 ± 0.29e-3) t]
total clay clover-N	6	N = -(33.0 ± 12.6) exp[-(0.0404 ± 0.0336) t] + (57.6 ±7.7) exp[-(1.28e-3 ± 0.51e-3) t]
fine silt clover-N	4	N = (11.8 ± 1.4) exp[-(0.0266 ± 0.0082) t] + (9.22 ± 1.44) exp[-(1.37e-3 ± 0.52e-3) t]
coarse silt clover-N	5	N = (27.7 ± 3.0) exp[-(0.160 ± 0.010) t] + (0.289 ± 0.014)
sand clover-N	4	N = (27.2 ± 0.8) exp[-(0.121 ± 0.003) t] + (0.466 ± 0.031) exp[-(1.97e-3 ± 0.33e-3) t]
whole soil native-N	-	no significant change during incubation
fine clay native-N	7	N = (0 ± 26.2) + (13.4 ± 25.5) {1 - exp[-(0.0651 ± 0.1600) t]}
coarse clay native-N	7	N = (0 ± 45.3) + (42.4 ± 44.5) {1 - exp[-(0.0870 ± 0.0949) t]}
total clay native-N	7	N = (0 ± 88.1) + (56.8 ± 86.8) {1 - exp[-(0.0965 ± 0.1399) t]}
fine silt native-N	5	N = (7.06 ± 3.94) exp[-(0.0153 ± 0.0230) t] + (27.4 ± 2.4)
coarse silt native-N	5	N = (27.8 ± 13.8) exp[-(0.115 ± 0.046) t] + (5.99 ± 0.30)
sand native-N	3	N = (17.4 ± 4.8) exp[-(5.07e-3 ± 3.74e-3) t]

† Units are as follows: respiration rate = % of initial whole soil C per day, C = % of initial whole soil C, clover-N = % of initial whole soil clover-N, native-N = % of initial whole soil native-N, rate constants = day^{-1}

Materials and Methods). The excess water from the leaching solution required one day or more to evaporate and was likely inhibitory to respiration. This inhibitory effect may have been most pronounced during the first month of the incubation; the soils were leached twice during this period and the otherwise high respiration rate may have been greatly decreased. Accordingly, the combustion data of Figure 5 reflect soil C dynamics more accurately than do the respiration data.

The combustion data of Figure 5 indicate that about 40% of soil C was lost after 14 months. Thus, C mineralization (40%) exceeded N mineralization (30%, Figure 3); the explanation is that some mineralized N was again immobilized whereas mineralized C was permanently lost from the soil (see Introduction - C Mineralization). If it is assumed that the loss of native C was 6%[6], then about 75% of the clover C was mineralized (Figure 5, right-hand vertical axis).

N and C Mineralization/Immobilization in Whole Soils and Particle-Size Fractions

Recovery of Mass in Particle-Size Fractions

The particle-size yields following ultrasonic fractionation are shown in Table 7. Although the particle-size distributions for the five incubation times are in general agreement with the expected distribution, some differences are evident. Most notably,

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[6] This assumption is reasonable as judged by the data of Collins et al. These researchers collected agricultural soils from Michigan, Minnesota, Ohio, and Wisconsin, removed visible plant fragments, incubated the soils at 25 °C with optimum water content, and measured respiration. The respiration data was fit to the two-pool constrained model of Paul et al. (in press) as in the present study. Based on their model parameters, the average C mineralization in topsoils after 14 months would be 11±3% of the initial C. In the present study, non-clover C comprised about 50% of total C, and thus the expected mineralization of native C would correspond to only about 6% (50% x 0.11) of total C.

there was incomplete dispersion on Day 11 and variable fine clay recovery on the other dates. These discrepancies reflect difficulties in methodology and almost certainly do not represent true differences in the particle-size distributions; N and C amounts in the size fractions were adjusted accordingly (Appendix 5). The N and C amounts in each fraction were also adjusted for mass losses during particle separation:

corrected N or C in fraction = measured N or C in fraction / (% recovery / 100)

The percent recovery in the above equation is equal to:

100 x soil mass recovered in all fractions / unfractionated soil mass

(See Table 7.)

Dynamics of Clover-N, Native-N, and C

The amounts of clover-derived N in the size fractions and whole soil as a function of incubation time are given in Table 8. On Day 11, about one third of the initial clover-N resided in the silts and sand; an additional one third of the clover-N was found in the clays. The N found in the coarse silt and sand almost certainly is macroorganic matter (MOM). Direct visual observation or microscopy (50 X) revealed the presence of relatively large pieces of organic matter in the coarse silt and sand; this type of material was absent from the clays and fine silt. The N associated with the clays and fine silt probably represents sorbed organic matter, and may also include small pieces of organic

Table 7. Recovery of mass in ultrasonic particle-size fractionations. †

fraction	expected distribution ‡	incubation time (d)				
		11	34	95	190	439
fine clay (< 0.2 μm)	3.81 ± 0.56	1.32	3.55	4.29	3.15	4.24
coarse clay (0.2 - 2 μm)	12.4 ± 0.7	7.90	12.1	12.1	13.4	12.1
fine silt (2 – 10 μm)	12.0 ± 0.4	13.8	12.4	12.1	11.9	11.6
coarse silt (10 – 53 μm)	15.4 ± 2.9	16.2	17.0	16.8	16.9	17.9
sand (53 – 2000 μm)	56.3 ± 3.1	60.8	54.9	54.7	54.7	54.1
SUM	100	100	100	100	100	100
recovery (%) §	-	93	93	93	91	93

† Data are percent of recovered mass, unless otherwise noted.

‡ Means and sample standard deviations from preliminary study as described in Materials and Methods and Table 3.

§ Recovery (%) = 100 x soil mass recovered in all fractions / unfractionated soil mass

material with entrapped mineral particles. This sorbed and fine OM likely originated from solutes and small particles of the clover and from recent microbial products. About 15% of the initial clover-N was mineralized during particle-size separation of the Day 11 soil (Table 8). The original location of this mineralized N is unknown. This N is likely part of an active organic matter pool; as evidence, note that the N mineralized during particle-size separation declined throughout the incubation (Table 8).

Table 8. Clover-derived N in whole soils and size fractions (as % of clover-N initially present in whole soil). †

N o attempt was made to separate inorganic and organic N on Day 439. Thus, the reported value probably includes a small amount of inorganic N. (Note that whole soil organic N ≈ sum of fractions on this day.)

†† Whole soil organic N minus sum of fractions.

Table 8. Clover-derived N in whole soils and size fractions (as % of clover-N initially present in whole soil). †

fraction	incubation time (d)					
	0 ‡	11	34	95	190	439
fine clay (< 0.2 μm)	-	6.16 (0.10)	7.84 (0.07)	11.1 (0.8)	6.39 (0.49)	7.22 (0.35)
coarse clay (0.2 – 2 μm)	-	26.3 (2.6)	38.7 (4.1)	40.5 (4.0)	33.2 (0.1)	25.8 (0.7)
total clay	-	35.9 (2.6)	46.1 (4.1)	53.0 (4.1)	42.1 (0.4)	33.8 (0.8)
fine silt (2 – 10 μm)	-	18.0 (0.5)	13.3 (0.4)	9.41 (0.27)	6.87 (0.20)	5.14 (0.17)
coarse silt (10 – 53 μm)	-	5.06 (0.26)	0.410 (0.014)	0.268 (0.023)	0.315 (0.024)	0.285 (0.014)
sand (53 – 2000 μm)	-	7.69 (0.38)	0.888 (0.057)	0.377 (0.022)	0.336 (0.022)	0.189 (0.015)
sum of fractions		66.6 (2.8)	60.7 (4.1)	63.0 (4.1)	49.6 (0.5)	39.5 (0.9)
whole soil organic N	100.0 (2.5)	82.0 § (1.2)	71.3 ¶ (1.7)	70.0 ¶ (4.9)	50.5 ¶ (2.9)	41.1 # (1.2)
N mineralized during particle-size separation ††	-	15.4 (3.0)	10.6 (4.4)	6.94 (6.43)	0.863 (2.927)	1.63 (1.48)

† Data are means with sample standard devations in parentheses. Data are based on isotope dilution equations, as described in Appendix 1. The data have been adjusted for (1) loss of soil mass during particle-size separation, (2) incomplete dispersion on Day 11, and (3) variable clay recovery. Because of these adjustments, total clay and sum of fractions cannot be directly calculated from the data in the table. See text of Results for details.

‡ Soil was not fractionated on Day 0.

§ No whole soil sample was taken on Day 11. Whole soil organic N for this day was estimated as described in the text.

¶ On Days 34, 95, and 190, whole soil organic N was determined as described in Materials and Methods.

The measured and modeled dynamics of clover-N are depicted graphically in Figure 6; model parameters and standard errors are summarized in Table 6. The whole soil organic clover-N was fit to a two-pool exponential decay model, constrained such that the sum of the two pools was equal to the initial clover N (Figure 6A). Given that the loss of native-N from the incubated soil was negligible (as will be shown later) and that clover-N comprised 51% of the initial soil N, the model equation for loss of both native and clover N from whole soil (Figure 3) is computed as:

$$N\ (\%\ \text{of initial}) = 12.6 \exp(-0.106\ t) + 38.4 \exp(-1.51e\text{-}3\ t) + 49 \qquad (12)$$

Although one might expect C and N mineralization to follow similar models (with perhaps slower decay constants for N), the pool sizes and rate constants in Equation 12 are substantially different from the equation for loss of C (as determined by combustion, Figure 5). The C equation is restated below for ease of comparison:

$$C\ (\%\ \text{of initial}) = 24 \exp(-0.0303\ t) + 54.1 \exp(-9.08e\text{-}4\ t) + 21.9$$

The differences probably are a result of the oversimplification in the C model; it was not possible to model the clover- and native-C separately. The large difference between the rate constants in the fast pool may be a result of the lack of data for whole soil C on Day 11; perhaps the initial decline in C in Figure 5 was faster than predicted by the modeled combustion data.

Despite the aforementioned differences between the modeled C and N losses from whole soil, Figure 6 and Table 6 indicate that the dynamics of clover-N within whole soil

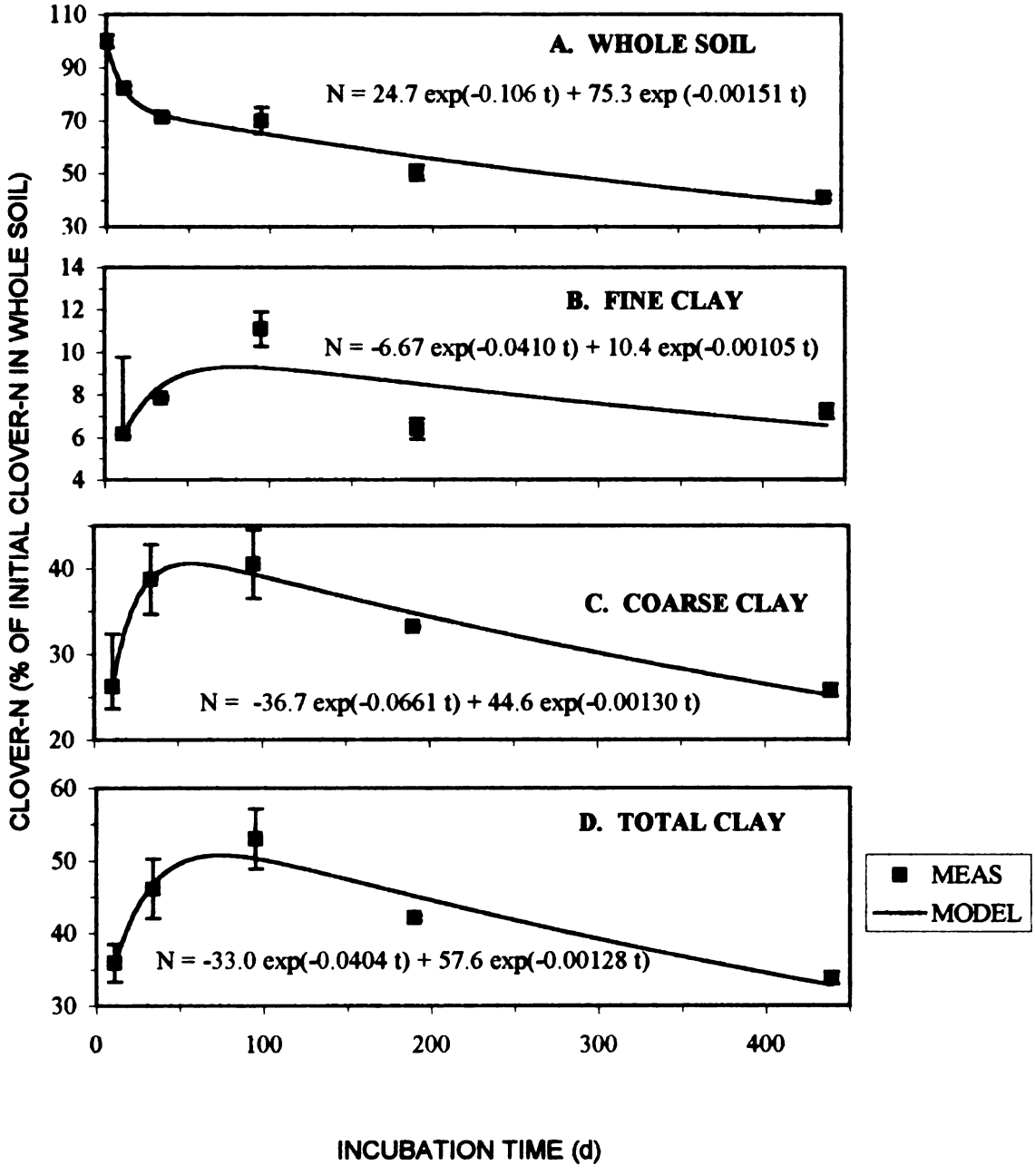

Figure 6. Clover-N amounts in incubated soil.

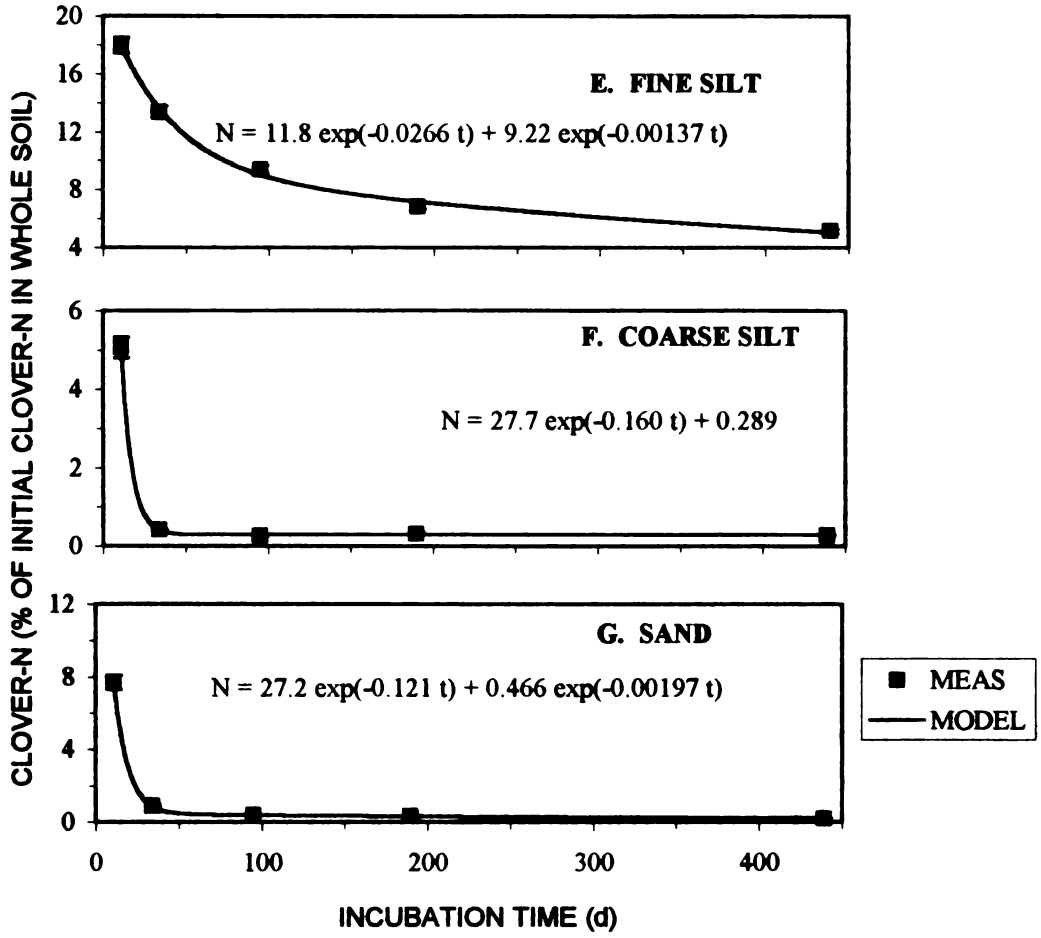

Figure 6 (cont'd.). Clover-N amounts in incubated soil.

and particle-size fractions are described reasonably well by simple kinetic models. Sand- and coarse silt-associated N were characterized primarily by a pool of fast-decaying organic N ($k = 0.12$ to $0.16\ d^{-1}$) which almost certainly represents macroorganic matter (MOM). The fine silt contained two clover-N pools; the faster pool had an intermediate decay rate ($k = 0.03\ d^{-1}$) and probably was MOM (with entrapped mineral particles) in an intermediate stage of decomposition. The slow pool within the fine silt ($k = 0.001\ d^{-1}$) is attributed to sorbed N. The clay fractions exhibited fast accumulation ($k = 0.04$ to $0.07\ d^{-1}$) followed by slow depletion ($k = 0.001\ d^{-1}$) of clover-N, and were modeled with the assumption that both processes followed an exponential trend (Appendix 2). (The large positive error bars for fine and coarse clay on Day 11 reflect incomplete soil dispersion (Appendix 5).) The accumulation is considered a result of the transfer of N from coarser fractions, and the depletion reflects the slow mineralization of sorbed N. The ratios of the standard errors to the parameter estimates are rather large for the clay fractions and for the fast-decaying pool of whole soil N (Table 6), but this probably is due to the inherent difficulty in fitting models with three or four unconstrained parameters to only five or six data points.

The overall dynamics of the clover-N in the size fractions can be summarized as follows: Upon amendment of soil with clover, soluble and particulate N rapidly distribute such that clover-N is found in all fractions. The coarser fractions (sand, coarse silt, and to a lesser extent fine silt) are dominated by macroorganic nitrogen and the finer fractions (clays, and to a lesser extent fine silt) are dominated by sorbed N. The macroorganic N is rapidly attacked ($k = 0.12$ to $0.16\ d^{-1}$), and the resulting microbial

products accumulate as sorbed N in the clays. Thereafter, the clay-sorbed N is slowly mineralized ($k = 0.001\ d^{-1}$), acting as a long-term nutrient source.

The dynamics of native (non-clover) native N are presented in Table 9 and Figure 7. The distribution of native N on Day 11 is similar to that of clover-N, except that the proportion of native N in the coarser fractions (> 2 μm) exceeded the proportion of clover-N in said fractions (compare Tables 8 and 9). This is attributed to the possible mineralization of relatively labile macroorganic clover-N during particle-size separation, as well as to the grinding of the clover prior to the incubation.

Figure 7A shows that there was little or no net mineralization of native organic N in the whole soil throughout the 14-month incubation. The large amendment of N-rich clover and the high concentrations of inorganic N during much of the incubation (see Figure 3) explain this observation. The measured and modeled data for the size fractions (Figure 7B-G) are similar to that of clover-N in that a rapid depletion of N in the coarse fractions is accompanied by an accumulation of N in the clays. The movement of apparently labile native N from the sand- and coarse silt-associated MOM to the clays *without subsequent decomposition of the clay-associated N* (in contrast to the case of clover-N) is difficult to explain. In the N-rich (C-limited) condition of this study, native MOM may have been attacked primarily to satisfy microbial C requirements. The disintegration of the MOM may have released resistant N compounds, which were then sorbed on clays. The relatively poor model fits for native N as compared to clover-N (Table 6, Figures 6 and 7) are probably due to the higher uncertainty of the native N amounts as well as the paucity of data points during times of native N accumulation or depletion within the fractions.

Table 9. Native N in whole soils and size fractions (as % of native N initially present in whole soil). † ‡

No attempt was made to separate inorganic and organic N on Day 439. Thus, the reported value probably includes a small amount of inorganic N. (Note that whole soil organic N ≈ sum of fractions on this day.)

†† Whole soil organic N minus sum of fractions.

Table 9. Native N in whole soils and size fractions (as % of native N initially present in whole soil). † ‡

fraction	incubation time (d)					
	0 §	11	34	95	190	439
fine clay (< 0.2 μm)	-	3.73 (0.41)	16.6 (0.8)	10.0 (1.5)	12.7 (1.1)	14.8 (2.1)
coarse clay (0.2 – 2 μm)	-	25.1 (4.4)	42.7 (11.6)	41.5 (7.4)	38.3 (1.8)	45.7 (3.9)
total clay	-	36.0 (4.4)	58.2 (11.6)	52.8 (7.6)	52.9 (2.2)	62.2 (4.6)
fine silt (2 – 10 μm)	-	31.9 (2.0)	34.4 (2.5)	27.5 (2.3)	26.8 (2.1)	28.6 (1.2)
coarse silt (10 – 53 μm)	-	13.8 (1.0)	6.55 (0.32)	6.07 (0.19)	6.47 (0.31)	5.44 (0.16)
sand (53 – 2000 μm)	-	22.3 (1.9)	7.85 (0.53)	9.69 (0.26)	8.01 (0.90)	3.82 (0.44)
sum of fractions		104.0 (5.5)	107.0 (11.9)	96.1 (8.0)	94.2 (3.2)	100.0 (4.7)
whole soil organic N	100.0 (2.5)	ND	103.9 ¶ (9.5)	110.2 ¶ (14.2)	104.2 ¶ (10.2)	103.2 # (6.4)
N mineralized during particle-size separation ††	-	ND	-3.03 (15.20)	14.1 (16.3)	10.0 (10.6)	3.18 (7.96)

† Data are means with sample standard devations in parentheses. Data are based on isotope dilution equations, as described in Appendix 1. The data have been adjusted for (1) loss of soil mass during particle-size separation, (2) incomplete dispersion on Day 11, and (3) variable clay recovery. Because of these adjustments, total clay and sum of fractions cannot be directly calculated from the data in the table. See text of Results for details.

‡ ND = not determined

§ Soil was not fractionated on Day 0.

¶ On Days 34, 95, and 190, whole soil organic N was determined as described in Materials and Methods.

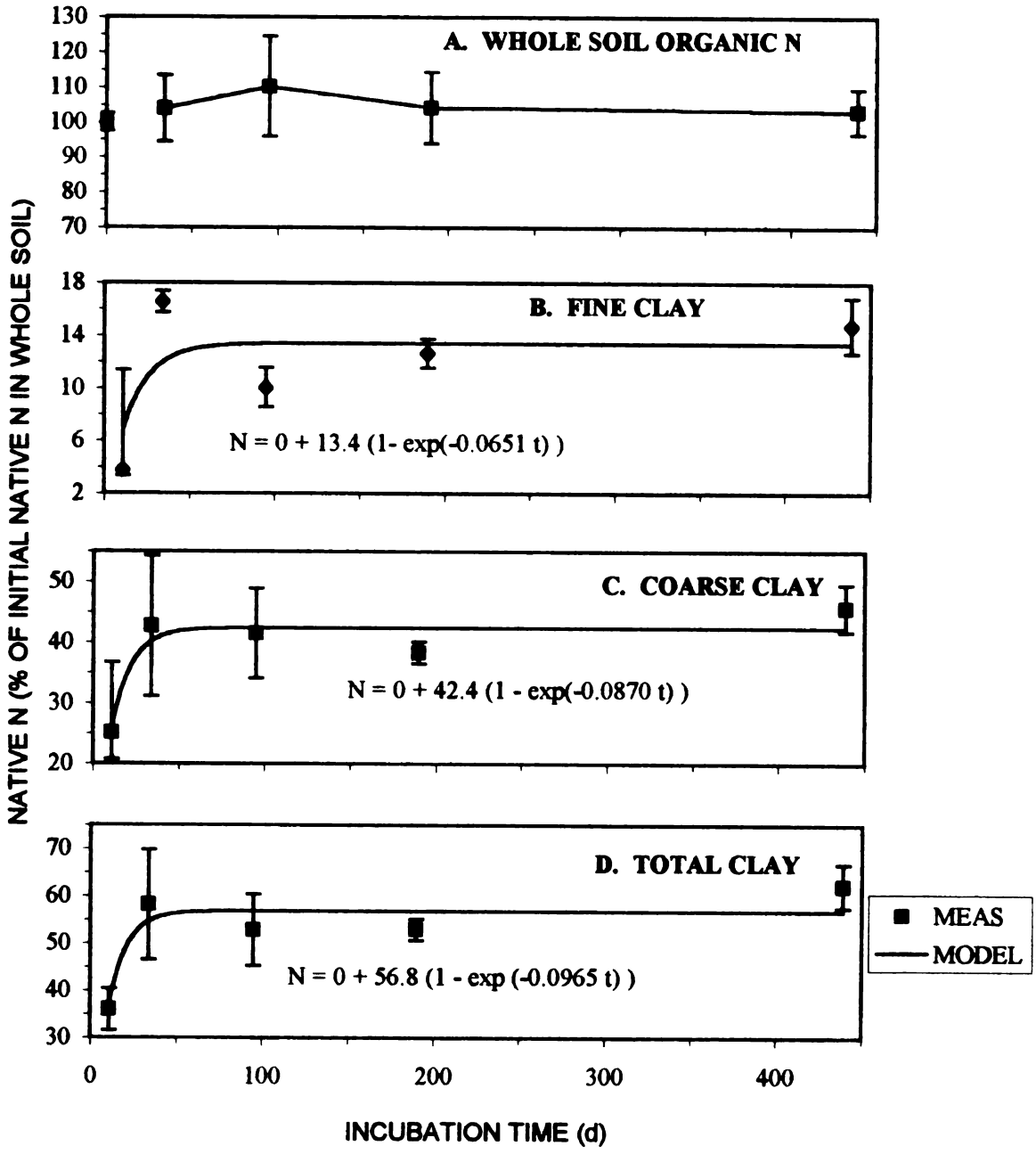

Figure 7. Native N amounts in incubated soil.

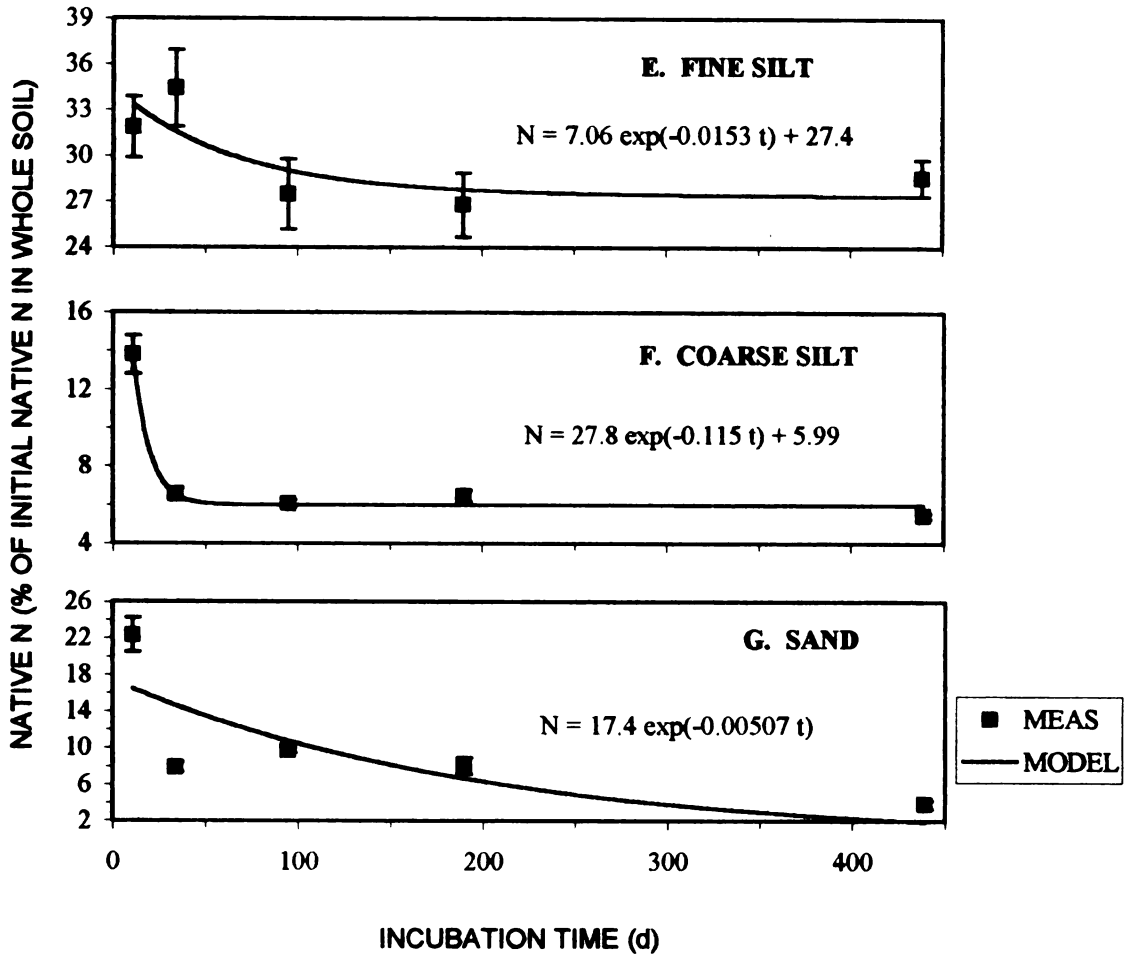

Figure 7 (cont'd.). Native N amounts in incubated soil.

The dynamics of C (clover plus native) within the soils and particle-size fractions are given in Table 10 and Figure 8. As in the case of N, significant amounts of C were mineralized during particle-size separation except on Day 439 (Table 10). The trends in C are similar to those of N; disappearance of C in the coarse fractions (> 2 μm) is accompanied by accumulation of C in the clays. Although no decomposition of clay-associated C is evident (Figure 8A-C), it may be that the mineralization of labile clover-derived C and the accumulation of resistant native C occurred simultaneously in the clay fractions. The net decrease in sand-associated C on each measurement date (Figure 8) suggests that POM could have provided C to the clay fractions throughout the incubation.

Mineralization of N in Whole Soils and Particle-Size Fractions in Aerobic Shaken Slurries

An aerobic shaken slurry N mineralization test was conducted on the whole soils and particle-size fractions from Days 34, 190, and 439 of the incubation of clover-amended soil, as described in Materials and Methods. The test provided additional detailed information about the N in the size fractions, and, ideally, reflected the capacity of the mineral-associated OM to supply inorganic N in the absence of aggregate

Table 10. C (native- plus clover-derived) in whole soils and size fractions (% of C initially present in whole soil). † ‡

fraction	incubation time (d)					
	0 §	11	34	95	190	439
fine clay (< 0.2 μm)	-	2.56 (0.06)	6.97 (1.02)	6.16 (0.93)	5.79 (0.86)	7.36 (1.11)
coarse clay (0.2 – 2 μm)	-	15.2 (0.3)	28.4 (1.3)	27.5 (0.6)	27.8 (0.3)	26.6 (0.1)
total clay	-	22.8 (0.3)	34.9 (1.3)	34.4 (0.6)	35.6 (0.4)	34.8 (0.3)
fine silt (2 – 10 μm)	-	19.9 (0.3)	25.3 (0.6)	19.2 (2.0)	16.2 (0.7)	17.6 (0.2)
coarse silt (10 – 53 μm)	-	9.16 (0.07)	5.18 (0.13)	3.93 (0.03)	4.34 (0.08)	4.67 (0.11)
sand (53 – 2000 μm)	-	18.9 (0.6)	8.24 (0.07)	7.10 (0.18)	5.74 (0.92)	4.03 (0.29)
sum of fractions		70.8 (0.8)	73.6 (1.5)	64.6 (2.1)	61.8 (1.2)	61.2 (0.5)
whole soil C	100.0 (5.8)	ND	83.3 (4.1)	72.0 (2.6)	68.4 (3.7)	58.0 (1.6)
C mineralized during particle-size separation ¶	-	ND	9.69 (4.36)	7.40 (3.31)	6.52 (3.87)	-3.15 (1.69)

† Data are means with sample standard deviations in parentheses. The data have been adjusted for (1) loss of soil mass during particle-size separation, (2) incomplete dispersion on Day 11, and (3) variable clay recovery. Because of these adjustments, total clay and sum of fractions cannot be directly calculated from the data in the table. See text of Results for details.

‡ ND = not determined

§ Soil was not fractionated on Day 0.

¶ Whole soil C minus sum of fractions.

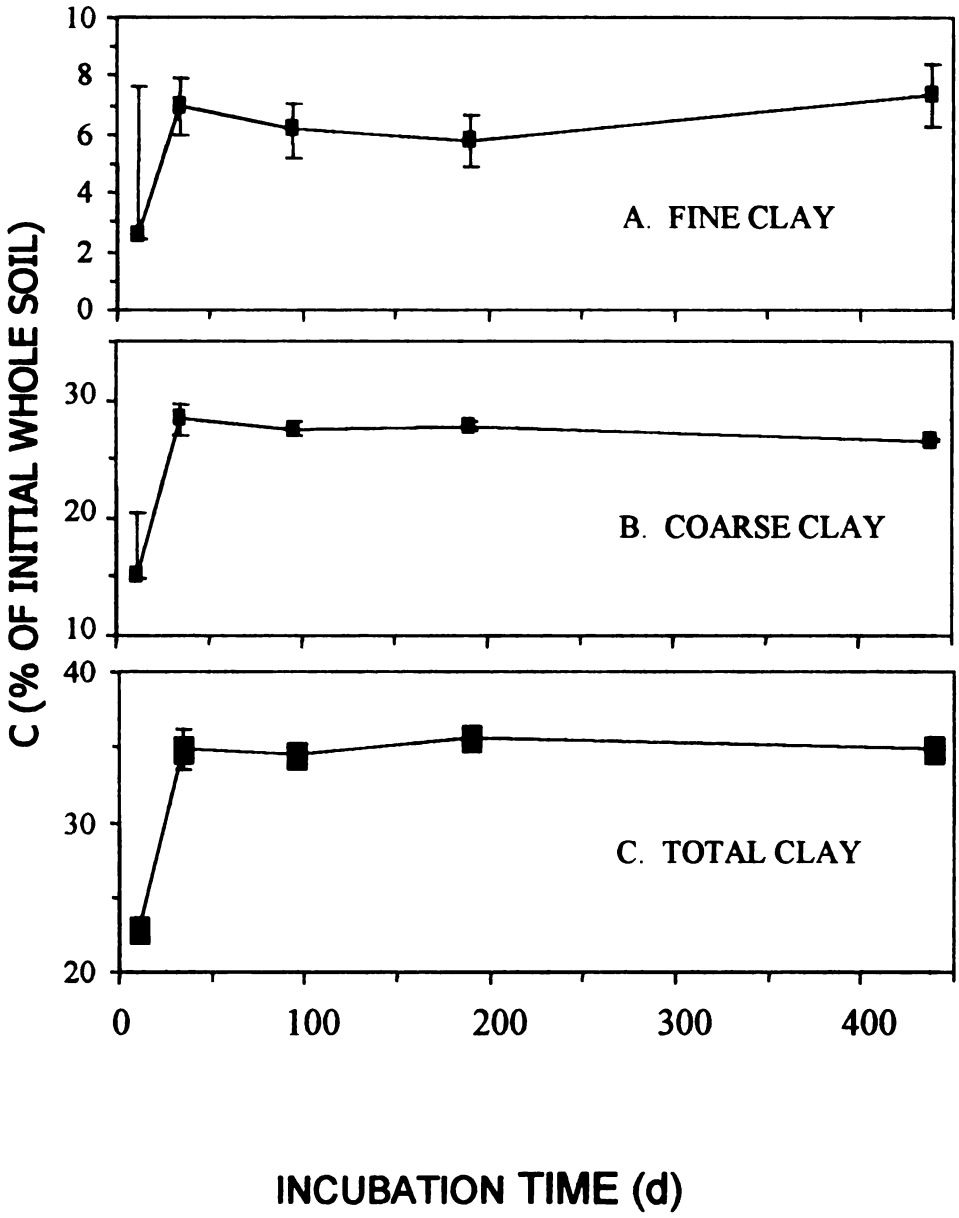

Figure 8. C amounts in incubated soil. About 51% of the initial soil C was due to clover.

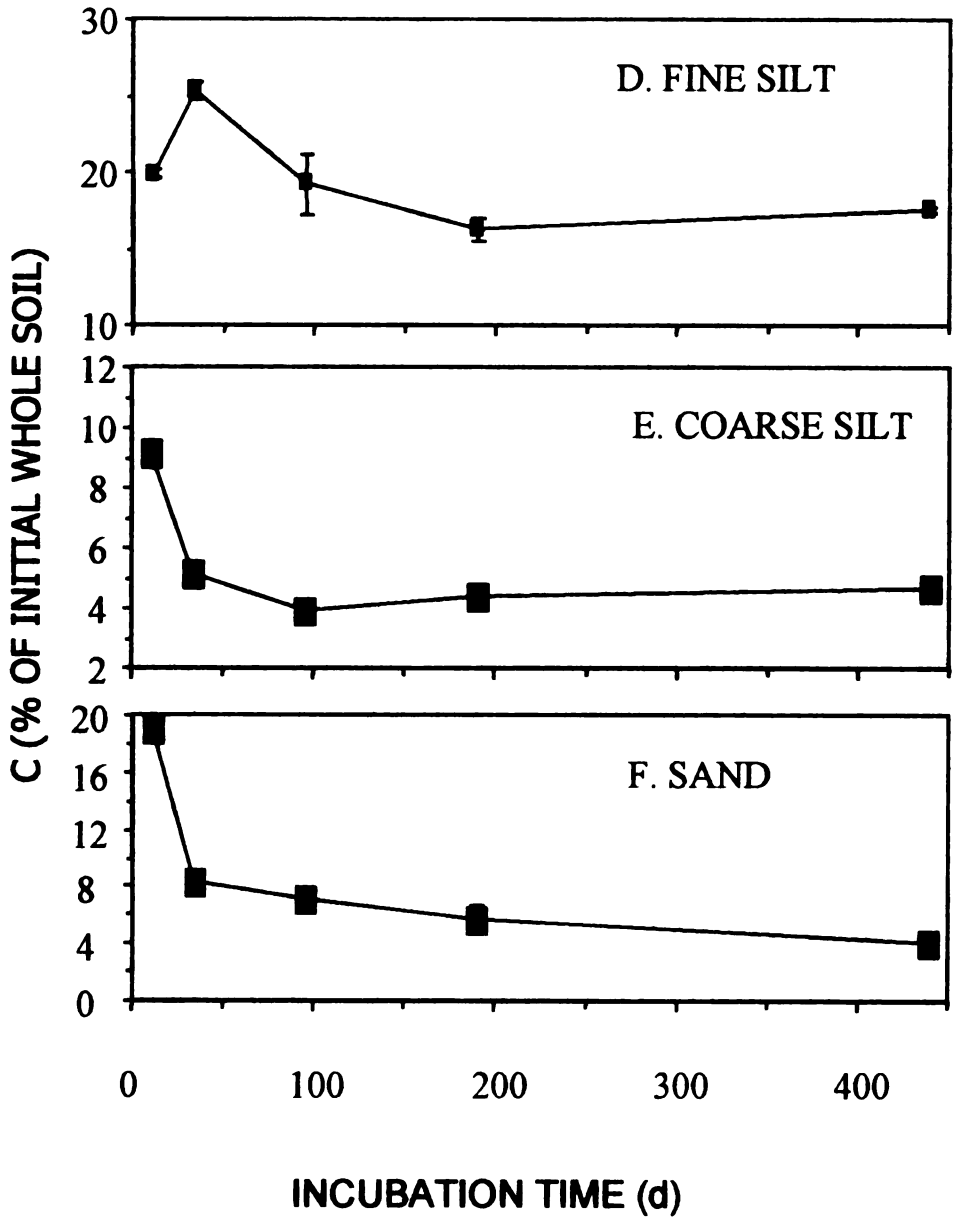

Figure 8. (cont'd.) C amounts in incubated soil. About 51% of the initial soil C was due to clover.

protection. One might argue that the shaken slurry N mineralization tests were ambiguous measures of aggregate protection because of greater diffusion of oxygen and inorganic nutrients in the shaken slurries relative to the undisturbed soil. However, oxygen did not limit biological activity in an undisturbed silt loam soil (Rovira and Greacen, 1957; see Introduction of present study), and would not be a likely limiting factor in the sandy loam soil of the present study. The undisturbed soil of this study was probably well-supplied with inorganic nutrients via the clover amendment, which was grown in Hoagland's nutrient solution. The periodic leachings of the undisturbed soil with N-free nutrient solution supplied additional inorganic nutrients.

The shaken slurry mineralization in whole soil is considered a measure of aggregate protection because prolonged shaking in water has been shown to disaggregate (disperse) soils (Edwards and Bremner, 1967). Edwards and Bremner (1967) found that shaking of soils for 10 days with 18 mL water per g soil resulted in clay yields that were, on average, 76% of those obtained with the highly effective resin method (shaking of soil with an Na-saturated exchange resin). No attempt was made to measure the dispersion of whole soil in shaken slurry in the present study, and the high water:soil ratios (24 to 30 mL per g) may have resulted in less than complete dispersion. Thus the aggregate protection results for whole soil should be viewed as semiquantitative.

Nitrogen mineralization in aerobic shaken slurry ranged from slightly negative numbers (immobilization) to 12% of the organic N initially present in the slurry (Table 11). Thus, one to twelve % of the N in the whole soil, fine clay, coarse clay, and fine silt was aggregate-protected. The non-mineralized N in these fractions was evidently stabilized by other mechanisms, such as adsorption per se or humification. There was

Table 11. Thirty-day N mineralization in shaken slurries (as percent of organic N in fraction). † ‡

incubation time prior to fractionation (d)	fraction					
	whole soil	fine clay	coarse clay	fine silt	coarse silt	sand
34	8.51 (2.29)	11.7 (0.9)	5.23 (0.73)	0.752 (0.090)	-0.448 (0.252)	-0.257 (0.478)
190	5.26 (5.24)	7.07 (1.15)	4.66 (0.29)	2.35 (0.45)	0.246 (0.234)	0.138 (0.255)
439	8.16 (2.22)	4.14 (0.91)	2.52 (0.15)	1.19 (0.22)	0.417 (0.376)	1.24 (1.25)

† Data are means in with sample standard deviations in parentheses.

‡ No separate information about clover-N and native N is available. (The ^{15}N content of mineralized N was not determined.)

very limited mineralization in the coarse silt and sand, which was probably a direct result of high C:N ratios in these fractions (Table 12). These fractions contained only a small portion of the soil N recovered on Days 34, 190, and 439 (Tables 8 and 9) and will be discussed no further.

The aggregate protection (N mineralization) of whole soil N between Days 34 and 439 did not change (Table 11). This is surprising, as one would expect aggregate-protected N to be humified with time. Perhaps real differences are masked by the variability of the whole soil data[7]. Alternatively, the high nitrate levels in the Day 34 whole soil (Figure 3) may have inhibited N mineralization.

The relationship of 30-day N mineralization in the shaken slurries to particle size, time (i.e. days of incubation prior to fractionation), and C:N ratio was investigated by analysis of variance (ANOVA) (GLM procedure of SAS; SAS Institute, 1989). Coarse silt, sand, and whole soil were excluded from the ANOVA because of inhibition of mineralization by high C:N ratios in coarse silt and sand, and high variability in the whole soil, as discussed earlier. Both 30-day N mineralization (NMIN) and its natural logarithm (ln(NMIN)) were subjected to analysis of variance because scatterplots and linear regressions revealed that the relationship between NMIN and C:N ratio was more exponential than linear (Figure 9). When only time and particle size were considered as sources of variation, ANOVA of both NMIN and ln(NMIN) revealed a highly significant interaction of time and particle size (Table 13). When C:N ratios were considered as well

[7] The high uncertainties in the whole soil data stem from the relatively large values of N_0 (initial inorganic N) in Equation 8. As discussed previously, inorganic N levels were high during much of the 14-month incubation (Figure 3); subtraction of the large N_0 value from the similarly large N_{30} value in Equation 8 results in considerable uncertainty.

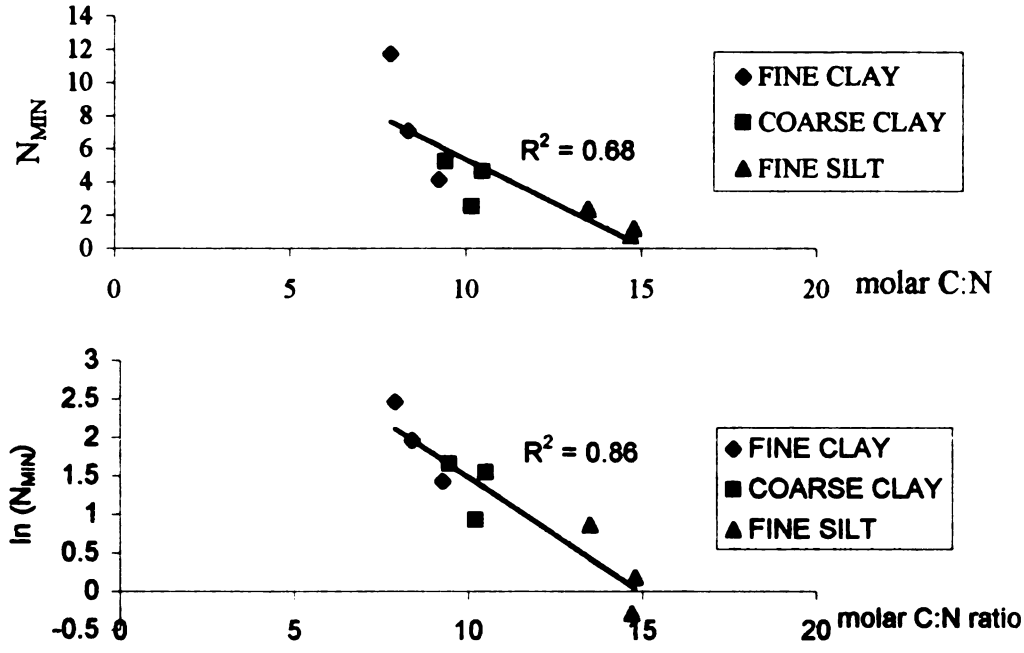

Figure 9. NMIN and ln(NMIN) versus molar C:N ratio. NMIN = 30-day N mineralization in aerobic shaken slurry as percent of organic N in fraction. Data points are averages of two or three replicates.

Table 12. Soil C/N ratio (molar basis) as a function of incubation time.

fraction	incubation time (d)					
	0 †	11	34	95	190	439
whole soil	13.47 (0.8)	ND ‡	11.1 (0.5)	9.4 (0.1)	8.63 (0.26)	11.2 (0.1)
fine clay (< 0.2 μm)	ND	6.84 (0.24)	7.90 (0.04)	7.82 (0.26)	8.39 (0.10)	9.27 (0.74)
coarse clay (0.2-2 μm)	ND	7.96 (0.21)	9.46 (0.87)	9.04 (0.12)	10.5 (0.1)	10.2 (0.3)
fine silt (2-10 μm)	ND	11.0 (0.3)	14.7 (0.2)	14.5 (1.0)	13.5 (0.0)	14.8 (0.2)
coarse silt (10-53 μm)	ND	13.5 (0.3)	21.4 (0.4)	17.9 (0.4)	18.5 (0.3)	23.5 (0.5)
sand (53-2000 μm)	ND	17.6 (0.4)	27.0 (1.5)	20.3 (0.6)	19.8 (1.2)	29.1 (2.7)

† The C/N ratio on Day 0 was computed from the C and N contents of the soil and clover amendment.

‡ ND = not determined

as time and particle size, ANOVA revealed that all three factors were significant sources of variation (Table 13). The important interpretation is that 30-day N mineralization is significantly related to (1) the balance of C and N availabilities, (2) the extent of previous decomposition (time), and (3) fundamental differences in the nature of each size class of organo-mineral complexes.

Specific pre-planned comparisons among the size fractions are presented in Table 14; these were derived from the SAS least-squares means option within the ANOVA of NMIN versus (time, particle size, time x particle size). For a given time, the data show that NMIN decreased significantly ($p < 0.05$) as follows: fine clay > coarse clay > fine silt.

Table 13. Analysis of variance of 30-day N mineralization in aerobic shaken slurries.†

dependent variable ‡	source of variation §	p-value
N_{MIN}	time	0.0001
	particle size	0.0001
	time x particle size	0.0001
N_{MIN}	time	0.0780
	particle size	0.0116
	C:N ratio	0.4708
$\ln(N_{MIN})$	time	0.0001
	particle size	0.0001
	time x particle size	0.0001
$\ln(N_{MIN})$	time	0.0016
	particle size	0.0166
	C:N ratio	0.0006

† Only fine clay, coarse clay, and fine silt were included in the analysis of variance per reasons stated in the text. For details of analyze of variance, see Appendix 6.

‡ $N_{MIN} = 100 \times (N_{30} - N_0 - N_{CTRL}) / (N_T - N_0)$ (See Materials and Methods.)

§ time = incubation time prior to fractionation

Table 14. Significance levels for pre-planned comparisons of 30-day N mineralization values in shaken slurries. †

incubation time prior to fractionation (d)	fine clay > coarse clay	fine clay > fine silt	coarse clay > fine silt
34	5e-5	5e-5	5e-5
190	0.03	0.0007	5e-5
439	0.003	5e-5	5e-5

† Tests are for differences *within* rows. Comparisons were derived from pre-planned one-tailed tests of least squares means in SAS. See text for additional details.

Aggregate protection factors (APF) are complementary to the above analyses of shaken-slurry N mineralization, and are useful for distinguishing among N stabilization mechanisms. The APF is defined as follows:

$$\text{APF} = \frac{\text{30-d N mineralization in shaken slurry}}{\text{30-d N mineralization expected in undisturbed soil}}$$

The expected N mineralization in the undisturbed soil was computed on the basis of the kinetic model equations for clover- and native-N given earlier; see Appendix 7 for details.

The whole soil APF showed no change with time (Table 15). (The apparent difference in the aggregate protection factor between Days 34 and 439 is not statistically significant ($p > 0.1$ for t-test).) This finding is surprising because one might expect APFs to decrease as humification proceeds. Possible reasons for unexpected findings for whole soil were given earlier (high nitrate, high variability).

The highest APFs were found in the fine clay (Table 15). This is reasonable as fine-clay associated N is expected to be less accessible to microbes compared to the N in other fractions. The decrease in APF with time for both fine and coarse clay suggests that humification was occurring in those fractions or that the organic N in those fractions was becoming more strongly bound to the mineral phase. The APF for fine silt on Day 34 is less than one. This result is difficult to explain, and may be due to uncertainty in the mineralization modeled for the undisturbed soil.

Table 15. Aggregate-protection factors for whole soil and particle-size fractions. † ‡

incubation time prior to fractionation (d)	whole soil	fine clay	coarse clay	fine silt
34	3.64 ± 0.98	10.6 ± 0.8 A a	2.67 ± 0.37 B a	0.0737 ± 0.0088 B
190	3.73 ± 3.75	6.20 ± 1.01 A b	2.44 ± 0.15 B a	1.63 ± 0.31 C
439	5.83 ± 1.59	3.70 ± 0.81 A b	1.67 ± 0.10 A b	1.69 ± 0.31 A

† Aggregate-protection factor = (30-day N mineralization in shaken slurry) / (30-day mineralization in undisturbed soil); see text and Appendices 7 and 8 for details.

‡ Different capital letters denote differences within rows. Different lowercase letters denote differences within columns. ($p = 0.10$) Whole soil was excluded from statistical tests due to its high variability, and no tests for time trend were performed on fine silt. These results are based on unplanned two-tailed t-tests; the stated p-value reflects the experimentwise error rate according to Bonferroni's inequality (Sachs, 1982).

The estimation of aggregate-protected pool sizes in the whole soils and size fractions constitutes another useful application of the shaken slurry N mineralization data. The aggregate-protected pool size is estimated as:

aggregate-protected pool (% of N in fraction) = (30-day shaken slurry N mineralization) – (30-day N mineralization in undisturbed soil)

This operational definition is similar to that used by Beare et al. (1994). The aggregate-protected pool sizes are shown in Table 16, and range from 0 to 11% of N in a given fraction or whole soil. The data indicate that the aggregate-protected pool sizes (as a percentage of N in fraction) decrease with increasing particle size. In the case of fine clay and coarse clay, the pool sizes decrease with time. These observations reinforce conclusions drawn from the APF data of Table 15. That is, aggregate protection is more pronounced in fine fractions, and its decrease with time in the fine fractions indicates that humification is occurring or that N is becoming more tightly bound to the mineral phase. No decrease in the aggregate-protected pool size was observed in whole soil; this may stem from the difficulty in detecting differences with highly variable data.

Table 16. Aggregate-protected pool sizes for whole soil and particle-size fractions (% of N in fraction). † ‡

incubation time prior to fractionation (d)	whole soil	fine clay	coarse clay	fine silt
34	6.17 ± 2.29	10.6 ± 0.9 A a	3.27 ± 0.73 B a	NC §
190	4.34 ± 5.96	5.93 ± 1.15 A b	2.75 ± 0.29 A a	0.91 ± 0.45 B a
439	6.76 ± 2.22	3.02 ± 0.91 A c	1.01 ± 0.15 A b	0.49 ± 0.22 B a

† Aggregate-protected pool size (% of N in fraction) = (30-day N mineralization in shaken slurry) – (30-day mineralization in undisturbed soil); see text and Appendices 7 and 8 for details.

‡ Different capital letters denote differences within rows. Different lowercase letters denote differences within columns. ($p = 0.10$) Whole soil was excluded from statistical tests due to its high variability. These results are based on unplanned two-tailed t-tests; the stated p-value reflects the experimentwise error rate according to Bonferroni's inequality (Sachs, 1982).

§ NC = not calculable (computed pool size is negative).

Test of Quantitative NMR

The CPMAS-^{15}N-NMR spectrum of the prepared mixture of ^{15}N-clover/^{15}N-uracil/unlabeled soil is shown in Figure 10. The two N atoms of uracil are evident as well as the amide, guanidinium, and amino N atoms of clover. The peak area percentages with and without correction for differential relaxation (per Equations 9, 10, and 11) are given in Columns 2 and 3 of Table 17. Differential relaxation effects were small for the sample since the relaxation-corrected peak area percentages are approximately equal to the uncorrected percentages; this is because (1) the delay time for the spectrum of Figure 10 (1.25 s) was substantially greater than the maximum value of T_{1H} (0.454 s, see Table 18), and (2) the correction factors for differential relaxation during the contact time were approximately equal for all functional groups (Table 18). With or without correction for relaxation, the NMR peak area percentages do not correspond to the known sample composition (Table 17, column 1). Peak overlap was considered a likely source of this error.

To correct for error due to peak overlap, the clover/uracil/soil spectrum was approximated as a linear combination of previous spectra taken of uracil/soil and clover/soil. After the statistical validity of this approach was confirmed, the relative contributions of uracil and clover to the clover/uracil/soil spectrum were computed (Table 17, Column 4 - see Appendix 9 for details). In this case, the peak area proportions correspond closely to the known sample composition. The important conclusion is that ^{15}N-NMR is quantitative in a complex soil sample for both heterocyclic and noncyclic N. However, significant errors may result from peak overlap in samples of unknown composition.

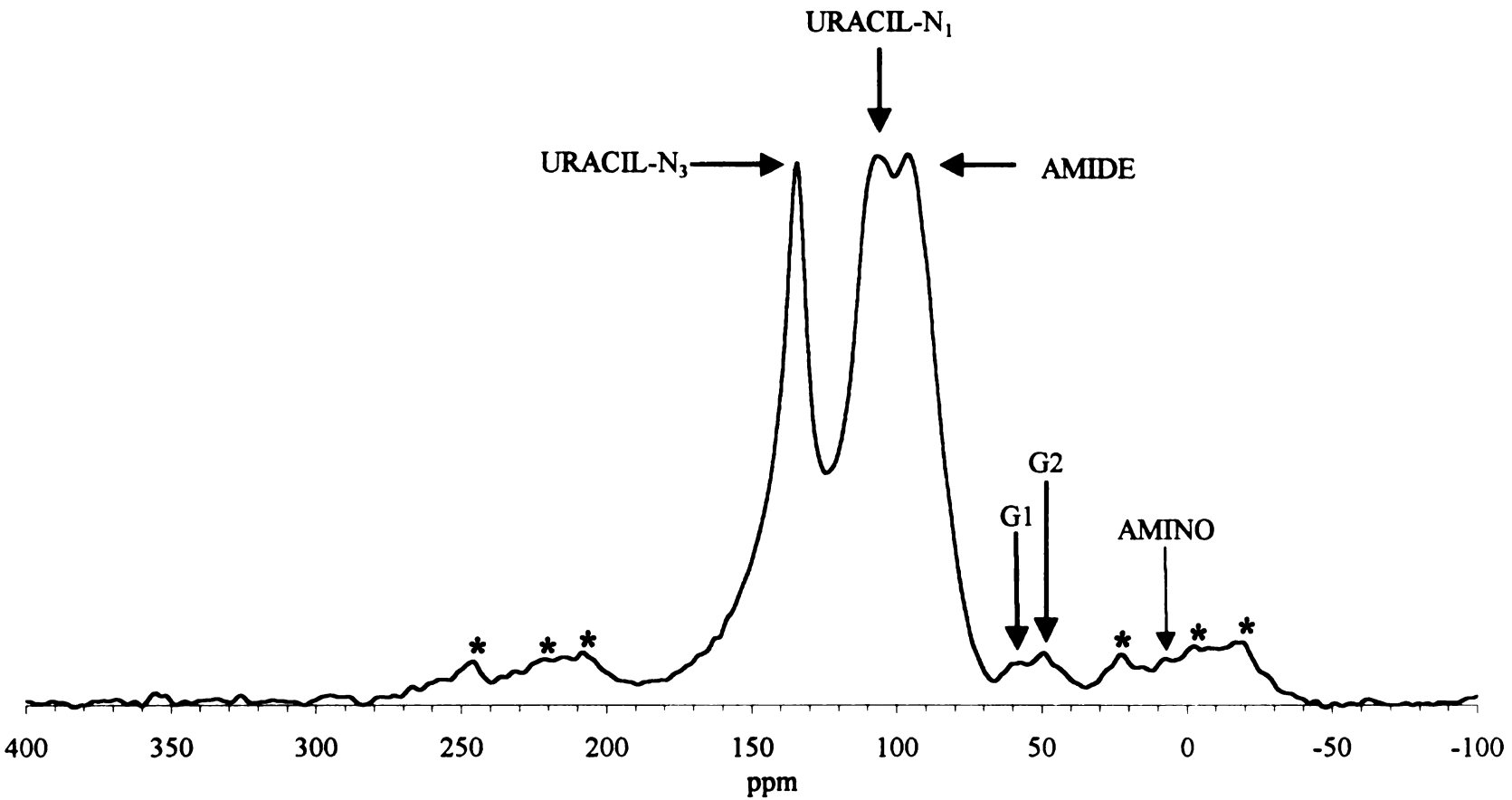

Figure 10. ^{15}N-NMR spectrum of prepared sample of ^{15}N-uracil + ^{15}N-clover + unlabeled soil. Contact time = 0.2 ms, delay time = 1.25 s. G1 = guanidinium -NH- of arginine, G2 = guanidinium -NH_2 of arginine, * = spinning sideband.

Table 17. Test of quantitative ^{15}N-NMR in prepared mixture of (^{15}N-uracil + ^{15}N-plant material + unlabeled soil).

^{15}N form	1. Known sample composition (percent of total N) ‡	2. ^{15}N-NMR peak area percentages (uncorrected) §	3. ^{15}N-NMR peak area percentages (corrected for differential relaxation)	4. ^{15}N-NMR peak area percentages (corrected for differential relaxation and peak overlap) ¶
uracil-N_3 †	25.2 (1.2)	33.1	34.2	22.2
uracil-N_1 †	25.2 (1.2)	30.3	30.4	24.7
clover (*Trifolium pratense* L.)	49.6 (3.7)	36.5	35.4	53.2
SUM	100	100	100	100

† Subscripts refer to ring positon of ^{15}N. (Uracil = $C_4H_4N_2O_2$, see Figure 2)

‡ Computed from ^{15}N concentrations in clover and uracil. Clover ^{15}N concentration was determined by mass spectrometer; uracil ^{15}N concentrations were provided by manufacturer; soil ^{15}N concentration was negligible. Sample standard devations (in parentheses) were computed based on uncertainty in masses, ^{15}N concentration, and water content of the mixture components.

§ NMR analyses were not replicated. The estimated uncertainties in peak area percentages due to phasing, baseline correction, and spectral noise were less than 0.5% of total signal intensity.

¶ Peak overlap corrections based on linear combination of uracil/soil and clover/soil spectra as described in Appendix 9.

Table 18. Relaxation data for prepared mixture of ^{15}N-clover + ^{15}N-uracil + unlabeled soil. †

functional group	T_{1H} (s)	T_{NH} (ms)	$T_{1\rho H}$ (ms)	correction factor for differential relaxation during contact time ‡
uracil-N_3 §	0.454 (0.256)	0.0882 (0.0164)	4.06 (0.91)	1.153
uracil-N_1 §	0.372 (0.084)	0.0813 (0.0117)	5.07 (0.98)	1.123
amide	NA ¶	0.0705 (0.0153)	4.75 (1.28)	1.094
guanidinium -NH-	NA	0.0479 (0.0265)	3.40 (1.74)	1.063
guanidinium -NH_2-	NA	0.0477 (0.0284)	1.57 (0.73)	1.121
amino	NA	0.0963 (0.0157)	5.10 (1.16)	1.173

† Standard errors in parentheses.

‡ Correction factor calculated per Equation 10, with contact time = 0. 2 ms.

§ Subscripts refer to ring position.

¶ NA = no dependence of signal intensity upon delay time was observed; functional group assumed to be fully relaxed between scans. (Minimum delay value tested was 0.75 s.)

NMR Spectroscopy of Incubated Soils

The CPMAS-NMR spectra of clover, whole soil, fine clay, coarse clay, and fine silt are shown in Figure 11. (No separate spectra were acquired for coarse silt and sand due to the low concentrations of ^{15}N in these fractions.) The peak area percentages (corrected for differential relaxation effects) are summarized in Table 19. Throughout the incubation, the N functional group composition of the whole soil and size fractions was not significantly different from that of pure clover; the organic N was approximately 90% amide, 5 - 10% guanidinium N of arginine, and 5% amino. (The identification of the guanidinium and amino peaks is explained below.)

The spectrum of pure clover (Figure 11A) is better resolved than the spectra of the soil samples and thus provides additional information on the organic N composition at the beginning of (and probably throughout) the incubation. The strong amide signal (95 ppm) suggests that the clover-N is mostly proteinaceous; therefore, the other peaks in the spectrum probably represent non-amide N within amino acids. The spectrum shows one peak at approximately 150 ppm, two peaks in the region 30-70 ppm, and two peaks in the region -10 to 20 ppm. The peak at 150 ppm is likely to be the heterocyclic N of histidine. Multiple functional groups have resonances in the region 30-70 ppm (Table 5), but stoichiometric considerations strongly suggest that the peaks at 58 and 45 ppm are the -NH- and $-NH_2$ units of the guanidinium group of arginine, respectively. The ratio of the relaxation-corrected peak areas (-NH- : $-NH_2$ = 1:2.5, data not shown) was close to the expected stoichiometric value (1:2) for the guanidinium group. The two peaks in the region -10 to 20 ppm probably represent the amino groups of free or amino-terminal amino acids; these peaks cannot be unambiguously assigned to specific amino acids

Table 19. NMR peak area percentages for clover and incubated soil. † ‡ §

material →	clover	whole soil			fine clay			coarse clay			fine silt		
incubation time (d) →	0	34	190	439	34	190	439	34	190	439	34	190	439
aromatic N of histidine	1.08	0	0	0	0	0	0	0	0	0	0	0	0
amide	85.3	85.1	85.6	84.5	85.6	83.9	86.0	91.5	91.1	89.6	89.4	87.1	88.4
guanidinium N's of arginine	7.98	8.35	8.37	8.81	8.35	9.23	7.68	5.64	5.97	7.15	7.28	8.01	7.28
amino	5.62	6.59	6.02	6.73	6.09	6.85	6.34	2.82	2.93	3.23	3.33	4.91	4.32
SUM	100	100	100	100	100	100	100	100	100	100	100	100	100

† Each data point is based on one (unreplicated) spectrum. Each spectrum underwent data processing twice (Fourier transformation + phasing + baseline correction), and final peak areas were caclulated as the means of the two peak areas.

‡ Due to peak overlap, spectral noise, and subjectivity in phasing and baseline correction, differences of less than 5 – 10 % of total signal intensity are probably not significant.

§ Data have been corrected for differential relaxation. These corrections altered the peak area percentages by no more than 3 % of total signal intensity.

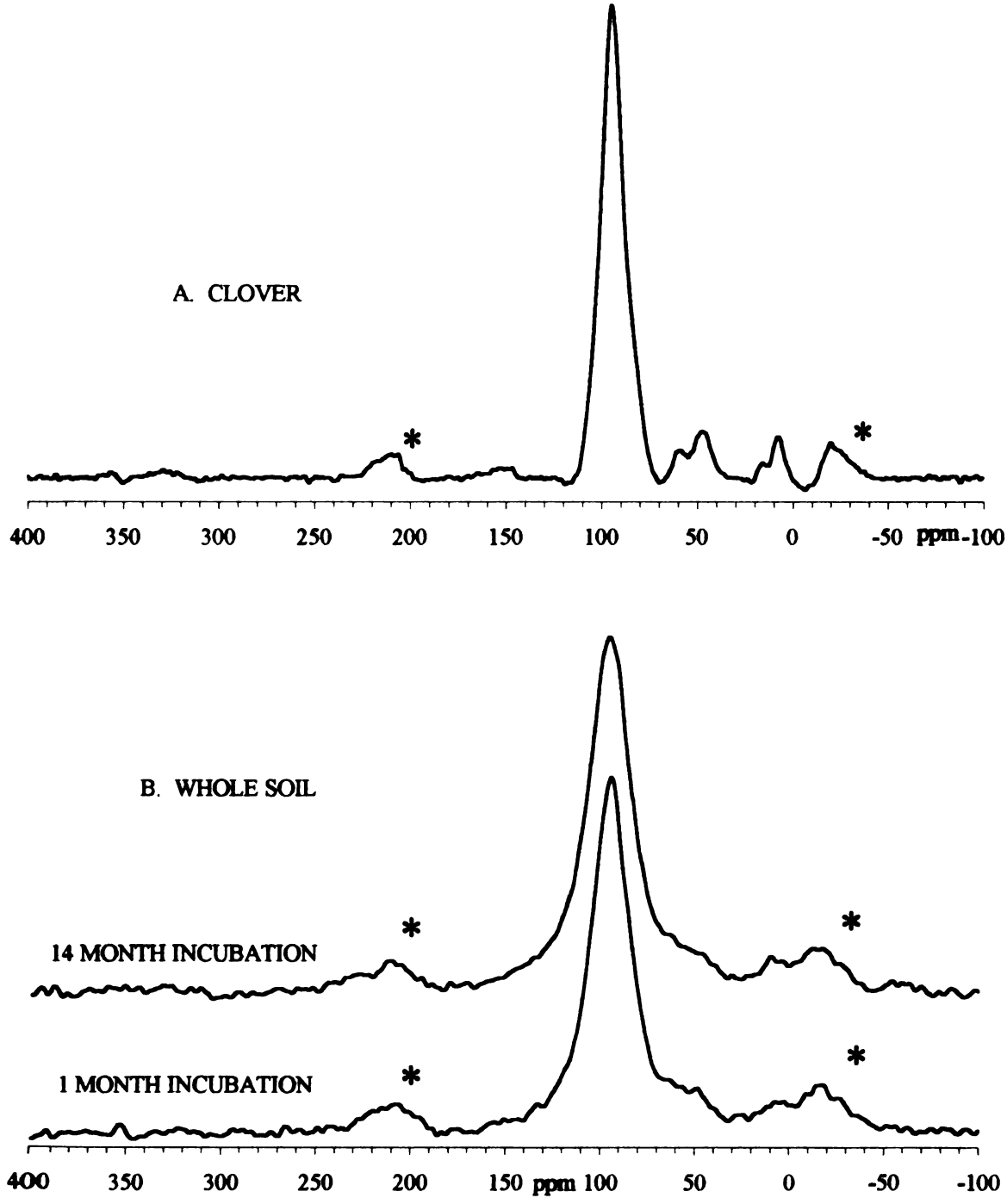

Figure 11. NMR spectra of clover and whole soil. * = spinning sideband Contact time = 0.4 ms, delay time = 1 s (clover) or 0.5 s (whole soil).

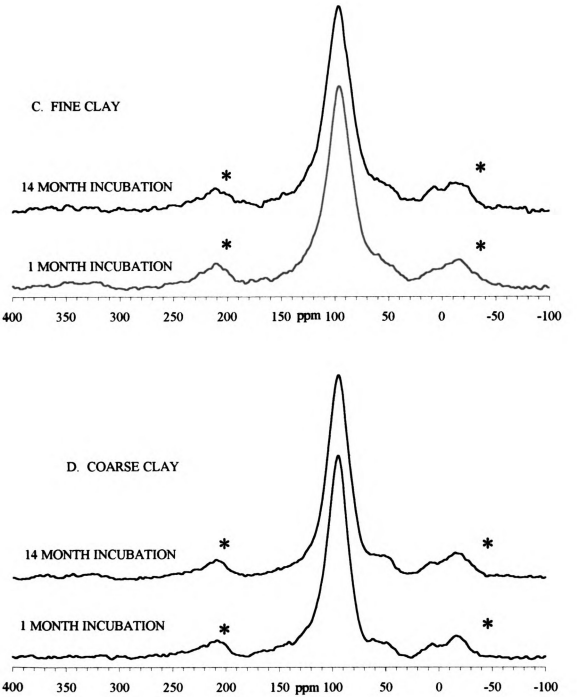

Figure 11 (cont'd). NMR spectra of fine clay and coarse clay. * = spinning sideband Contact time = 0.4 ms, delay time = 0.9 s (coarse clay, 1 month), 1 s (coarse clay, 14 month), or 0.5 s (fine clay).

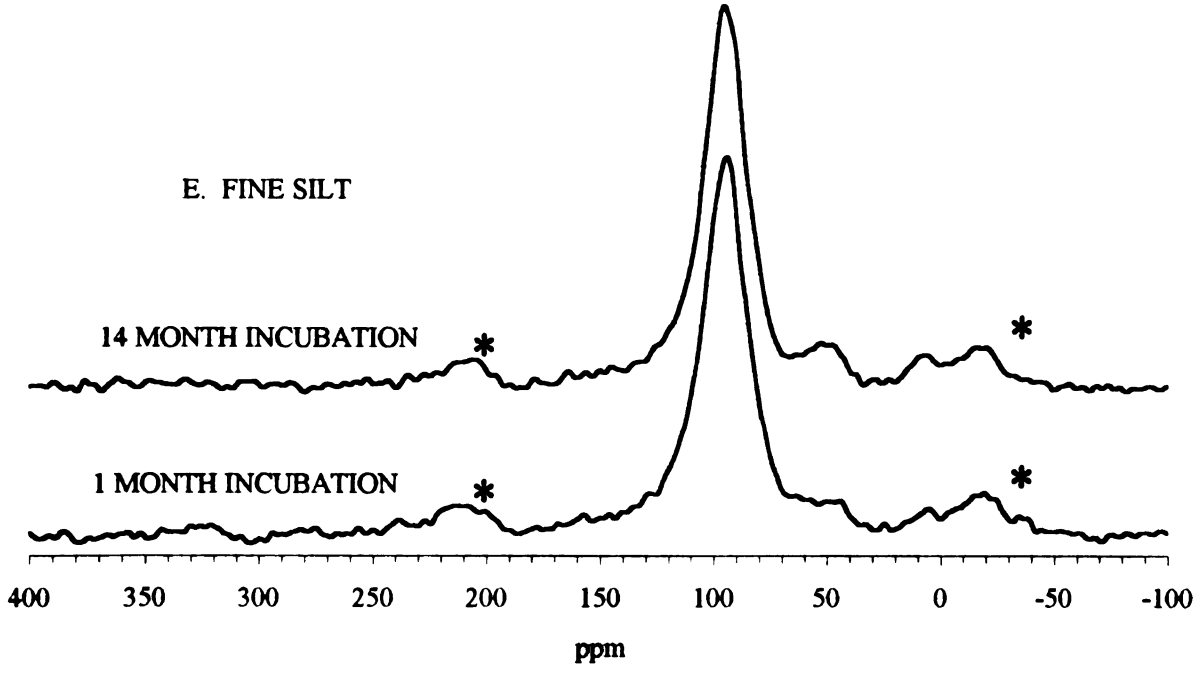

Figure 11 (cont'd). NMR spectra of fine silt. * = spinning sideband
Contact time = 0.4 ms, delay time = 0.75 s.

because the α-amino groups of all the common amino acids (except proline) have resonances in or near this region (Table 5 and Appendix 4).

As mentioned above, the NMR peak area percentages for clover and incubated soils were presented after correction for differential relaxation effects (Table 19). The overall corrections for differential relaxation effects were small; the maximum difference between corrected and uncorrected peak area percentages for each sample corresponded to no more than 3% of the total signal intensity. The nature and validity of the corrections is discussed below.

Relaxation during the delay time was characterized by the parameter T_{1H} (Equation 11). The ratios of the standard errors to the estimated values for T_{1H} were generally low, indicating that the T_{1H} model is correct (Table 20). A notable exception is the high-frequency amino group of clover. The standard error was high for this relatively small peak, which represented < 1% of the total spectral intensity.

The T_{1H} values (Table 20) were generally small compared to the delay times used in the NMR spectra of Figure 11 (t = 1 s for clover, 0.5 to 1 s for soil); thus the differences between the measured and T_{1H}-corrected signal intensities were small. The T_{1H} values for soil were in all cases lower than those for clover; this is attributed to the relatively high concentrations of paramagnetic species in soil (e.g. Fe^{3+}) (Pfeffer et al., 1984).

Differential relaxation during the contact time was also considered, and was characterized by the parameters T_{NH} and $T_{1\rho H}$ (Equation 9). The relatively low ratios of standard errors to parameter values (Tables 21 and 22) and the close correspondence between measured and modeled values (Figures 12, 13, and 14) indicate that the contact

Table 20. T_{1H} values (s) for clover and incubated soils. †

material	functional group					
	aromatic N of histidine	amide	guanidinium N's of arginine		amino	
			-NH	$-NH_2$		
clover ‡	0.365 (0.425)	0.531 (0.095)	NA §	0.520 (0.103)	0.972 (1.556)	0.829 (0.082)
whole soil	ND ¶	0.187 (0.006)	NA		0.523 (0.168)	
fine clay	ND	0.140 (0.047)	NA		NA	
coarse clay	ND	0.200 (0.051)	NA		NA	
fine silt	ND	0.175 (0.019)	NA		0.210 (0.056)	

† Standard error in parentheses. For the incubated soils, model parameters were generated only for samples that had been incubated for 34 d. The parameters were assumed to be equally applicable to soils that had been incubated for different times.

‡ For clover, two peaks were observed in both the guanidinium and amino regions. Values on the left correspond to the peaks with the higher NMR frequency.

§ NA = no dependence of signal intensity upon delay time was observed; functional group assumed to be fully relaxed. Minimum delay values tested were as follows: clover = 1 s, whole soil = fine clay = coarse clay = fine silt = 0.5 s.

¶ ND = functional group not detected.

Table 21. T_{NH} values (ms) as a function of material and functional group. †

material	functional group					
	imidazole N of histidine	amide	guanidinium N's of arginine		amino	
			-NH	$-NH_2$		
clover ‡	0.128 (0.053)	0.0588 (0.0089)	0.0496 (0.0181)	0.0599 (0.0056)	0.0605 (0.0583)	0.292 (0.103)
whole soil	ND §	0.0784 (0.0076)	0.0888 (0.0093)		0.187 (0.043)	
fine clay	ND	0.0757 (0.0089)	0.0846 (0.0219)		0.162 (0.100)	
coarse clay	ND	0.0623 (0.0035)	0.0553 (0.0224)		0.130 (0.060)	
fine silt	ND	0.0724 (0.0053)	0.0823 (0.0110)		0.268 (0.081)	

† Standard errors in parentheses. For the incubated soils, model parameters were generated only for samples that had been incubated for 34 d. The parameters were assumed to be equally applicable to soils that had been incubated for different times.

‡ For clover, two peaks were observed in both the guanidinium and amino regions. Values on the left correspond to the peaks with the higher NMR frequency.

§ ND = functional group not detected

Table 22. $T_{1\rho H}$ values (ms) as a function of material and functional group. †

material	functional group					
	imidazole N of histidine	amide	guanidinium N's of arginine		amino	
			-NH	$-NH_2$		
clover ‡	3.15 (1.48)	2.14 (0.28)	1.96 (0.58)	1.41 (0.11)	12.6 (29.3)	1.65 (0.59)
whole soil	ND §	3.24 (0.33)	1.95 (0.19)		1.95 (0.46)	
fine clay	ND	2.15 (0.23)	1.67 (0.40)		0.932 (0.558)	
coarse clay	ND	2.29 (0.12)	1.80 (0.60)		2.83 (1.42)	
fine silt	ND	3.17 (0.24)	2.19 (0.28)		2.60 (0.88)	

† Standard errors in parentheses. For the incubated soils, model parameters were generated only for samples that had been incubated for 34 d. The parameters were assumed to be equally applicable to soils that had been incubated for different times.

‡ For clover, two peaks were observed in both the guanidinium and amino regions. Values on the left correspond to the peaks with the higher NMR frequency.

§ ND = functional group not detected.

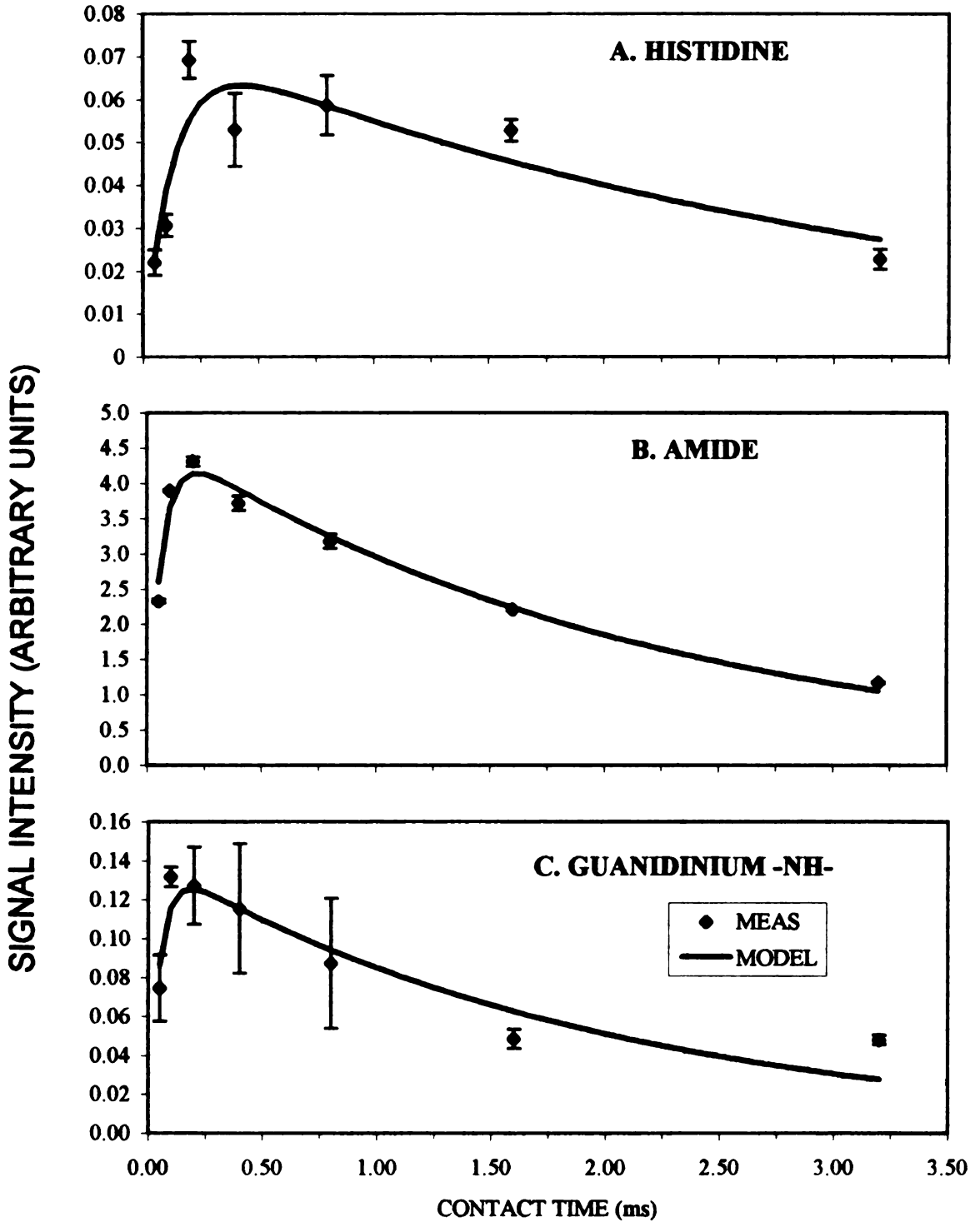

Figure 12. Measured and modeled signal intensities for clover as a function of contact time. Error bars represent uncertainty due to spectral noise and subjectivity in phasing/time. Error bars represent uncertainty due to spectral noise and subjectivity in phasing/baseline correction as described in Appendix 10.

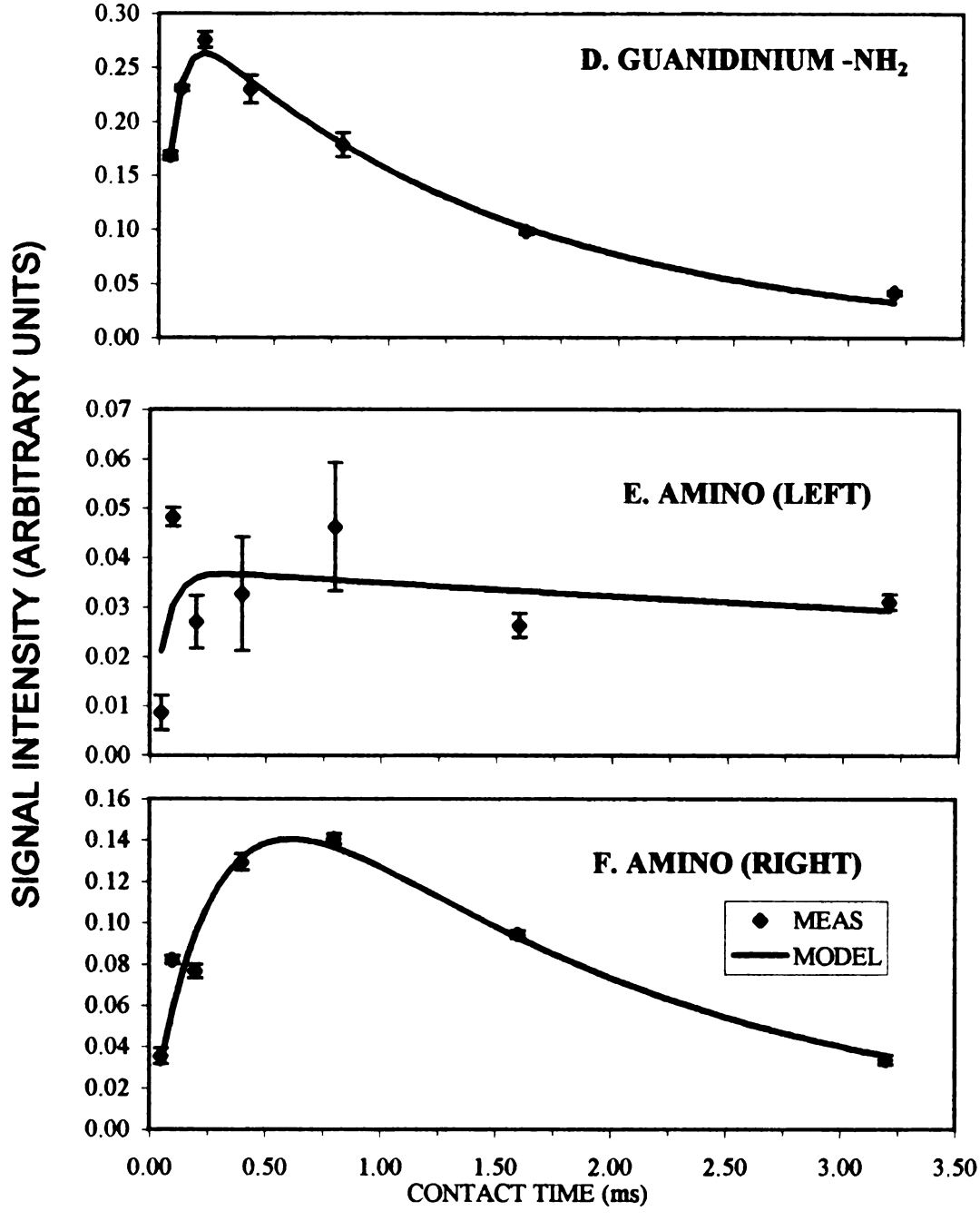

Figure 12. (cont'd) Measured and modeled signal intensities for clover as a function of contact time. Error bars represent uncertainty due to spectral noise and subjectivity in phasing/time. Error bars represent uncertainty due to spectral noise and subjectivity in phasing/baseline correction as described in Appendix 10.

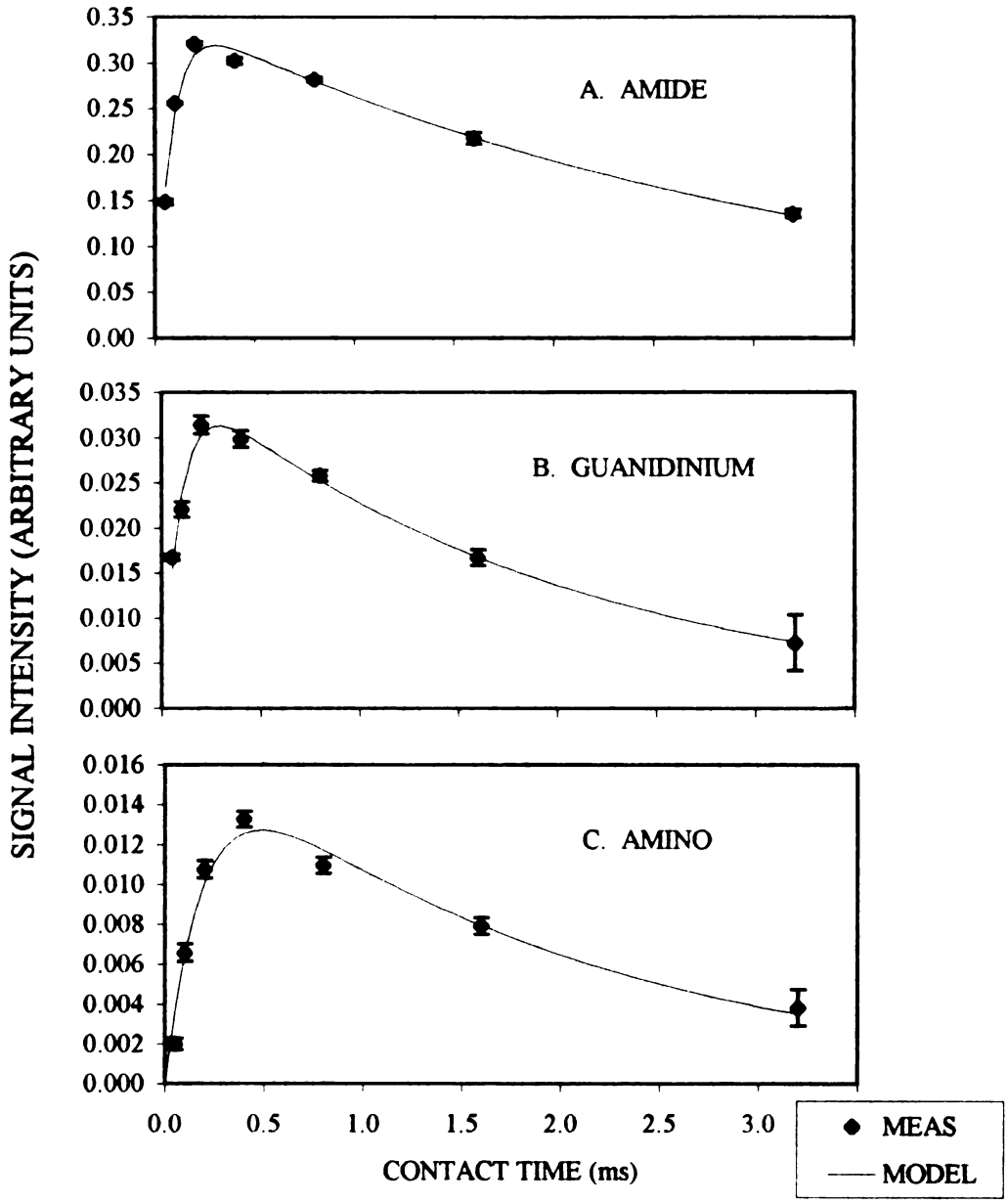

Figure 13. Measured and modeled signal intensities for whole soil as a function of contact time. Error bars represent uncertainty due to spectral noise and subjectivity in phasing/baseline correction as described in Appendix 10.

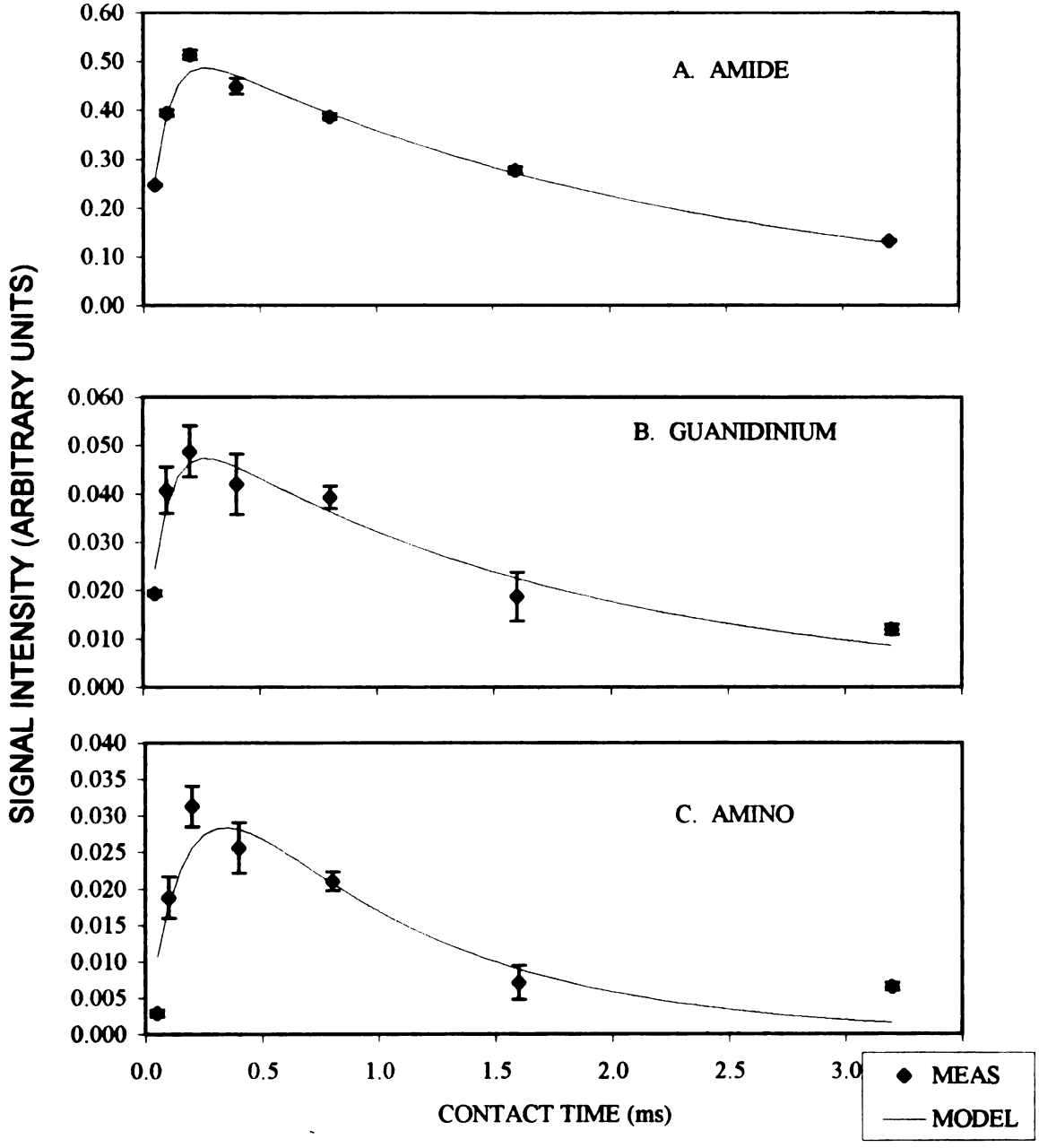

Figure 14. Measured and modeled signal intensities for fine clay as a function of contact time. Error bars represent uncertainty due to spectral noise and subjectivity in phasing/baseline correction as described in Appendix 10.

time model (Equation 9) was appropriate. The poorer fits for the amino group relative to other functional groups are likely a result of the relatively small peak area of amino as well as peak overlap (Figure 11). The correction factors for differential relaxation during the contact time were computed from T_{NH} and $T_{1\rho H}$ (Equation 10, Table 23). Within each sample, the ratio of the maximum to the minimum correction factor among the various functional groups varied from about 1 to 1.5. (A high maximum:minimum ratio indicates that differential relaxation effects may be significant.) The highest correction factor usually corresponded to the amino group, probably as a result of high T_{NH} values for amino (Table 21). High T_{NH} values are expected in relatively mobile functional groups such as amino (Kinchesh et al., 1995).

Correlation of the NMR spectral intensities with the amounts of ^{15}N in the incubated soil samples constitutes additional evidence of the quantitativeness of CPMAS-NMR. Linear regressions were run with ^{15}N amount and total NMR signal intensity per scan as dependent and independent variables, respectively. Regressions were run separately for each soil fraction with spectra that had been collected in a single NMR session; thereby confounding effects due to soil fraction (e.g. differential Fe contents) and variation in spectrometer performance were avoided. The correlations for whole soil, fine clay, coarse clay, and fine silt were all significant ($p \leq 0.05$, Figure 15). The lowest R^2 value was obtained for fine clay, and a plot of detectability (NMR signal intensity per scan per μmol ^{15}N) versus incubation time revealed an apparent decrease in the detectability of ^{15}N with increasing incubation time (Figure 16). One might argue that the decrease in detectability suggests that a form of undetectable N, such as heterocyclic N in slow-relaxing rigid aromatic structures (or perhaps clay-fixed NH_4), may have

Table 23. Correction factors for NMR peak areas due to differential relaxation during the contact time. †

material	functional group						max. correction factor / min. correction factor
	imidazole N of histidine ‡	amide	guanidinium N's of arginine		amino		
			-NH	$-NH_2$			
clover §	1.146	1.174	1.196	1.274	1.029	1.551	1.51
whole soil	-	1.112	1.188		1.298		1.17
fine clay	-	1.169	1.220		1.459		1.25
coarse clay	-	1.161	1.212		1.161		1.04
fine silt	-	1.114	1.166		1.418		1.27

‡ Imidazole N was observed in clover only.

† Correction factors were applied to spectra acquired with 0.4 ms contact time per Equation 9. No correction was made if correction factors were approximately equal for all functional groups (max / min < 1.05). For the incubated soils, model parameters were generated only for samples that had been incubated for 34 d. The parameters were assumed to be equally applicable to soils that had been incubated for different times.

§ For clover, two peaks were observed in both the guanidinium and amino regions. Values on the left correspond to the peaks with the higher NMR frequency.

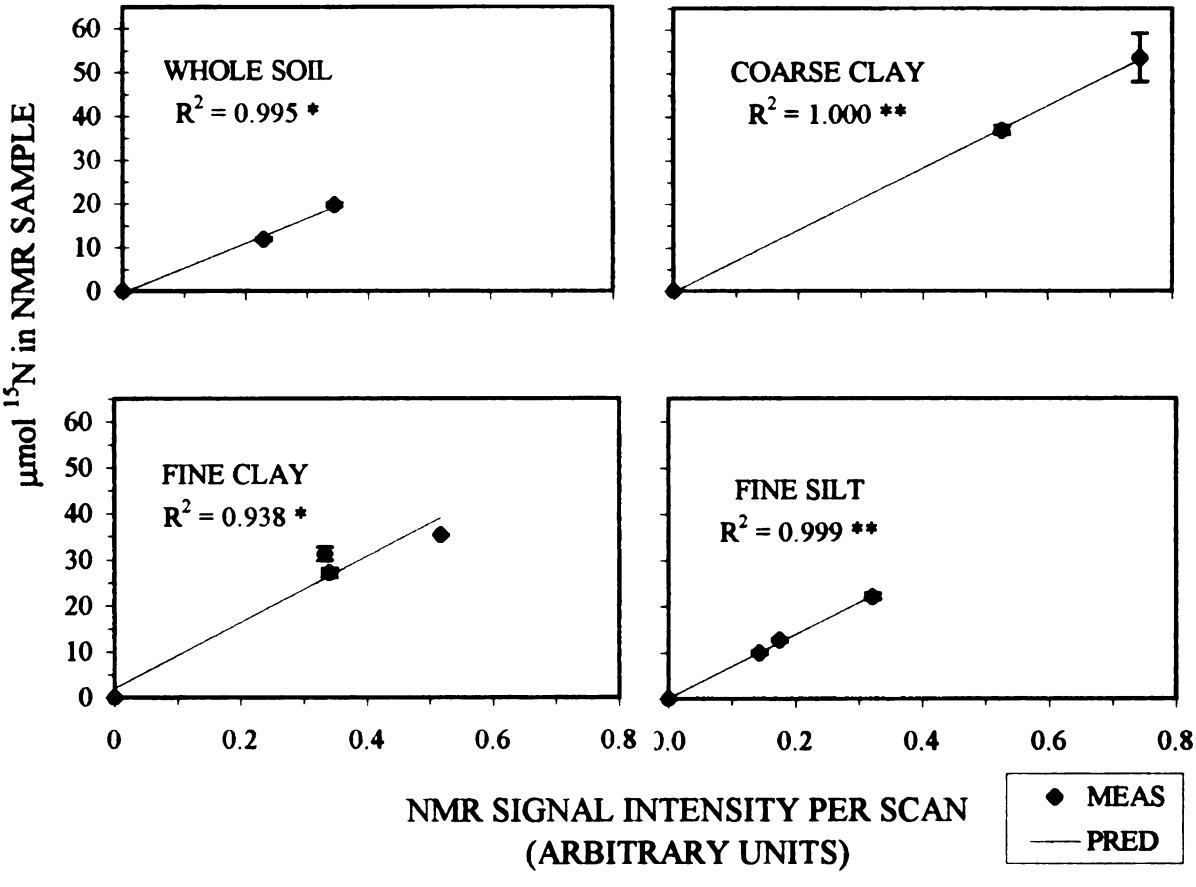

Figure 15. Linear regressions of ^{15}N amounts versus NMR signal intensity for soil samples. Each point represents a soil sample collected after 1, 6, or 14 months of incubation. The point (0,0) is not a measured point, but was included in the input data for the regressions since no signal would be expected for a blank sample. Error bars represent sample standard deviations from mass spectrometric measurement of ^{15}N. * and ** indicate significance at the 0.05 and 0.01 probability levels, respectively.

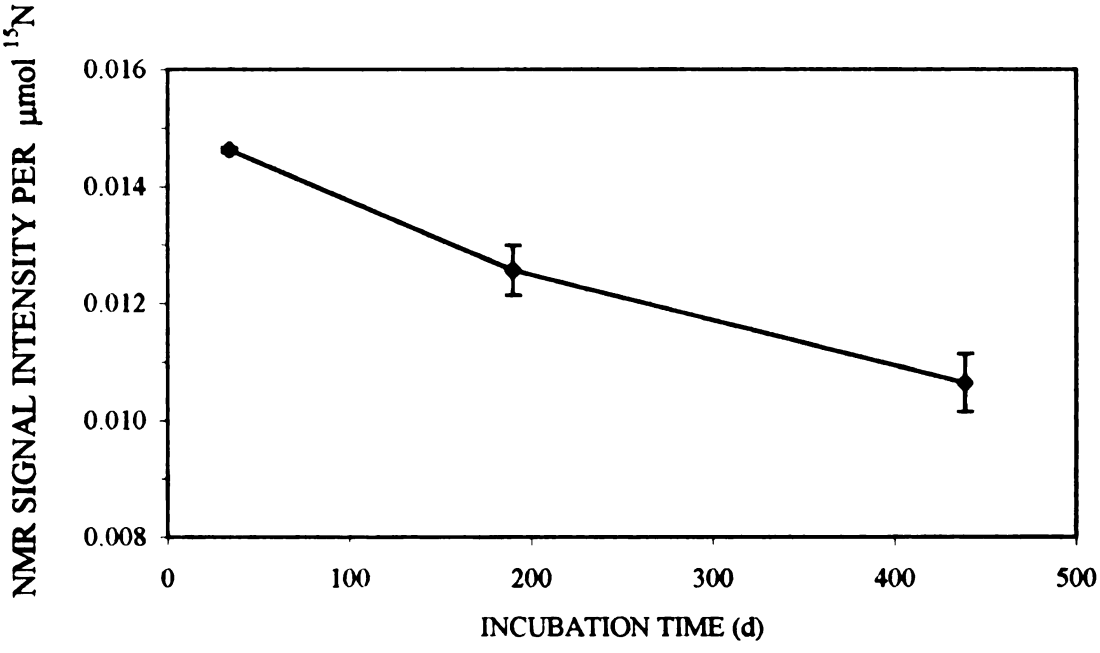

Figure 16. NMR detectability of fine-clay associated 15N after various incubation times.

slowly formed in the fine clay. If it is assumed that the ^{15}N is 100% detectable at Day 34, Figure 16 indicates that about 20% of the fine-clay ^{15}N may have been undetectable at Day 439; this undetectable ^{15}N corresponds to about 4% of the remaining ^{15}N in the whole soil.

The above analysis of NMR data suggests that very little, if any, heterocyclic ^{15}N was formed during the soil incubation. However, the broad high-frequency (left-hand) shoulder of the amide peak observed here (Figure 11) and in other studies (e.g. Knicker et al., 1993; Knicker and Lüdemann, 1995) may be a result of heterocyclic N. Comparison of the spectra of ^{15}N-clover in the presence and absence of soil (Figure 17) indicates that the shoulder is mainly a consequence of soil-induced line broadening. The shoulder does obscure a small signal due to histidine (near 150 ppm), but this signal represents only about 1% of the clover-^{15}N (Table 19). The NMR peaks in these soil samples were too broad to identify fungal glucosamine or bacterial purine as per Bedrock et al. (1998, see Introduction).

Relationship between N Mineralization and Organic N Functional Groups During the 14-Month Incubation

Throughout the 14-month incubation of soil amended with ^{15}N-clover, ^{15}N-NMR demonstrated that the composition of soil organic ^{15}N in whole soils and size fractions invariably was about 90% amide, 5-10% guanidinium N of arginine, and 5% amino. No separate NMR spectra were obtained for coarse silt and sand, but it is likely that their relatively short-lived ^{15}N pools resembled that of the initial clover.

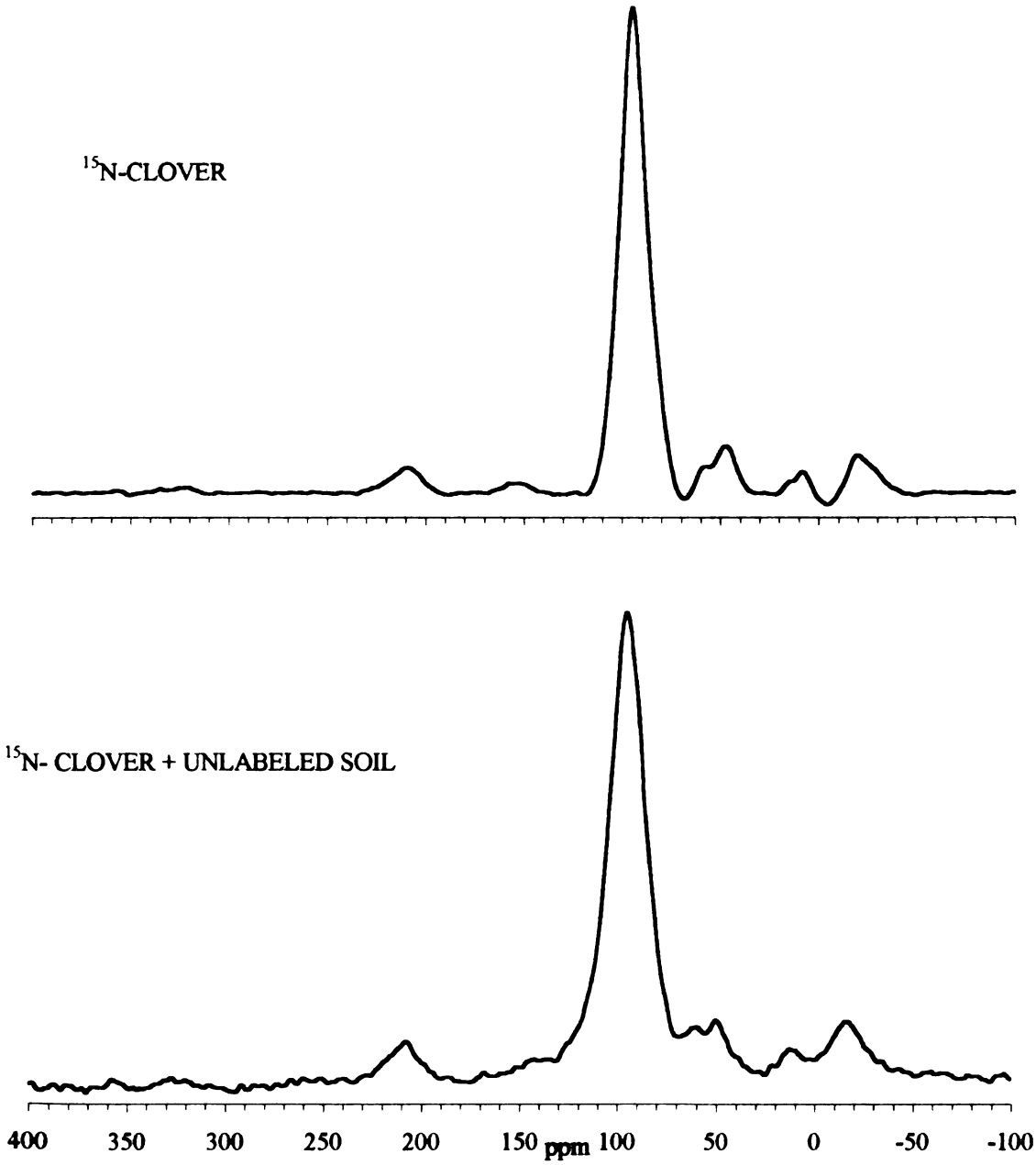

Figure 17. NMR spectra of 15N-labeled clover, with and without soil. In both spectra, contact time = 0.2 ms, delay = 1 s, line broadening = 120 Hz.

Despite the lack of change in organic ^{15}N functional groups, important differences in mineralization behavior were observed among size fractions. Kinetic analysis of clover-derived N (i.e. ^{15}N) indicated the presence of sorbed N in clays and fine silt ($k \approx 0.001\ d^{-1}$), processed macroorganic matter in fine silt ($k \approx 0.03\ d^{-1}$), and relatively unprocessed macroorganic matter in coarse silt and sand ($k \approx 0.1$ to $0.2\ d^{-1}$). When particle-size fractions from the 14-month incubation were incubated separately in shaken slurries, differences in N mineralization were observed and linked to C:N ratio, size class, and the time of incubation prior to fractionation. However, the shaken slurry results were measured for total N ($^{15}N + {}^{14}N$) and cannot be assumed to be completely applicable to ^{15}N. Nevertheless, the overall results indicate that for the entire 14-month incubation the clover-derived N was almost entirely proteinaceous, and that differences in N mineralization behavior were not related to the composition of organic N. Rather, clover-N mineralization in the relatively N-rich soil of this study appeared to be driven by the microbial requirement for C. The stability of clover-N was linked to humification or adsorption per se, and perhaps aggregate protection, as will be presented in the Discussion section.

DISCUSSION

Dynamics of Clover-N in Particle-Size Fractions: Agronomic Implications

The dynamics of clover-derived N turnover are best discussed in relation to microbial degradation. A vigorous microbial attack ensued immediately after the amendment of soil with ^{15}N-clover. After 1 and 5 d of incubation, the respiration rate corresponded to 5 and 3% of total soil C, respectively. Proteinaceous ^{15}N (as revealed by ^{15}N-NMR) was released from the original plant material and probably from the fast-cycling microbial biomass, and accumulated in the clay fractions (and perhaps in the fine silt) until about 95 d. (Evidently, ultrasound at 38 kJ per 28 g soil does not rupture plant cells; ultrasonic rupture of plant cells would have resulted in greater recovery of ^{15}N in size fractions after 11 d.) The slopes of the curves for clover-N loss indicate that most of the labile clover constituents had been transformed after 34 d. (See Figure 6, especially 6F and 6G.) Microbe-on microbe attack was likely a prominent process between 34 and 95 d; the maximum clover-N accumulation in the clays occurred at about 95 d. This interpretation of microbial dynamics is supported by McGill et al. (1975). These researchers added ^{14}C-acetate and ^{15}N-ammonium sulfate to soil and observed maximal fungal and bacterial populations at 5 and 10 d of incubation, respectively. They considered that the fungal decline was due to either autolysis or bacterial attack.

After 95 d, the clover-N associated with the fine soil fractions exhibited a slow degradation. The stabilization of clover-N in the fine fractions is emphasized by plotting clover-N amounts for all fractions as percent of remaining (Figure 18), and is supported

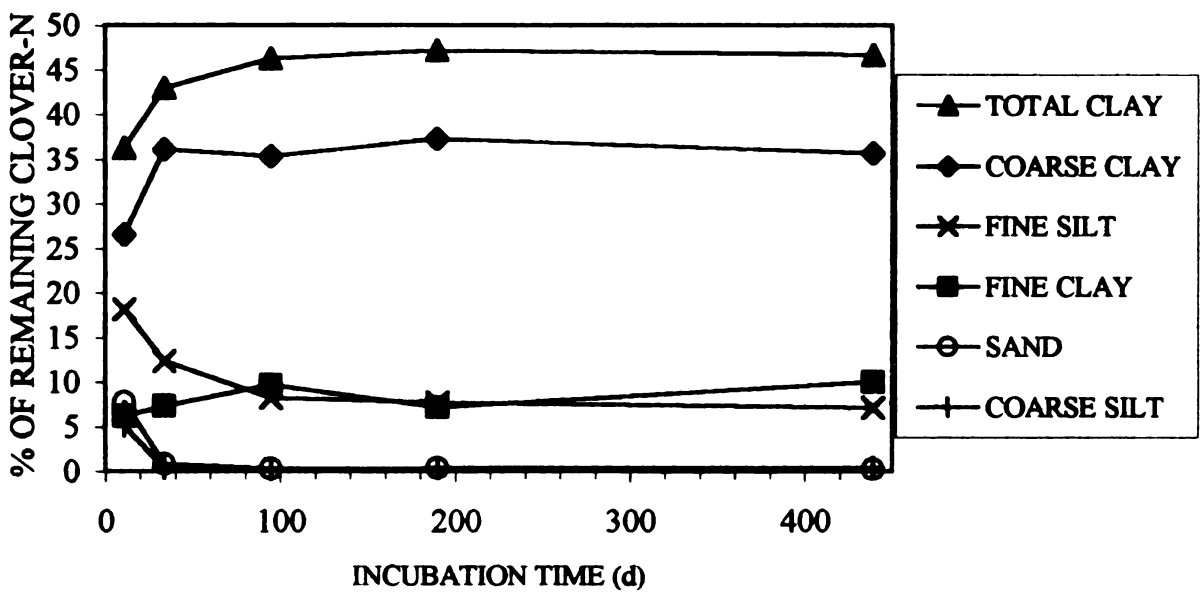

Figure 18. Clover-derived N in fractions as a function of time (% of remaining.)

by the mechanistic model of Hassink and Whitmore (1997). Their model characterizes clays as saturable adsorptive surfaces, which quickly adsorb and slowly desorb OM.

The rapid transformation of clover-derived, and perhaps native, light-fraction material in the coarse silt and sand supports the correlation between 70- and 150-d nitrogen mineralization potential (NMP) and macroorganic N and C found by Willson et al (in press). (These authors defined macroorganic N and C as the N and C in the 53 – 2000 μm fraction; this fraction was obtained by sieving a dispersed soil suspension and is thus similar to the macroorganic fraction of the present study.) Although the correlations of Willson et al. (in press) were statistically significant, no more than 41% of the variability in NMP was explained by macroorganic N or C. They noted that macroorganic matter did not increase immediately after plant residue incorporation; this was perhaps because the residue particle size was > 2000 μm. For a better prediction of NMP, they suggested that macroorganic matter measurements could be combined with information about recently incorporated plant residues.

Fresh plant residues may contain appreciable amounts of soluble organic N (e.g. Aita et al., 1997). This N pool is almost certainly a significant short-term N source and was not determined in the present study. The pool was probably lost (mineralized) during particle-size separation (Table 8). In future studies, this pool might be preserved by performing all fractionation steps in a chilled environment and sampling for dissolved organic compounds.

The agronomic relevance of the apparently long-term stabilization of clover-N observed here can be assessed by comparing the N retention in this study to that observed in the field by others (Table 24). For these comparisons, the rate constants for clover-N

decomposition were adjusted to the field based on a Q_{10} value of 2. (See Kätterer et al. (1998) for details.) No attempt was made to account for variations in moisture that would be expected in the field, as moisture was not correlated with CO_2 fluxes in a field study in southwest Michigan (Paul et al., 1999). The overall plant decomposition in the present study closely matches that of Aita et al. (1997), but is less than that observed by Harris and Hesterman (1990). The higher decomposition (lower retention) observed by Harris and Hesterman (1990) is likely a result of the presence of growing plants (Cortez and Cherqui, 1991). Roots are known to be a major source of soil C and lead to N immobilization followed by mineralization (Jansson and Persson, 1982). This analysis suggests that some but not all field mechanisms of N mineralization and immobilization were operating in the present study.

Table 24. ^{15}N retention in present study compared to field studies.

reference	agronomic system	soil texture	conditions	soil organic ^{15}N as % of initial (per reference)	soil organic ^{15}N as % of initial (present study, corrected to environmental conditions of the reference)
Aita et al. (1997)	^{15}N-wheat straw decomposition in absence of growing plants	silt loam	365 d, 10 °C, 45 kg straw-N per ha	62	60
Harris and Hesterman (1990)	^{15}N-alfalfa decomposition in presence of corn	loam	332 d, 10 °C, 112 kg alfalfa-N per ha	38	61
Harris and Hesterman (1990)	^{15}N-alfalfa decomposition in presence of corn	sandy loam	332 d, 10 °C, 112 kg alfalfa-N per ha	40	61

The stabilization of considerable quantities of plant-derived N observed by Aita et al. (1997) and Harris and Hesterman (1990) is likely due in large part to association of N with clays via mechanisms discussed in the next section. Calculations of the degree of saturation of the soil protective capacity per the model of Hassink and Whitmore (1997) indicate that the soils of Aita et al. (1997), and Harris and Hesterman (1990) were undersaturated with organic matter both before and after addition of plant residues (Table 25). The soil of the present study was undersaturated before addition of clover and at saturation immediately after addition of clover.

Table 25. Comparison of soil C with soil protective capacity.

reference	soil texture (USDA system)	percent clay (< 2 μm) †	soil C prior to addition of plant residue (g C kg^{-1}) ‡	soil C immediately after addition of plant residue (g C kg^{-1}) ‡	calculated soil protective capacity (g C kg^{-1}) §
Aita et al. (1997)	silt loam	15	9.9	11.3	27
Harris and Hesterman (1990)	loam	25-50	14	14.3	30 – 40
Harris and Hesterman (1990)	sandy loam	0-20	8	8.3	21 – 29
present study	sandy loam	16	14	27	27

† Exact values of percent clay were not given by Harris and Hesterman (1990); the range of percent clay was deduced from their reported soil textural class.

‡ Exact values of native and added C were not given by Harris and Hesterman (1990) but were estimated from OM content of soil and from C:N ratio of plant residue.

§ Soil protective capacity per Hassink and Whitmore (1997):
protective capacity (g C kg^{-1}) = 21.1 + 0.375 x (% particles < 2 μm)

The retention of N in cover crop systems or other systems with high organic inputs is important in the long-term stabilization or buildup of SOM levels. High levels of SOM may eventually lead to increases in the native N mineralized during a growing season.

Mechanisms of Short- to Medium-Term Stabilization of Soil Organic N

Aggregate protection of organic N in this study was assessed by (1) the extent of mineralization in shaken slurry, (2) the ratio of shaken slurry to undisturbed mineralization (aggregate protection factor, or APF), and (3) the difference between shaken slurry and undisturbed mineralization (aggregate-protected pool size). The aggregate protection factors (APF) measured for whole soil (APF $\approx$ 4, Table 15) correspond roughly to those measured by other authors for soils with much higher clay contents than the sandy loam soil (15% clay) of this study. Edwards and Bremner (1967) and Craswell and Waring (1972) assessed aggregate protection by comparing two-week N mineralization (30 °C, anaerobic or aerobic) of coarse-ground (2000 μm) and fine-ground (50 μm) soils. In nine soils with clay contents ranging from 26 – 72%, the APFs ranged from 1.3 to 4.3, with the exception of one observation (APF = 25). (APFs were calculated from their data as the ratio of fine-ground to coarse-ground N mineralization.) For soils with clay contents ranging from 10-17%, no enhancement of N mineralization was observed (Craswell and Waring, 1972). The apparent difference between the results of Craswell and Waring (1972) and those of the present study is explicable on the basis of methodology. All soils studied by Craswell and Waring (1972) were air-dried prior to grinding, and the dried clayey soils undoubtedly required considerable grinding energy

before passing through the 50 μm sieve. The dried sandy soils, on the other hand, were probably loosely aggregated; the small aggregates containing the protected OM probably passed through the sieve with minimal grinding energy. In contrast, the sandy loam soil of the present study was probably dispersed to a considerable degree during shaking as explained in Results.

Aggregate-protected N accounted for no more than 11% of N (clover + native) stabilization in this study. Therefore, the most prominent N stabilization mechanisms were probably humification or clay adsorption per se. APF values and aggregate-protected pool sizes decreased with time for fine clay and coarse clay; the explanation is that the strength of adsorption increased with time (e.g. increased number of covalent bonds between organic N compounds and minerals) and/or that the organic N compounds were being humified.

Due to lack of ^{15}N data in the shaken slurry mineralization tests, the aggregate protection of clover-derived N cannot be directly evaluated. Nonetheless, the *maximum* possible mineralization of clover-N was calculable, and the results indicated that at most 33% of the clover-N was mineralizable in shaken slurry (Table 26). Therefore, humification or adsorption per se, and perhaps aggregate protection, were important mechanisms for the stabilization of clover-N. The distribution of organic ^{15}N during this study was invariably 90% amide, 5-10% guanidinium N of arginine, and 5% amino; incorporation of N into heterocycles was not observed. Overall, these findings support the notion that proteinaceous N is incorporated into soil humus as reviewed in the Introduction. In the short term, the NMR data of this study show that this incorporation is probably not accompanied by change in the overall distribution of N functional groups.

Table 26. *Maximum* possible mineralization of clover-N in shaken slurry.

days of prior incubation	fraction	*maximum* possible mineralization of clover-N (%) †
34	whole soil	20
	fine clay	33
	coarse clay	10
	fine silt	2.4
190	whole soil	17
	fine clay	19
	coarse clay	9.3
	fine silt	10
439	whole soil	25
	fine clay	11
	coarse clay	6.4
	fine silt	6.9

† (μmol mineralized in shaken slurry / μmol organic clover-N present at beginning of shaken slurry) x 100

Chemical Structure of Soil Organic N as Determined by NMR: A Critical Analysis

The overall quantitativeness of NMR was demonstrated by characterization of differential relaxation effects, analysis of a complex soil-organic mixture with known composition, and correlation of NMR spectral intensities with ^{15}N concentrations as measured on a mass spectrometer. The relatively small differential relaxation effects observed here should not be generalized; samples that contain appreciable amounts of both high-mobility N (e.g. amino) and limited-mobility N (e.g. amide) would be expected to exhibit relatively large differential relaxation effects. The analysis of the mixture of known composition showed that peak overlap is an important though not fatal weakness of the NMR analysis of soil samples.

Although ^{15}N-CPMAS NMR has been used to distinguish fungal versus bacterial biomass in pure culture and in decaying organic materials (Bedrock et al., 1998), NMR peaks in the presence of soil are too broad to make such distinctions. Moreover, a peak coinciding with the fungal N-acetyl glucosamine peak reported by Bedrock et al. (1998) was observed in pure clover (Figure 11A).

Improvements in N-NMR spectroscopy of soils seem possible. Density fractionation leads to isolation of fractions low in specific gravity and relatively high in organic content; such schemes were used in a ^{13}C-NMR study by Baldock et al. (1992). Christensen (1992) has questioned some density fractionation schemes, and considers that density fractions of clay and silt particles may not represent truly distinct pools of organic matter. If this is true, one might argue that the NMR spectrum of the "light" fraction within clay and silt would be similar to that of the "heavy" fraction within said particles. A further suggestion for improvement of NMR spectra is the dissolution of mineral matter by HF (Schmidt et al., 1997). This approach would seem to forfeit the advantage of the noninvasiveness of NMR. Finally, the recently developed technique of high-resolution solid-state ^{14}N-NMR may help resolve the current controversy surrounding the chemical structure of native soil organic N (Jeschke and Jansen, 1998). However, this approach requires high spinning speeds and further advances in broadband spectral excitation, and may not be immediately applicable to soil systems.

The composition of ^{15}N in whole soils and particle-size fractions in this study (90% amide, 5-10% guanidinium N of arginine, and 5% amino) is in good agreement with other ^{15}N-CPMAS-NMR studies of soil but at variance with the composition of soil N as presented by Schulten and Schnitzer (1998). The composition of soil N per

Schulten and Schnitzer is: proteins + peptides + amino acids, 40 %; amino sugars, 5 - 6 %; heterocyclic N, 35 %; and NH_3, 19 %. (NH_3 is an operationally defined pool; see Table 1.) Schulten and Schnitzer (1998) argue that the incubation times of ^{15}N-amended soils in studies such as the present one may be too short for the formation (or selective preservation) of resistant heterocyclic N. Although this caveat may certainly apply to this study, the discrepancy in N chemical composition may stem from assumptions used by Schulten and Schnitzer. Their analysis appears to rely on the assumption that no proteinaceous N is contained in the N fraction that is resistant to hot acid hydrolysis (Sowden et al., 1977). This assumption may be reasonable in some studies, as hot acid hydrolysis is a traditional method for decomposing proteins into their constituent amino acids (Creighton, 1993). However, ^{15}N-CPMAS-NMR of the nonhydrolyzable residue of a soil incubated for two years with ^{15}N-ammonium sulfate + crop residues + powdered filter paper revealed that about 80% of the N in the residue was in the form of amide and amino groups; about 20% could be assigned to either amide or heterocyclic N of indole (Zhuo et al., 1992). Similarly, the ^{15}N-NMR analysis (at natural abundance levels) of the hydrolysis residue from an organic-rich lake sediment produced an amide-dominated spectrum quite similar to the spectra acquired in the present study (Knicker and Hatcher, 1997). Further analysis of the residue (thermochemolysis followed by gas chromatography/mass spectrometry) identified the N as proteinaceous (Knicker and Hatcher, 1997). Based on the unchanging proteinaceous NMR signature of clover-derived N throughout the 14-month soil incubation and the shaken slurry mineralization tests, the considerable differences in the mineralization/immobilization behavior of

clover-N among the different particle-size fractions appear to be linked to adsorption per se or humification and possibly aggregate protection.

CONCLUSIONS

This study confirmed that soil particle-size fractions contain biologically distinct pools of organic nitrogen, and strongly suggested that fundamental differences in the short- to medium-term behavior of legume-derived N among these fractions are not a result of differences in the chemical structure of N. A transient macroorganic legume-derived N pool was found in coarse silt and sand, with a decay rate of about 0.1 d^{-1} in the laboratory. This rate corresponds to a steady-state mean residence time (MRT) of 10 d in the laboratory or 25 d in the field (10 °C). A stable pool of legume-derived N was found in the clays and fine silt and corresponded to about 60% of added legume-N; its decay rate was 0.001 d^{-1}, corresponding to an MRT of 3 years in the laboratory or 7 years in the field (10 °C). The large size of this stable N pool despite a high rate of legume addition was explained in terms of the soil protective capacity of Hassink and Whitmore (1997); the amount of native plus added C was roughly equal to the protective capacity. Evidence from ^{15}N-CPMAS-NMR suggested that both the transient and stable pools of legume-derived N were almost entirely proteinaceous. The aggregate-protected N pool (legume-derived plus native N) was operationally defined as the 30-day shaken slurry N mineralization minus the 30-day mineralization in undisturbed soil. This pool comprised between 0 and 11% of the N within the size-fractions and whole soil, and increased (as a percentage of N in the fraction) with decreasing particle size. These findings support the notion that some organic N is resistant by virtue of its entrapment in pores inaccessible to microbes (Edwards and Bremner, 1967). The generally low amounts of N mineralized in

shaken slurries (maximum of 12% of total N, 33% of legume-N) indicate that substantial amounts of N are stabilized via adsorption per se or humification.

Key weaknesses of ^{15}N-CPMAS-NMR are peak overlap and its impracticality in establishing the chemical structure of native (unlabeled) soil organic N. Nevertheless, ^{15}N-CPMAS-NMR was a useful tool for the noninvasive study of labeled soil organic N. The technique was able to detect both heterocyclic and noncyclic N forms in a prepared mixture of unlabeled soil and ^{15}N-labeled organic materials, and was quantitative for incubated soils to within 5-10% of the total spectral intensity.

Overall, the mechanistic and kinetic information of the present study are important to both the fundamental and practical understanding of soil N availability. Mechanistically, the short- to medium-term mineralization/immobilization of legume-derived N was controlled by the physical location of this N within the soil matrix rather than by the organic functional group composition of this N. The kinetic rate constants determined in the laboratory, after correction to field temperatures, reflected some but not all of the mechanisms operating in the field. These kinetic parameters along with other key information, such as percent clay, degree of saturation of soil protective capacity, magnitude and timing of crop N needs, and mean annual air temperature, will allow us to better predict N mineralization/immobilization in the field. Such prediction is important in designing agricultural systems in which organic N is mineralized in synchrony with plant needs.

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APPENDICES

Appendix 1. Isotopic Tracer Equations

Clover-derived N and native N were calculated for the various N pools (whole soils and particle-size fractions) throughout the incubation on the basis of the following equations:

$$N_{POOL} = N_{CLOV}\, f + N_{NTV}\, g \qquad (1)$$

$$^{15}N_{POOL} = {}^{15}N_{CLOV}\, f + {}^{15}N_{NTV}\, g \qquad (2)$$

N_{POOL} and $^{15}N_{POOL}$ are the amounts of N and ^{15}N, respectively, in the pool of interest. N_{CLOV} and $^{15}N_{CLOV}$ are the amounts of clover-N and clover-^{15}N, respectively, present in the whole soil on Incubation Day 0. N_{NTV} and $^{15}N_{NTV}$ are the amounts of native-N and native-^{15}N, respectively, present in the whole soil on Incubation Day 0. The values f and g are defined as follows:

f = clover-derived N in pool of interest / clover N in whole soil on Day 0

g = native N in pool of interest / native N in whole soil on Day 0

All N amounts in these equations were expressed in moles. The equations assume that isotopic fractionation effects are negligible.

Simultaneous solution of (1) and (2) yields:

$$f = \frac{{}^{15}N_{POOL} - \frac{{}^{15}N_{NTV}}{N_{NTV}} N_{POOL}}{{}^{15}N_{CLOV} - \frac{{}^{15}N_{NTV}}{N_{NTV}} N_{CLOV}} \qquad (3)$$

$$g = \frac{{}^{15}N_{POOL} - \frac{{}^{15}N_{CLOV}}{N_{CLOV}} N_{POOL}}{{}^{15}N_{NTV} - \frac{{}^{15}N_{CLOV}}{N_{CLOV}} N_{NTV}} \qquad (4)$$

The values of f and g appear in Tables 8 and 9.

The sample standard deviations of f and g were computed with two simplifying assumptions. First, the uncertainty in the denominators of f and g in Equations 3 and 4 were assumed to be zero. Second, errors involving differences were assumed to be additive (a worst-case assumption). The second assumption avoided complications due to the interdependence of $^{15}N_{POOL}$ and N_{POOL}. (Theses two terms are not independent because the measurement of $^{15}N_{POOL}$ relied on the value of N_{POOL}; see "C, N, and ^{15}N Analyses" in Materials and Methods.)

Appendix 2. Modeling of Mineralization-Immobilization of Clover-Derived N in Clay Fractions

In the clay fractions, clover-N was observed to accumulate in the initial stages of the incubation and to decline in the latter stages. This behavior was modeled by the following equation:

$$N_t = -A \exp(-k_A t) + B \exp(-k_D t) \qquad (1)$$

where A and B are (positive) constants, k_A is the accumulation rate constant, and k_D is the depletion rate constant. The derivation of the model is described below.

Assume that both the accumulation and depletion of clover-N in the clay fractions follow first-order kinetics:

$$dN/dt = k_A N' - k_D N \qquad (2)$$

where N is the amount of clay-N, k_A and k_D are rate constants ($time^{-1}$) for accumulation and depletion, respectively, and N′ is the amount of N in an unspecified pool from which N is being transferred. Accordingly,

$$dN'/dt = -k_A N' \qquad (3)$$

Equations 2 and 3 constitute a system of simultaneous linear differential equations and were solved for N as described by Spiegel (1971) to yield Equation 1.

Appendix 3. Brief Overview of NMR Concepts

Chemical Shift

This is a measure of the frequency at which a ^{15}N nuclei resonates in a magnetic field. The units are ppm. Electron-deficient N atoms (e.g. nitrate) have high chemical shift (high frequency) and appear at the left edge of the spectrum. Electron-rich N atoms (e.g. ammonium or amino) have low chemical shift and appear at the right edge of the spectrum.

Cross-Polarization Magic-Angle Spinning (CPMAS)

In the solid state, ^{15}N NMR spectra of soils are usually acquired with cross-polarization magic-angle spinning (CPMAS). In CPMAS, magnetization is transferred from magnetically sensitive ^{1}H to relatively insensitive ^{15}N. Simultaneously, the sample is spun 4000 - 5000 times per second about an angle of 54.7°. Without these methods, solid state ^{15}N NMR spectra would be impractically slow and the peaks obtained would be excessively broad.

Spinning Sidebands

These are produced on both sides of a prominent peak in a CPMAS experiment. Though they are sometimes a nuisance (they might overlap with other peaks), their peak areas need to be added to the area of the parent peak for correct quantitation.

Differential Relaxation Effects

There are two times during an NMR scan in which such effects can occur. The first time is the *contact time*, during which magnetization is transferred from ^{1}H to ^{15}N. The rate of transfer is characterized by two relaxation (equilibration) times: T_{NH} and $T_{1\rho H}$. The parameter T_{NH} is a measure of the rate of magnetization transfer from ^{1}H to ^{15}N, and $T_{1\rho H}$ is a measure of how fast the ^{15}N nuclei loses the transferred magnetization. These parameters are not necessarily the same for all ^{15}N functional groups, and correction of signal intensities (peak areas) may be necessary.

The second time in which differential relaxation effects can occur is the *delay time*. This is the time between NMR scans in which ^{15}N nuclei return to equilibrium; without the delay time, the nuclei would become saturated and give no signal. (Multiple scans are usually required to obtain an acceptable spectrum because of the low concentration of ^{15}N.) The rate of return to equilibrium is characterized by T_{1H}. This parameter may differ by functional group, and correction of signal intensities may be necessary. Further, ^{15}N nuclei with very long T_{1H} values (say, > 5 s) may be undetectable in a typical (delay time $\approx$ 1 s) CPMAS-NMR experiment.

* Explanation of chemical shift scale:

The chemical shift scale here defines $^{15}NH_4NO_3$ in 2 M HNO_3 as 0.00 ppm. The solid ammonium salts $^{15}NH_4NO_3$ and $(^{15}NH_4)_2SO_4$ are expected to have a chemical shift value within about 1 ppm of $^{15}NH_4NO_3$ in 2 M HNO_3; thus, these solids are equivalent to $^{15}NH_4NO_3$ in 2 M HNO_3 in all but the most exact studies.

Conversions from ammonium nitrate or ammonium sulfate reference to NH_3 (l,25 °C) reference can be made with data based on Levy and Lichter (1979):

Table A-2 Chemical shifts conversion.

compound	chemical shift (ppm)
NH_3 (l,25 °C)	-21.60
$^{15}NH_4NO_3$ in aqueous HNO_3	0.00

a

1. Blomberg and Ruterjans, 1983.
2. Cross et al., 1982. Data are for solid state.
3. Levy and Lichter, 1979.
4. Thorn and Mikita, 1992.
5. Wuthrich, 1976.

b Hawkes et al., 1977; as cited by Blomberg and Ruterjans (1983).

c

A = adenine, C = cytosine, G = guanine, T = thymine; these are the bases of DNA. Adenine also occurs in the adenosine energy-transfer molecules AMP, ADP, and ATP, as well as in NAD. Guanine occurs as a reactant in the citric acid cycle in the form of guanosine phosphate.

Appendix 4. Detailed Table of ^{15}N Chemical Shifts

Table A-1. ^{15}N chemical shifts of biologically important molecules

compound	chemical shift (ppm)	ref.[a]
ammonium sulfate or ammonium nitrate	0	-
free amino acids (data probably applicable to amino-terminal amino acids in proteins), amino groups	5-34 (including Gly and Pro)	5
	ca. 20 (most common shift)	5
	12-20 (most common shifts in solvent and pH conditions reasonable for soil)	1
guanidine NH2 -NH- (epsilon)	42-53 60-65	1,5
-NH2 of nucleic acids	76 (cytosine); 59 (adenine); 53 (guanine)	2
tryptophan –NH- (indole)	61	1
peptide-N	83-116 (including Gly and Pro) 95-110 (common amino acids; excluding Gly and Pro)	1,5
uracil[b]	111.2 (N1) 138.8 (N3)	1
histidine ring N (imidazole)	145-155 cation; 157 & 211 amphiion; 173 & 197 anion	1
aromatic N in nucleic acids[c]		
G3, T3, C1, G1, T1	120.6-139.6	2
A9, G9	147.5	2
C3, A3, A1, A7, G7	174-211	2
pyrrole	136 (in DMSO-d_6)	4
pyrrole as part of chlorophyll A	173, 175, 192, 233 (in acetone-d_6)	1
flavin (isoalloxazine)	128-140 (N3); 142 (N10, ox.); 170-179 (N1); 312-320 (N5, ox.)	1
nitrate	354	3

The reason for the chemical shift-equivalence of these reference compounds can be explained in terms of H-bonding. According to Briggs and Randall (1973), the chemical shift of the ammonium-^{15}N in these compounds in the solution state is greatly influenced by the H-bonding of the ammonium hydrogens to the lone pairs of electrons on nearby O atoms. Thus, the electron density around the ^{15}N atom should be similar regardless of whether the H-bond donor is NO_3^-, SO_4^{2-}, or H_2O. In support of this argument, Briggs and Randall (1973) found variation in chemical shift of 1 ppm at most when $^{15}NH_4NO_3$ or $(^{15}NH_4)_2SO_4$ was dissolved in its parent acid with concentrations of the acid anion ranging from 1 to 10 molal. Their results were confirmed for $^{15}NH_4NO_3$ in its parent acid by Srinivasan and Lichter (1977). The invariance of the chemical shifts with increasing concentration suggests that the solid-state salts have the same absolute chemical shift as the dissolved salts; this is confirmed by the very plausible peak positions detected for biological materials in the present study.

Appendix 5. Adjustment of N and C Amounts for Incomplete Dispersion on Day 11 and for Variable Clay Recovery

Methodological difficulties led to some variability in particle-size distributions. (See Table 7 and "Recovery of Mass in Particle-Size Fractions" in Results.) This variability and the associated corrections of N and C amounts in the size fractions are explained below.

On Day 11, incomplete dispersion resulted from failure to hand-shake the incubated soil with water prior to sonication. Clay amounts were less than expected, and fine silt and sand amounts were greater than expected (Table 7). The N and C recoveries in the fine silt and sand were adjusted accordingly:

> adjusted N or C in fine silt or sand = (measured N or C in fraction) x
> (expected mass of particles in fraction) / (measured mass of particles in fraction)

The *total clay* N and C were adjusted by adding the "excess" N or C from the fine silt and sand to the N and C measured in the total clay. (The "excess" N or C in fine silt and sand were taken as the adjusted minus the measured in said fractions.) The N and C measured in the fine clay and coarse clay were not adjusted (individually) because it was felt that there was insufficient information for such a computation.

The variability in fine clay yields on Days 34-439 (Table 7) presented a further challenge in determining the true N and C distributions among the size fractions. This variability was attributed to the difficulty of siphoning a centrifuged suspension without altering the vertical distribution of fine particles. The amounts of N or C in fine clay on these days were adjusted as follows:

adjusted N or C on Day X = (measured N or C on Day X) x

(average mass of fine clay for Days 34-439) / (mass of fine clay on Day X)

Finally, the coarse clay recovery for Day 190 (Table 7) was determined to be a statistical outlier relative to Days 34, 95, and 439 per Dixon's gap test (Bliss, 1967). Therefore, the coarse clay on Day 190 was adjusted as follows:

adjusted N or C = (measured N or C) x

(average mass of coarse clay for Days 34, 95, 439) /

(mass of coarse clay on Day 190)

Appendix 6. Details of Analysis of Variance of Shaken Slurry N Mineralization

Nitrogen mineralization in shaken slurry (N_{MIN}) was a function of the measurements N_{30}, N_0, N_{CTRL}, and N_{TOTAL} as per Equation 8 in Materials and Methods. These destructive measurements were performed twice or more on subsamples derived from a single bulk sample of material. For purposes of the analysis of variance (ANOVA), N_{MIN} was computed as:

$$N_{MIN,i} = 100 \times (N_{30,i} - \langle N_0 \rangle - \langle N_{CTRL} \rangle) / (\langle N_{TOTAL} \rangle - \langle N_0 \rangle)$$

where i denotes replicate number and <> denotes mean value. This computation of N_{MIN} reflects the fact that N_0 and N_{TOTAL} are pseudoreplicates (simple chemical tests repeated on subsamples of the same material). The values N_{30} and N_{CTRL}, in contrast, were considered true replicates (since they reflected the outcome of a biological process). However, the mean value of N_{CTRL} was used for simplicity; N_{CTRL} values were generally very small compared to N_{30}. Within the ANOVA, each value of N_{MIN} was weighted by $(VAR^*)^{-1}$, where VAR^* is the variance in N_{MIN} due to variability in N_0, N_{CTRL}, and N_{TOTAL} as computed by the rules of statistical error propagation (Meyer, 1975). The ANOVA was implemented with PROC GLM within the SAS software (SAS Institute, 1989).

Appendix 7. Computation of 30-Day Mineralization in the Undisturbed Soil (For Comparison With Shaken Slurry Mineralization Data)

The thirty-day N mineralization in shaken slurry was compared to the N mineralization in the undisturbed clover-amended whole soil as a means of assessing physical protection. The shaken slurry samples were derived from ultrasonic fractionation of the clover-amended whole soil after 34, 190, and 439 d of incubation; unfractionated whole soil samples from 34, 190, and 439 d of incubation were also subjected to the shaken slurry test. The thirty-day mineralization in the undisturbed soil was computed on the basis of the kinetic models of clover-derived and native N within the particle size fractions (Table 6).

For whole soil and fine silt, the computations of thirty-day N mineralization in undisturbed soil were as follows:

$$\text{moles native-N mineralized in undist. soil} = N_{MIN,NTV} = [1 - (N_{NTV,t+30,PRED})/(N_{NTV,t,PRED})] \times N_{NTV,t,MEAS}$$

where $N_{NTV,t+30,PRED}$ is the molar amount of native N in fine silt or whole soil as predicted by the kinetic model thirty days after fractionation, $N_{NTV,t,PRED}$ is the molar amount of native N in fine silt or whole soil as predicted by the kinetic model on the day of fractionation, and $N_{NTV,t,MEAS}$ is the molar amount of native N as measured in fine silt or (unfractionated) whole soil on the day of fractionation. For whole soil, $N_{MIN,NTV}$ was assumed to be zero (Table 6, Figure 7). The moles of *clover*-N mineralized in undisturbed soil were computed by an analogous equation:

$$\text{moles clover-N mineralized in undist. soil} = N_{MIN,CLOV} = [1 - (N_{CLOV,t+30,PRED})/(N_{CLOV,t,PRED})] \times N_{CLOV,t,MEAS}$$

The expected thirty-day mineralization (percent) for clover- plus native-N was then computed as follows:

$$N_{MIN,CLOV+NTV} = [(N_{MIN,NTV} + N_{MIN,CLOV})/(N_{NTV,t,MEAS} + N_{CLOV,t,MEAS})] \times 100$$

For fine clay and coarse clay, accumulation of N as per Equations 6 and 7 was neglected for the sake of the comparison; this is because no transfer of material between fractions was possible in the shaken slurry tests. Accordingly the undisturbed mineralization was computed as follows:

$$N_{MIN,CLOV} = [1 - (N_{CLOV,t+30,PRED}/N_{CLOV,t,PRED})]\ N_{CLOV,t,MEAS}$$

Taking $N_{CLOV,t+30,PRED} = N' \exp[- k_D (t +30)]$ and $N_{CLOV,t,PRED} = N' \exp(- k_D t)$ with N' being an arbitrary constant and k_D being the decay rate constant from Equation 6, the above equation simplifies to:

$$N_{MIN,CLOV} = [1 - \exp(-30\ k_D)]\ N_{CLOV,t,MEAS}$$

Numerically, k_D was equal to 0.00105 d^{-1} (fine clay) or 0.00130 d^{-1} (coarse clay); see Table 6. The value of $N_{MIN,NTV}$ (native N mineralized from undisturbed clays) was assumed to be zero because accumulation with no depletion was observed for native N in

the clays (Figure 7). The expected thirty-day mineralization (percent) for clover- plus native-N was then computed as follows:

$$N_{MIN,CLOV+NTV} = [(N_{MIN,NTV} + N_{MIN,CLOV})/(N_{NTV,t,MEAS} + N_{CLOV,t,MEAS})] \times 100$$

Appendix 8. Calculations of Aggregate Protection Factors and Aggregate-Protected Pool Sizes

Table A-3. Aggregate Protection Factors And Pool Sizes.

incubation time prior to fractionation (d)	30-day N mineralization as measured in shaken slurry (%) †	30-day N mineralization as modeled in undisturbed soil (%) † ‡	aggregate protection factor §	aggregate-protected pool (%) ¶
whole soil				
34	8.51 ± 2.29	2.34	3.64 ± 0.98	6.17 ± 2.29
190	5.93 ± 5.96	1.59	3.73 ± 3.75	4.34 ± 5.96
439	8.16 ± 2.22	1.40	5.83 ± 1.59	6.76 ± 2.22
		AVERAGE =	4.40	5.76
fine clay				
34	11.7 ± 0.9	1.10	10.6 ± 0.8	10.6 ± 0.9
190	7.07 ± 1.15	1.14	6.20 ± 1.01	5.93 ± 1.15
439	4.14 ± 0.91	1.12	3.70 ± 0.81	3.02 ± 0.91
coarse clay				
34	5.23 ± 0.73	1.96	2.67 ± 0.37	3.27 ± 0.73
190	4.66 ± 0.29	1.91	2.44 ± 0.15	2.75 ± 0.29
439	2.52 ± 0.15	1.51	1.67 ± 0.10	1.01 ± 0.10
fine silt				
34	0.752 ± 0.090	10.2	0.0737 ± 0.0088	NC #
190	2.35 ± 0.45	1.44	1.63 ± 0.31	0.91 ± 0.45
439	1.19 ± 0.22	0.704	1.69 ± 0.31	0.49 ± 0.22

† (N mineralized / N present at beginning of 30-d period) x 100

‡ Calculated per kinetic models as explained in Appendix 6. For simplicity, error in modeled mineralization was assumed to be zero.

§ Aggregate protection factor = shaken slurry / undisturbed

¶ Aggregate-protected pool (% of N in fraction) = shaken slurry - undisturbed

Not calculable (computed pool size is negative).

Appendix 9. Correction for Error Due to Peak Overlap in ^{15}N-NMR Spectrum of Clover-Uracil-Soil

To correct for error due to peak overlap, the spectrum of ^{15}N-clover + ^{15}N-uracil + unlabeled soil (CUS) was approximated as a linear combination of previous spectra taken of (1) ^{15}N-uracil + unlabeled soil and (2) ^{15}N-clover + unlabeled soil. Upon showing the linear combination to be statistically valid, the relative contributions of clover and uracil in the CUS spectrum were estimated. The details of the computations are explained below.

Mathematically, for each NMR frequency f in the spectrum:

$$S_{CUS,MEAS}(f) \approx S_{CUS,SIM}(f) = k_{US}\, S_{US,MEAS}(f) + k_{CS}\, S_{CS,MEAS}(f) \qquad (1)$$

where $S_{CUS,MEAS}$ is the measured signal intensity in the clover/uracil/soil spectrum, $S_{CUS,SIM}$ is the simulated signal intensity in the clover/uracil/soil spectrum, $S_{US,MEAS}$ and $S_{CS,MEAS}$ are the measured signals in the uracil/soil and clover/soil spectra, respectively, and k_{US} and k_{CS} are positive constants. The statistical validity of Equation 1 was tested by adjusting k_{US} and k_{CS} so as to minimize chi-square as defined below:

$$\chi^2 = \frac{\sum_{f=f_L}^{f_R} \left(S_{CUS,MEAS}(f) - S_{CUS,SIM}(f)\right)^2}{\left(VAR_{CUS,MEAS} + VAR_{CUS,SIM}\right) df} \qquad (2)$$

where f_L and f_R are the NMR frequencies of the left and right edges of the spectral region of interest, df is the degrees of freedom (df = number of NMR frequencies in spectral

region of interest minus one), $VAR_{CUS,MEAS}$ is the variance of the measured spectrum of clover/uracil/soil and $VAR_{CUS,SIM}$ is the variance of the simulated spectrum of clover/uracil/soil. According to Bevington and Robinson (1992), a sufficiently small value of χ^2 indicates that the two distributions in the numerator of Equation 2 are not significantly different.

The variance of the measured spectrum in Equation 2 was estimated as the variance in signal intensity in 25 points chosen from a portion of the spectrum without peaks. The variance of the simulated spectrum was based on the rules of statistical error propagation:

$$VAR_{CUS,SIM} = k_{US}^2 VAR_{US,MEAS} + k_{CS}^2 VAR_{CS,MEAS} \qquad (3)$$

where k_{US} and k_{CS} are the constants from Equation 1, and $VAR_{US,MEAS}$ and $VAR_{CS,MEAS}$ are the variances of the measured spectra of uracil/soil and clover/soil. $VAR_{US,MEAS}$ and $VAR_{CS,MEAS}$ were computed per the aforementioned 25-point method.

The chi-square minimization per Equation 2 yielded $\chi^2 = 49.41$, corresponding to $p = 1.000$; thus the simulated and measured spectra (not shown) were not significantly different. Therefore, the linear combination of spectra was considered valid and the peak area percentages of the clover/uracil/soil sample were calculated as:

$$PAP_{U-N3} = \frac{k_{US} PA_{U-N3,US}}{PA_{TOTAL}} \times 100$$

$$PAP_{U-N1} = \frac{k_{US} PA_{U-N1,US}}{PA_{TOTAL}} \times 100$$

$$PAP_{CLOV} = \frac{k_{CS} PA_{CLOV,CS}}{PA_{TOTAL}} \times 100$$

where $PAP_{U\text{-}N3}$ and $PAP_{U\text{-}N1}$ are peak area percentages in the simulated clover/uracil/soil spectrum due to uracil N at ring positions 3 and 1, respectively. $PA_{U\text{-}N3,US}$ and $PA_{U\text{-}N1,US}$ are peak areas in the measured uracil/soil spectrum of uracil-N_3 and uracil-N_1, respectively. PAP_{CLOV} is the peak area percentage in the simulated spectrum due to clover, and $PA_{CLOV,CS}$ is the peak area in the measured clover/soil spectrum PA_{TOTAL} is defined as:

$$PA_{TOTAL} = k_{US} (PA_{U\text{-}N3,US} + PA_{U\text{-}N1,US}) + k_{CS} PA_{CLOV,CS}$$

Appendix 10. Estimation of Uncertainty in NMR Spectra Due to Noise and Subjectivity in Phasing/Baseline Correction

Phasing and baseline correction are somewhat subjective (dependent upon visual observation), and were performed twice for each spectrum. A standard deviation due to processing (sd_{PROC}) was computed for each functional group in the spectrum:

$$sd_{PROC} = \sqrt{\frac{\sum_{i=1}^{n} (PA_i - \langle PA \rangle)^2}{n-1}}$$

where n = 2, PA_i is the peak area of the functional group in the *i*th replicate of processing, and <PA> is the mean peak area of the functional group.

The error due to noise was also estimated for each spectrum. Twenty-five regions of 1 ppm width in the non-peak portion of each spectrum were integrated separately; the standard deviation of these twenty-five areas was defined to be the noise present per 1 ppm ($sd_{NOISE,1}$). From this quantity, a standard deviation due to noise (sd_{NOISE}) was calculated for each functional group in the spectrum based on the rules of statistical error propagation:

$$sd_{NOISE} = \sqrt{(WIDTH) \times sd^2_{NOISE,1}} = sd_{NOISE,1} \sqrt{(WIDTH)}$$

where WIDTH is the width in ppm of the horizontal base of the peak. Finally, the overall standard deviation was computed as:

$$sd_{OVERALL} = \sqrt{sd^2_{NOISE} + sd^2_{PROC}}$$

with the assumption that sd_{NOISE} and sd_{PROC} are independent.