Does the Acid Hydrolysis–Incubation Method Measure Meaningful Soil Organic Carbon Pools?

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ABSTRACT

The literature was reviewed and analyzed to determine the feasibility of using a combination of acid hydrolysis and CO_2–C release during long-term incubation to determine soil organic carbon (SOC) pool sizes and mean residence times (MRTs). Analysis of 1100 data points showed the SOC remaining after hydrolysis with 6 *M* HCl ranged from 30 to 80% of the total SOC depending on soil type, depth, texture, and management. Nonhydrolyzable carbon (NHC) in conventional till soils represented 48% of SOC; no-till averaged 56%, forest 55%, and grassland 56%. Carbon dates showed an average of 1200 yr greater MRT for the NHC fraction than total SOC. Long-term incubation, involving measurement of CO_2 evolution and curve fitting, measured active and slow pools. Active-pool C comprised 2 to 8% of the SOC with MRTs of days to months; the slow pool comprised 45 to 65% of the SOC and had MRTs of 10 to 80 yr. Comparison of field ^{14}C and ^{13}C data with hydrolysis–incubation data showed a high correlation between independent techniques across soil types and experiments. There were large differences in MRTs depending on the length of the experiment. Insertion of hydrolysis–incubation derived estimates of active (C_a), slow (C_s), and resistant pools (C_r) into the DAYCENT model provided estimates of daily field CO_2 evolution rates. These were well correlated with field CO_2 measurements. Although not without some interpretation problems, acid hydrolysis–laboratory incubation is useful for determining SOC pools and fluxes especially when used in combination with associated measurements.

S OIL ORGANIC MATTER (SOM) is a complex mixture of plant- and microbiologically-derived compounds (Stevenson, 1994). Fractionation schemes have been employed to (i) isolate meaningful pools that provide information on C and nutrient cycling; (ii) test hypotheses on soil formation and ecosystem functioning; (iii) relate SOM characteristics to soil management and global change; and (iv) aid in developing models describing SOM dynamics. Observed differences in MRTs from months to centuries to millennia for different SOM fractions prompted the development of models with multiple soil C pools with differing MRTs (Jenkinson and Rayner, 1977; Van Veen and Paul, 1981; Parton et al., 1987). To date, no single biological, physical, or chemical fractionation technique has been developed that adequately describes the continuum of SOC that exists in nature. Mismatches between measurements of ^{14}C-labeled plant materials using three size-density fractions and model outputs have been identified (Magid

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et al., 1996). Motavalli et al. (1994) and Smith et al. (2002) were not successful in their attempts to equate the active pool to microbial biomass plus soluble C fraction or the light fraction. Similar problems were found when attempts were made to equate the size of the slow pool in models with the particulate organic matter fraction (Metherell et al., 1995). However, more recent work investigating free and aggregate-associated SOC has produced more promising results (Kong et al., 2005; Sohi et al., 2005)

This paper reviews the degree to which a combination of acid hydrolysis and long-term laboratory incubation can be used to define SOC dynamics. Three pool models, with first order kinetics, have most often been found to best describe SOC dynamics (Paustian et al., 1992) with the general equation

$$C_{TSOC} = C_a e^{-k_a t} + C_s e^{-k_s t} + C_r e^{-k_r t}, \qquad [1]$$

where C_{TSOC} = the soil organic carbon released as CO_2 at time t, a = active, s = slow, r = resistant, k = decomposition rate constant (1/MRT), and t = time (days).

The coupling of long-term incubation with chemical digestion of SOC by acid hydrolysis has been reported by Collins et al. (2000), Haile-Mariam et al. (2000), Fortuna et al. (2003), and Paul et al. (2001a, 2001b). Resistant soil C (C_r) was measured by chemical fractionation using acid hydrolysis; its decomposition rate factor (k_r) was measured by ^{14}C-dating the NHC. Long-term incubation enabled endogenous soil microorganisms to biologically fractionate the SOC with their enzymes with a resultant release of CO_2 under aerobic conditions. Statistical analysis of the rate of CO_2 evolution during long-term incubation (curve fitting) estimated the pool size and turnover time for the active fraction (C_a). This corresponded to the rapid, initial release of CO_2–C during the initial phases of the incubation. The turnover time for the slow fraction (k_s) was estimated by statistical analysis from the slope of the CO_2–C release curves during extended incubation. The size of the slow fraction (C_s) was determined by the difference between the total SOC and the sum of C_a and C_r.

In this literature review, which is part of a series on the determination of meaningful pools of C and N in soil, we evaluate the degree to which acid hydrolysis and C mineralization during incubation can be used to describe SOC dynamics. This involves a description of the analytical procedures used and the rationale for selecting them. The efficacy of this approach has been determined using three approaches: (i) by assessing how well data

obtained by hydrolysis–incubation help interpret SOC dynamics under a range of management scenarios across time; (ii) by testing how well a combination of independent tracer and hydrolysis–incubation analyses on the same soil samples compare in their description of SOC dynamics; and (iii) a review of a paper that discusses how well the DAYCENT model (Parton et al., 1998) portrayed CO_2 evolution in the field when the parameters analytically determined by acid hydrolysis–incubation were inserted into the model.

REVIEW OF THE METHODS
Acid Hydrolysis

Feller (1997) reports that in 1804, De Saussure used boiling HCl to fractionate humus. De Saussure determined that humus contained more C and less O and H than the original plant residues from which it was thought to be derived. Acid hydrolysis, involving refluxing in 6 M HCl at 116°C, has been extensively used to characterize soil N with 65 to 80% of the N being solubilized (Bremner, 1967). The hydrolysis reaction, through the process of protonation and hydration, solubilizes compounds with O- and N-containing functional side-groups (Barriuso et al., 1987). Fatty acids, proteins, and polysaccharides are susceptible to acid hydrolysis treatment, while long-chain alkyls, waxes, lignin, and other aromatics are resistant to hydrolysis.

The size of the resistant pool (C_r of Eq. [1]) is measured by acid hydrolysis as NHC and its decomposition rate constant (k_r) is measured by ^{14}C-dating. Where carbon dates are unavailable, in most agricultural soils it is possible to substitute a value of 1000 yr for the MRT ($1/k$) without significantly altering the outcome of the curve fitting for the active and slow pools (Paul et al., 2001a). The hydrolyzability of samples is often expressed as % NHC, and calculated (Plante et al., 2006) using the following equation that corrects for mass loss of the mineral portion of the sample during hydrolysis:

$$\% \text{ NHC} = \frac{\left(\dfrac{\text{gC}}{\text{kg sample}}\right)_{\text{after}} \times \dfrac{\text{mass}_{\text{after}}}{\text{mass}_{\text{before}}}}{\left(\dfrac{\text{gC}}{\text{kg sample}}\right)_{\text{before}}}, \qquad [2]$$

where the terms represent the organic C concentrations and masses of the sample before and after acid hydrolysis. Significant sample mass loss has been observed during the hydrolysis procedure. This could not be accounted for by the mass loss of the hydrolyzed organic matter and is likely attributable to the solubilization of inorganic constituents such as carbonates, metal oxides, and some clay minerals.

Incubation

Waksman (1932) reported that as far back as 1891, the CO_2 evolution rate was used as a measure of SOC oxidation taking place in soils. By 1896, mois-

ture and temperature controls had been recognized. In 1924, Lemmerans and Weissman (see Waksman, 1932) stated that under aerobic and constant environmental conditions, CO_2 formation proceeded according to the equation

$$x = a \times k \times \text{t}^m, \qquad [3]$$

where x is the amount of CO_2 produced in time (t), a is the initial C content of the soil, and k and m are constants. Löhnis and Fred (1923) and Lundegärdh (1927) pointed out that CO_2 is produced by soil microorganisms, and related rates of soil CO_2 evolution to plant growth and SOC changes. Mathematical analysis of long-term incubation data were first conducted by Stanford and Smith (1972) in their analysis of N-mineralization curves.

Early incubation methodology for SOC mineralization was described by Minderman and Vulto (1973) and Stotsky (1965). The CO_2 evolution rate during the initial incubation period is indicative of the size and decomposition rate constant (1/MRT) of the active pool. Methods for analysis of CO_2 data are presented by Paul et al. (2001a), Robertson et al. (1999), and Zibilske (1994). The parameters C_a, k_a, and k_s are determined using nonlinear regression (NonLIN, Systat Inc., Evanston, IL; or PROC NLIN, SAS Institute, Cary, NC) of the CO_2 incubation data (Paul et al., 2001a). After measurement of C_a and C_r, the C_s is determined by difference:

$$C_s = C_{\text{SOC}} - C_a - C_r,$$

where C_{SOC} is the total soil C at the time soil is removed from the field. It is possible to determine soil C dynamics for both labeled and unlabeled C where the evolved CO_2 contains a useable ^{13}C signal. This can occur in soils with crops that have had a plant switch between C_3 and C_4 photosynthesis types, such as when continuous corn (C_4) is grown after a previously mixed cropping system on soils that were developed under trees (C_3) or when wheat (C_3 plant) is grown on warm-season, grassland (C_4 plants) derived soils. Similar possibilities exist where fossil-fuel derived CO_2 with a specific label is added during plant growth such as in an elevated-CO_2 study (Haile-Mariam et al., 2000).

The moisture and temperature at which the incubation is conducted affects the CO_2 release rates. Incubation conditions using a temperature of 25°C and a water content of 60% of water holding capacity (Elliott et al., 1994) have most often been used (Paul et al., 2001a). Other temperatures and moisture contents could be used depending on the hypothesis being investigated. The MRTs determined in the laboratory have generally been corrected to reflect the measured mean annual temperature of field sites by assuming a Q_{10} of 2 (Collins et al., 2000). Soils are best incubated immediately after collection. Where dried samples are necessary, a preincubation period can be used to minimize the flush of SOC mineralization attributable to drying and rewetting. Publications have reported incubation times of 200 to 800 d depending on the soil type and incubation temperature (Haile-Mariam et al., 2000; Collins et al.,

2000). In general, incubations should be performed until the CO_2 evolution rate is low and rate curves have declined to approach an asymptote.

RESULTS OBTAINED WITH HYDROLYSIS–INCUBATION

Acid Hydrolysis

Our review of the literature and analysis of available data consisted of approximately 1100 data points on NHC contents or %NHC across a range of soil textures, climates, land use histories, and methods of analyses. The most commonly used method was to reflux with 6 M HCl at 116°C for 16 h (Campbell et al., 1967; Collins et al., 2000; Follett et al., 1997; Haile-Mariam et al., 2000; Jenkinson and Rayner, 1977; Martel and Paul 1974a, 1974b; Martel and LaSalle, 1977; McLauchlan and Hobbie, 2004; Paul et al., 1997, 2001a). Some studies used 6 M HCl at 96°C (Plante et al., 2006) or 100°C (Tan et al., 2004), while one study refluxed soil in 2 M H_2SO_4

at 116°C (Vlassak et al., 1969). Consistent differences were observed based on the method used ($P < 0.001$), but in all cases, the amount of NHC was strongly correlated to the amount of total SOC (Fig. 1). The most frequently used method (6 M HCl at 116°C for 16 h) had a regression slope of 0.51, while the use of 6 M HCl at 96 and 100°C resulted in a regression slope of 0.58, and the use of 2 M H_2SO_4 at 116°C resulted in a regression slope of 0.75 between NHC and SOC.

Acid hydrolysis generated reproducible measurements on single sites regardless of edaphic characteristics. The NHC expressed as a percentage of total C (Fig. 2) tended to decrease with depth in the cultivated-conventional tillage and forested plots. The range of hydrolyzability also increased with depth as different soils were analyzed. There were not enough samples reported for grassland to verify the apparent large increase in NHC > 50 cm. Also, the literature did not report enough data at deeper depths in no-till plots to ascertain depth effects in that treatment.
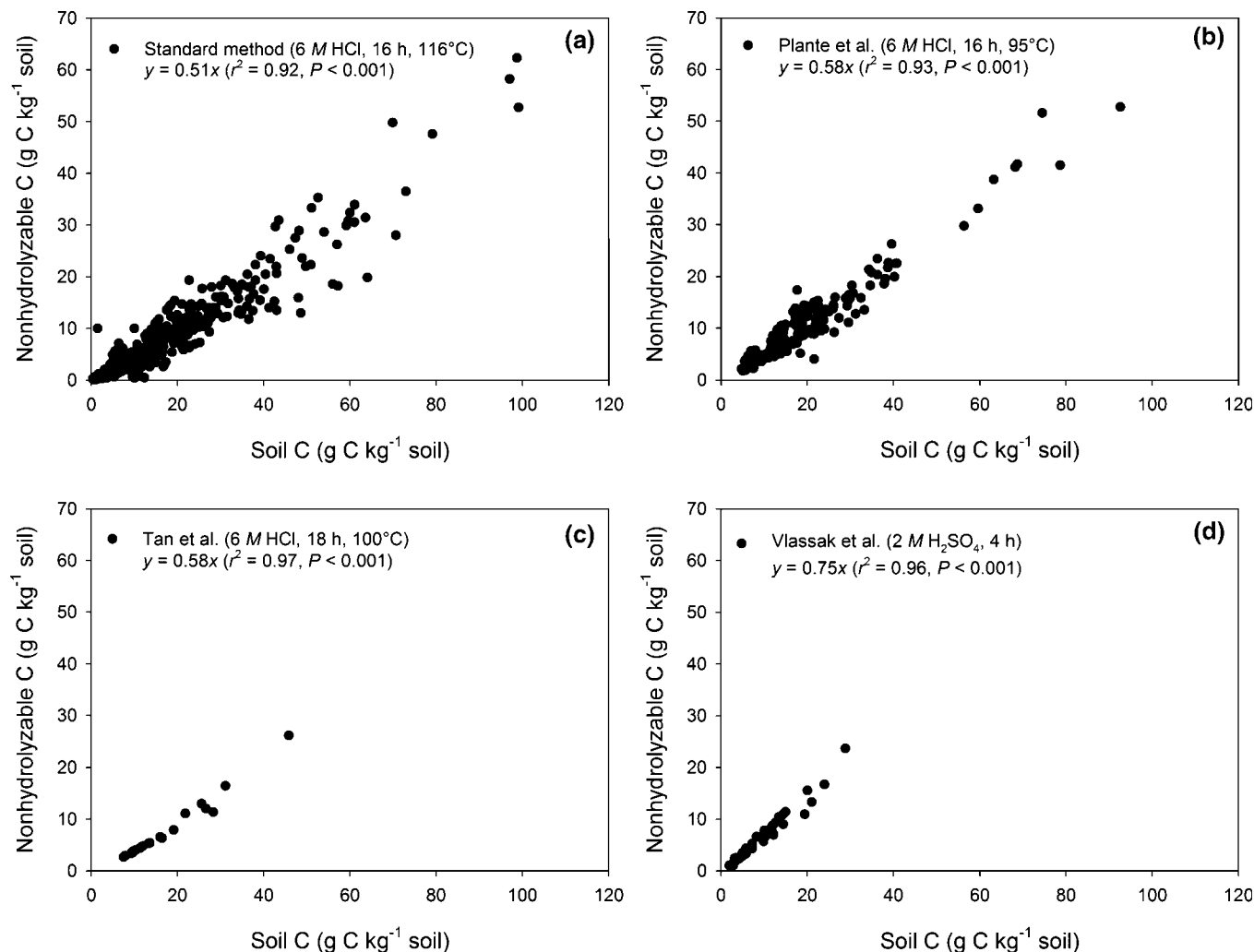

Fig. 1. Nonhydrolyzable soil C as a function of total soil C for each of four different methods of acid hydrolysis: (a) standard method, 6 M HCl for 16 h at 116°C (data from Campbell et al., 1967; Collins et al., 2000; Follett et al., 1997; Haile-Mariam et al., 2000; Jenkinson and Rayner, 1977; Martel and Paul, 1974a, 1974b; Martel and LaSalle, 1977; McLauchlan and Hobbie, 2004; Morris et al., 2006, unpublished data; Paul et al., 1997, 2001a, 2001b); (b) 6 M HCl for 18 h at 96°C (Plante et al., 2006); (c) 6 M HCl for 18 h at 100°C (Tan et al., 2004); and (d) 2 M H_2SO_4 for 4 h (Vlassak et al., 1969).
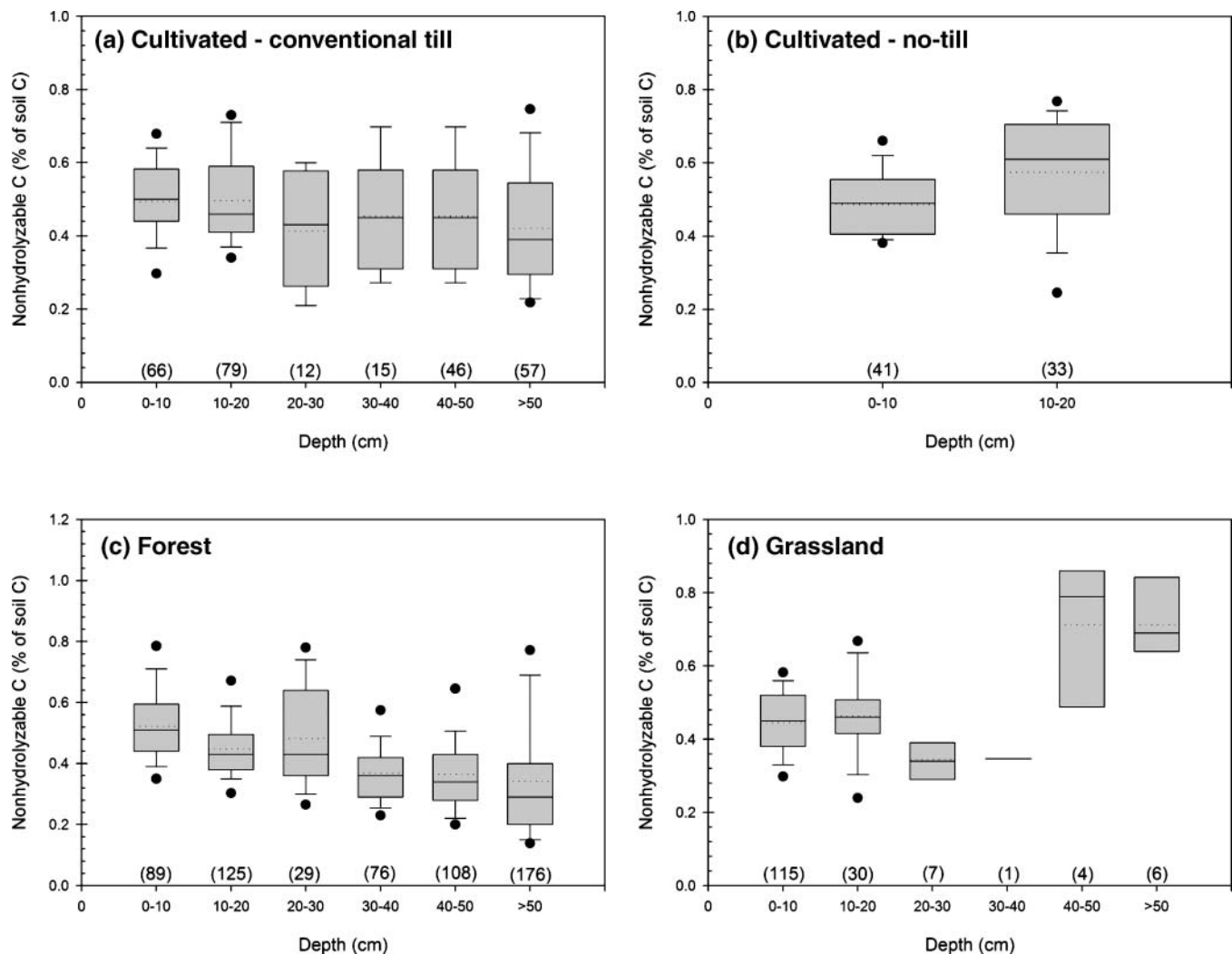
Fig. 2. Proportion of nonhydrolyzable C as a function of depth for (a) cultivated conventional till, (b) cultivated no-till, (c) forest, and (d) grassland samples. Median values are represented by the solid line in each box, mean values by the dotted line, 25th and 75th percentiles by the upper and lower bounds of the box, 10th and 90th percentiles by the upper and lower whiskers, and fifth and 95th percentiles by dots above and below boxes, where available. The number of data points within each depth–management combination is indicated in parentheses above the x axis. Data obtained with other methods were normalized to those obtained with 6 M HCl at 116°C for 16 h using data from Fig. 1.

The slope of the regression lines for NHC vs. SOC for no-till, native forest, and grassland sites was 0.56, and did not differ among land use types (Fig. 3). The NHC in conventionally tilled soils differed significantly from other land-use types ($P < 0.001$), with a slope of 0.48. This result is the opposite of what would be expected from modeling exercises such as with the Century Model (Parton et al., 1987). Passive soil C, as used in Century, should be a greater proportion of the total SOC under cultivation as a result of a loss of labile C due to enhanced mineralization and loss of protection. Martel and Paul (1974a, 1974b) observed a significant decrease in SOM content and a significant increase in radiocarbon age of both the SOC and the NHC after 60 yr of cultivation of virgin grassland. They did not, however, observe an appreciable change in the %NHC on cultivation. A decrease in inputs of recent C to the resistant pool, under cultivation, together with the possibility that factors other than biochemical recalcitrance help control

trol the proportion and radiocarbon age of NHC, were thought to be responsible for this observation. Poirier et al. (2005) recently suggested that the great resistance of NHC to acid hydrolysis is not paralleled by a great resistance to biodegradation triggered by changes in land use.

The data of Campbell et al. (1967), Martel and Paul (1974a, 1974b), Trumbore (1993), Leavitt et al. (1997), and Paul et al. (2001b) indicate that, on average, the NHC of surface soils is 1200 to 1400 yr older than that of the total SOC. Falloon and Smith (2000) summarized estimates of soil ^{14}C ages from different studies and found the average ^{14}C age of NHC was 1827 yr before present. Subsurface samples show greater increases in the MRT of the NHC with depth (Anderson and Paul, 1984; Stout et al., 1981). Yet, under land-use change, the amount of NHC in soils may be lost or gained during relatively short periods of time relative to the age of the resistant C pool. This is inconsistent with its assumed
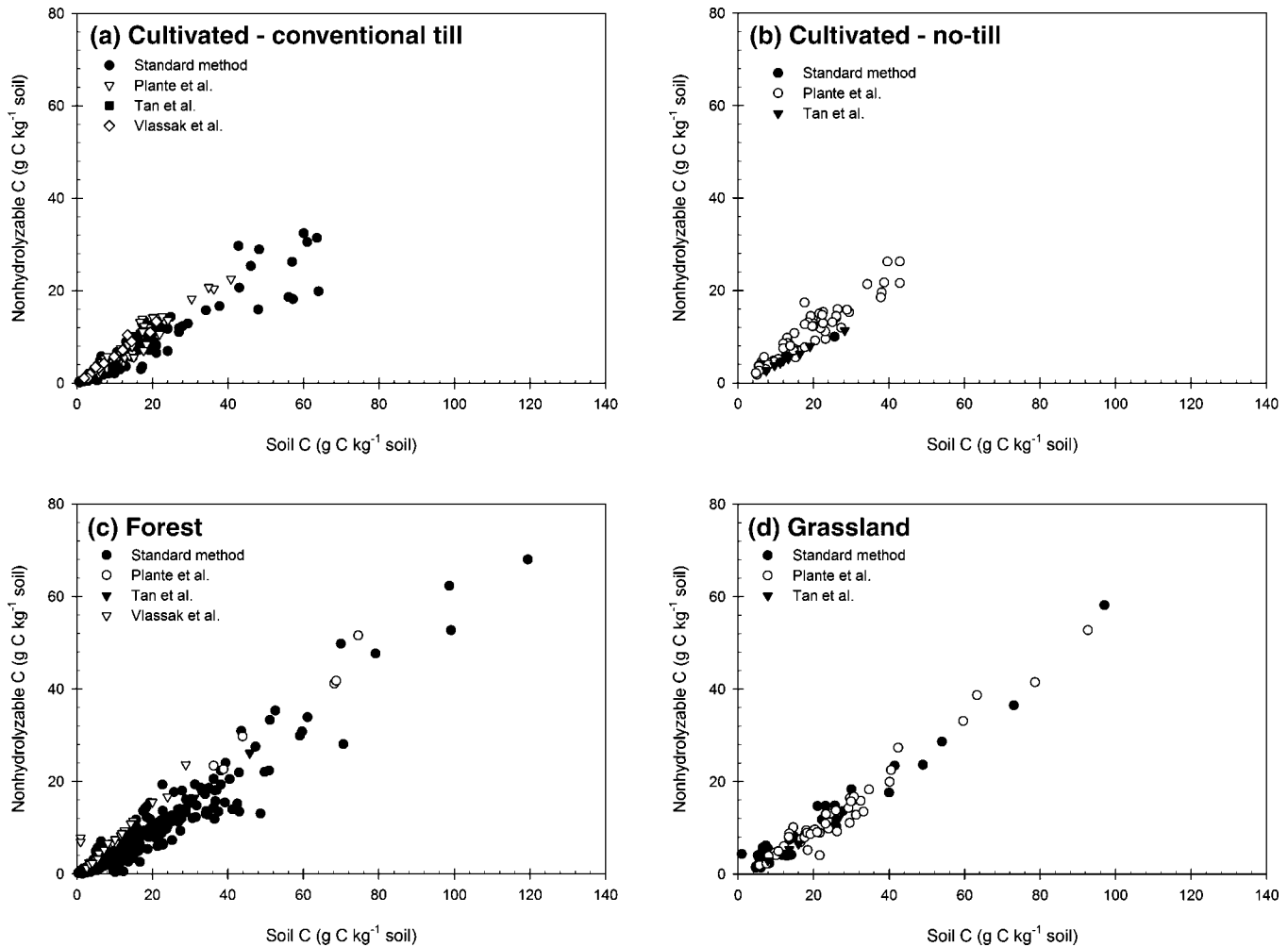
Fig. 3. Nonhydrolyzable soil C as a function of total soil C for samples from four different land-use types: (a) cultivated conventional till, (b) cultivated no-till, (c) native forest, and (d) native grassland (data from Campbell et al., 1967; Collins et al., 2000; Follett et al., 1997; Haile-Mariam et al., 2000; Jenkinson and Rayner, 1977; Martel and LaSalle, 1977; Martel and Paul, 1974a, 1974b; McLauchlan and Hobbie, 2004; Morris et al., 2006, unpublished data, Paul et al., 1997, 2001b; Plante et al., 2006).

recalcitrant-chemical nature and its observed age. Accumulation of C in the NHC pool would suggest that physical protection, association with soil minerals, and chemical recalcitrance are important for accumulation in soils.

An analysis of the MRT of three depths of seven cornbelt soils under various management practices (Paul et al., 2001b) produced negative regressions between the ^{14}C MRT of the total soil with its sand contents at depth (Table 1). Clay content had a positive effect on the MRT values. As with the effect of sand contents, there was greater statistical significance with depth with clay contents. The silt content had no statistically significant effect on the MRT of the SOC. Silt contents also were not associated with MRTs of the NHC at the surface of the seven sites, but silt affected the MRT of the NHC at depth. The intercepts show the general increase in MRT with depth. The great ages (high MRTs) of the NHC fraction are indicated by the intercepts, with silt having generally higher values than clays (Table 1).

Plante et al. (2006) showed that the proportion of NHC in silt and clay derived from microaggregates

ranged from 25 to 90% (Fig. 4). The silt-sized components of the 53- to 250-μm-sized microaggregates had more NHC than the clay components. This is in agreement with the data on MRTs of the various textural classes in Table 1 and with Paul and Van Veen (1978) who stated that NHC had much greater MRTs than

Table 1. The effect of sand, silt, and clay (g kg^{-1}) on the mean residence time (MRT, yr) of total and nonhydrolyzable soil C (data from Paul et al., 2001b).

| Regression	Depth	Equation (^{14}C MRT =)	R^2	P
	cm			
Total ^{14}C vs. sand	0–20	16 (sand) + 1015	0.26	0.09
	25–50	−42 (sand) + 3402	0.57	0.002
	50–100	−49 (sand) + 6109	0.56	0.002
Total ^{14}C vs. clay	0–20	16 (clay) + 131	0.34	0.03
	25–50	45 (clay) + 850	0.42	0.01
	50–100	71 (clay) + 2093	0.50	0.004
Total ^{14}C vs. silt	0–20	−20 (silt) + 2890	0.36	0.02
	25–50	−7 (silt) + 2892	0.08	0.76
	50–100	27 (silt) + 3970	0.07	0.40
Nonhydrolyzable ^{14}C vs. silt	0–20	15 (silt) + 1234	0.05	0.48
	25–50	68 (silt) + 2661	0.10	0.47
	50–100	155 (silt) + 3930	0.71	0.01
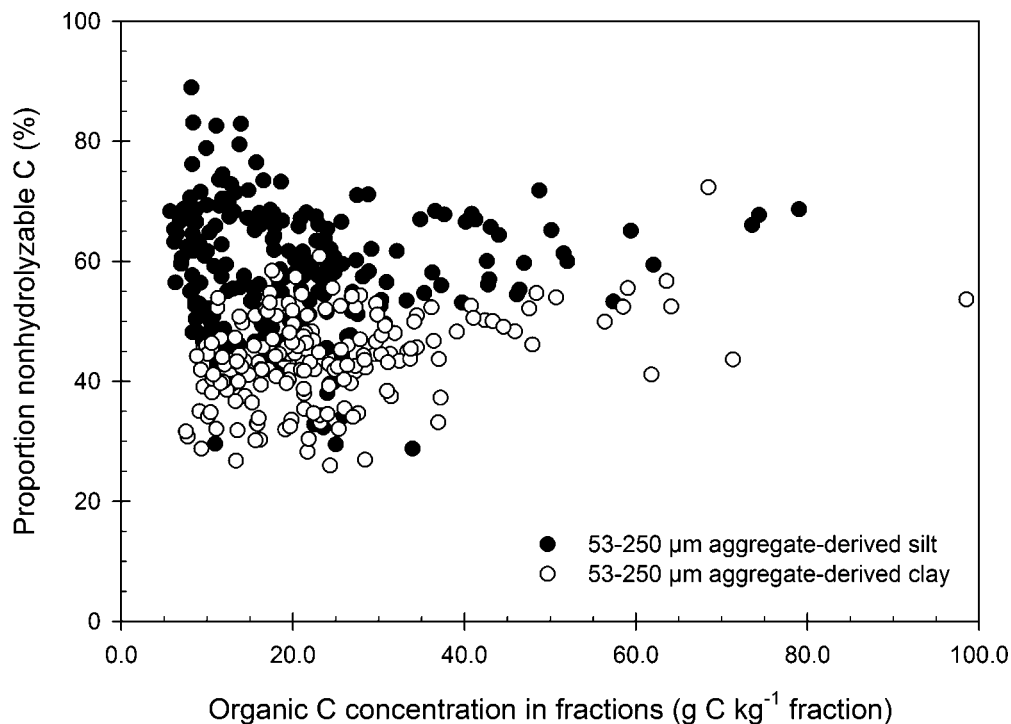
Fig. 4. Proportion of nonhydrolyzable C (%NHC) in silt- and clay-sized fractions isolated from 53- to 250-μm microaggregates (data from Plante et al., 2006).

SOC and that clay-associated C generally had smaller MRTs than C associated with silts.

Incubation

The quantification of C mineralization processes during incubation has been utilized to provide information about (i) the physiological status or catabolic potential of soil microbial populations; (ii) decomposition of specific organic substrates and soil C availability; (iii) soil biomass size and activity; (iv) the relative contributions of microbes, fauna, and abiotic sources (e.g., the dissolution of carbonate minerals); and (v) pool sizes and fluxes of organic matter (Robertson et al., 1999; Zibilske, 1994). Several investigators have used incubation to effectively identify an active fraction of soil C. McLauchlan and Hobbie (2004) found strong, direct relationships between several methods to estimate the size of the labile SOC fraction, including incubation. Alvarez and Alvarez (2000) also found strong relationships between the active C fraction measured using incubation, the size of the light fraction (density < 1.59 g mL^{-1}), and the size of the microbial biomass fraction. They evaluated five decay models (exponential, hyperbolic, double exponential, exponential plus linear, exponential plus constant) and found that all models explained some of the variation observed in CO_2 fluxes. Exponential and hyperbolic models were the only two able to perform well for all of their experimental cases.

Incubations in which CO_2 evolution rates approached an asymptote have been used to identify treatment effects on C dynamics in management studies involving crop rotations and composting (Fortuna et al., 2003).

Paul et al. (2003) analyzed the effect of management on the SOC dynamics of six long-term Canadian Prairie sites, and found rates of CO_2 evolution to be very useful in interpreting SOC dynamics, especially during the early stages of management when it is difficult to obtain statistical differences in total SOC levels. This was especially the case when differences in CO_2 evolution rates were expressed relative to differences in total C accumulation rates at a particular site. This approach normalized soil type effects and showed a high correlation of CO_2 evolution with particulate organic matter measured on the various treatments.

In a long-term incubation of corn-belt soils, Collins et al. (2000) found that 4.0 to 9.4% of the SOC was evolved during 800 d of incubation with little effect of depth when the CO_2 was expressed as a percentage of the SOC. Haile-Mariam et al. (2000) analyzed CO_2 evolution from ponderosa pine (*Pinus ponderosa* C. Lawson) soils subjected to increased levels of atmospheric CO_2 containing an identifiable ^{13}C label. They found that 4% of the surface horizon SOC was evolved in soils incubated at 37°C for 150 d. Three percent of the SOC was evolved from samples at depth. They measured the ^{13}C content of the evolved CO_2 and of the soil after incubation to determine the fraction derived from the labeling during growth in an enhanced CO_2 environment. In this soil, the ^{13}C-label associated with the clay-sized fraction decreased less (2.9% decrease) during incubation than did the ^{13}C-label associated with the silt-sized fraction (9.7% decrease). The particulate organic matter plus sand fraction lost 10.7% of the label whereas the most active pool, the light fraction, lost 35.6% of its label-induced ^{13}C during incubation.
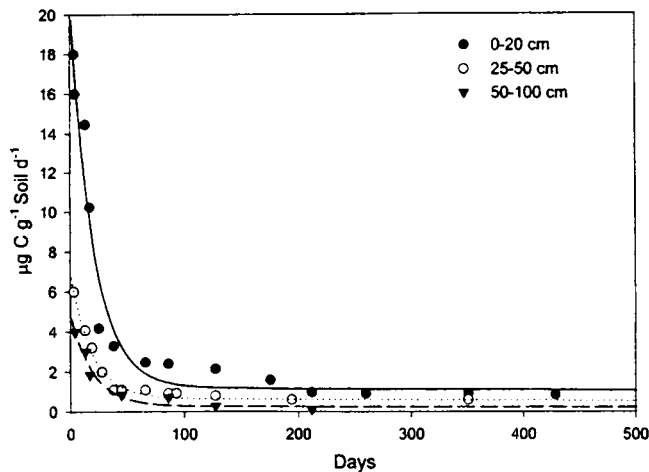
Fig. 5. Carbon dioxide evolution rate curves (μg C g soil^{-1} d^{-1}) for three depths (0–20, 25–50, and 50–100 cm) from a Michigan corn-belt soil (from Collins et al., 2000).

The general shape of incubation curves, when expressed as a rate function, is exemplified by the data from the Kellogg Biological Station–Long Term Ecological Research (KBS–LTER) site in Hickory Corners, MI (Fig. 5). The shoulder at approximately Day 100 has been found in many of the curves analyzed and could be an indication of a change in the soil biota responsible for decomposition during this period. Soil fauna have been shown to develop slowly during incubation (Coleman et al., 2004) and changes in bacterial to fungal ratios during incubation have been found (McGill and Paul, 1976; McMahon et al., 2005). Another explanation for the CO_2 shoulder could be a change in substrate availability at this time. The CO_2 evolved from the slow pool is measured as the gradual drop to the asymptote. Atypical CO_2 evolution curves, where the evolution is better expressed as zero order than first order kinetics,

have been occasionally found, especially in tundra soils (Weintraub and Schimel, 2003).

Analysis of Hydrolysis–Incubation Combined

Results from acid hydrolysis and incubation can be combined to provide an analytical determination of the pool sizes and kinetics of the active, slow, and resistant pools (Collins et al., 1999; Haile-Mariam et al., 2000). Table 2 gives an example of the type of data obtained by the hydrolysis–incubation method for six sites. The pool sizes for KBS–LTER in Michigan were obtained from the analysis of CO_2 evolution curves shown in Fig. 5. The NHC (C_r) with a MRT of 979 yr represented 45% of the SOC in the surface. In this soil, NHC decreased to 31% at depth where it had a MRT of 4400 yr. The slow pool (C_s) represented 49% of the SOC at the surface and 69% at depth with MRTs of 27 yr at the surface and 17 yr at depth. Some of the sites in Table 2 showed a decrease in the %NHC with depth; however, some sites did not. In all cases, the MRT of the NHC increased with depth. In sites such as Wooster, where ^{14}C-dates were unavailable, the MRT of the NHC was assigned a value of 1000 yr (Paul et al., 2001a) for calculation purposes. The slow pool represented the largest pool in the subsurface soils, but represented the largest pool in only three of the six surface samples and generally had MRTs of decades. The active pool varied most with soil type, being particularly small as a percentage of the SOC in the Placerville, CA, site under a ponderosa pine plantation. In all soils, it had MRTs of months rather than the years measured for the slow pool.

The analysis of hydrolysis–incubation data used to determine pools and fluxes utilizes first order equations. The modeling of such equations dictates that there are interrelationships between the decomposition rate constants and pool sizes utilized (Campbell et al., 2000;

Table 2. Pool sizes and soil organic C mineralization kinetics (mean resident time, MRT)† determined using acid hydrolysis and long-term incubations of samples from five different sites for the 0- to 20-, 25- to 50-, and 50- to 100-cm depths.

Site	Depth	Total C	Resistant pool (C_r)		Slow pool (C_s)		Active pool (C_a)	
			%	MRT	%	MRT	%	MRT
	cm	g kg^{-1}		yr		yr		d
KBS–LTER, MI	0–20	10.7a‡	45	979	49	27a	6.0a	78a
	25–50	2.6b	30	895	61	15b	7.6b	67a
	50–100	1.3c	31	4400	69	17b	7.6b	5b
Lamberton, MN	0–20	17.9a	49	1100	45	41a	3.3a	148a
	20–50	8.7b	40	3100	45	34b	3.4a	66b
	50–100	4.3c	28	6100	67	28c	4.6b	11c
Arlington, WI	0–20	17.6a	50	485	47	28a	2.2a	90a
	20–50	8.5b	35	2620	62	43b	2.4a	40b
	50–100	3.0c	30	4412	66	36b	3.3c	11c
Hoytville, OH	0–20	17.8a	45	1770	51	45a	3.3a	95a
	20–50	8.6b	44	5660	50	37a	4.6b	66b
	50–100	4.3c	39	9875	51	21b	4.6b	10c
Wooster, OH	0–20	10.9	39	N.D.§	55	39a	5.5a	145a
	20–50	7.0	44	N.D.	51	39a	3.3a	102b
	50–100	2.2	32	N.D.	64	23b	4.5a	49c
Placerville, CA	0–18	18.1a	52	985	47	34a	0.7a	34a
	18–30	15.3a	47	1480	52	39a	0.7a	24a
	30–60	7.3b	48	2013	50	37a	1.8b	53b

† Laboratory incubation MRTs converted to field using $Q_{10} = 2^{[(25-T)/10]}$, where T = mean annual temperature.
‡ Values within a column, by site, followed by the same letter, are not significantly different at P = 0.05. Data recalculated from Collins et al. (2000) and Haile-Mariam et al. (2000).
§ N.D., not determined.

Paustian et al., 1992). The hydrolysis–incubation method establishes the size of the slow pool as the difference between the sum of C_a and C_r from

$$C_{SOC}[C_s = C_{SOC} - (C_a + C_r)].$$

The curve fitting used to determine the best fit relates C_a and C_s with k_a and k_s. Solving for C_s and k_s by this method dictates that they are inversely related to C_r. The data in Fig. 1 and 3 show a high correlation between NHC and SOC with approximately 48 to 56% of the SOC being hydrolyzed. The primary effect of the %NHC in the curve-fitting calculations is to change the value for C_s.

Collins et al. (2000) related the pool sizes of the C_3 (noncorn) derived SOC measured directly from soil samples to their MRTs, which were measured with hydrolysis–incubation, in plots where corn (C_4) had been grown for the last 10 to 30 yr (Fig. 6). This diagram does not differentiate whether the MRT or the size of the pool is the factor controlling the relationships. It does, however, show that the corn-belt soils derived from forested soils (Hoytville, OH; Hickory Corners, MI; and Wooster, OH) had larger pool sizes relative to their MRTs than those derived from the prairie soils (Lamberton, MN, and Arlington, WI). Both curvilinear plots of Fig. 6 show that this approach separates out original vegetation type with a high correlation between size of the pool and its MRT. Fortuna et al. (2003) utilized the hydrolysis–incubation method to investigate the effects of fertilization, green manure, crop rotations, and compost on pool sizes and MRTs of both C and N at a

KBS–LTER site. They found that compost derived from oak leaves and cattle manure resulted in the largest changes during a 6-yr field experiment. Compost additions increased total soil C by 14% relative to the fertilizer N treatment. The proportion of NHC (C_r) increased by 30% and the slow pool by 10%. Both the size and MRT of the active pool decreased with oak–manure compost additions, showing the recalcitrant nature of the compost. This was reflected in N availability: compost additions did not supply enough N for the crop except in first-year corn following wheat–fallow cover crop treatments.

Validation of the Methodology

Approaches for isolating soil C fractions that are functionally meaningful should be (i) repeatable with low errors; (ii) amenable to soils with varying textures and mineralogical characteristics; (iii) have a high signal to noise ratio; and (iv) be useful in hypothesis testing. Measurements that are inexpensive, simple, and rapid are preferred over those that are expensive, complicated, or slow. Data from the fraction that is isolated should provide meaningful information on SOC dynamics. Theoretically, the size of the resistant fraction should be related to litter quality, intrinsic soil properties, climate, and management and change slowly on land-use change such as cultivation or afforestation. To be most useful, active and slow fractions should have characteristics that differ from the resistant fraction and from the whole soil. Acid hydrolysis can be conducted during a short time frame with automated equipment, and the
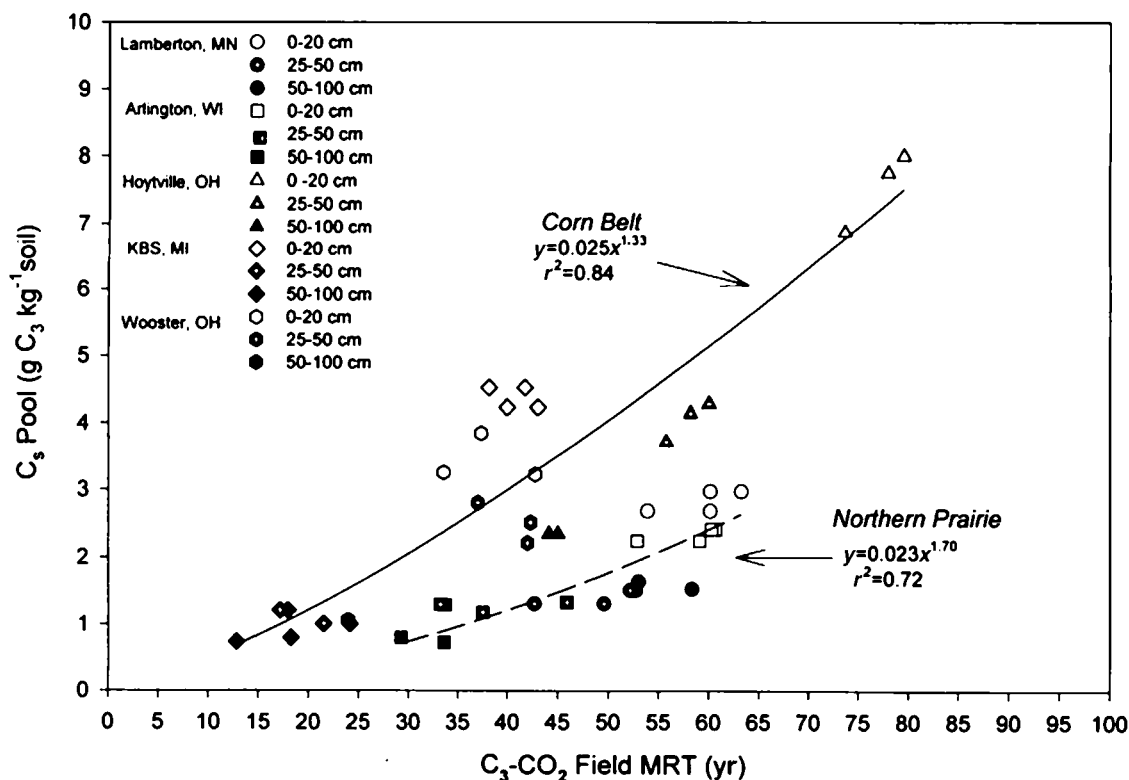

Fig. 6. Size of the slow soil C pool (C_s) determined using the hydrolysis–incubation method as a function of mean residence time (MRT) determined using ^{13}C content of soil for Corn Belt and Northern Prairie soils (from Collins et al., 2000).

results for individual soils are reproducible. Laboratory incubation is more challenging because of its extended nature. These challenges are not insurmountable because the methodology is relatively straightforward. The extended incubations conducted during long intervals require periodic sampling, oversight, and maintenance during long periods, take up room in climate-controlled facilities, and delay the interpretation of data. However, later stages of incubation are sampled less frequently than earlier stages.

The use of ^{13}C studies (Collins et al., 1999), ^{14}C dating (Paul et al., 2001b), and hydrolysis–incubation experiments (Collins et al., 2000) on the same soil samples made it possible to compare the results from three independent analyses on the same soil samples. Paul et al. (2001b) compared ^{13}C MRT data with data obtained using ^{14}C carbon dating on the same site with 30 yr of continuous corn (Collins et al., 1999). Their data (Fig. 7) demonstrates that the ^{14}C MRTs were related to the ^{13}C measurements by the equation

$$^{14}C \text{ MRT} = 176(^{13}C \text{ MRT})^{0.54}, R^2 = 0.70. \quad [4]$$

They suggested that the curvilinear equation reflected the thousands of years of exposure time for the ^{14}C MRTs relative to the 10 to 30 yr exposure for the ^{13}C MRTs measured during $C_4 \leftrightarrow C_3$ replacement in the continuous corn plots. Given sufficient time, the two tracers should provide similar data. On the same plots, Collins et al. (2000) observed a linear relationship with a high degree of correlation between the sizes of the C_3 soil MRT as determined in the field with the $C_4 \leftrightarrow C_3$ plant switch and that measured by an 800-d incubation and curve fitting with the equation

$$^{13}CO_2 \text{ MRT} = 0.33(C_3 \text{ field MRT}) + 34.3, R^2 = 0.55. \quad [5]$$

The 800-d incubation produced one third of the MRTs of those of the 30-yr-old field-derived measurements.

The fact that ^{14}C, ^{13}C, and the hydrolysis–incubation method gave highly correlated data gives credence to the hypothesis (Paul et al., 2003) that SOC is comprised of a continuum of closely related products with different MRTs. The MRTs measured in tracer experiments will depend on inherent controls on SOC dynamics as well as on the length of the experiment. Short-term experiments or weaker extractants (Martel and Paul, 1974b) identify the parts of the SOC continuum with faster turnover rates. The large differences in the MRTs of the SOC, depending on the time of tracer exposure, lead to the conclusion that data from soil-tracer experiments should be reported relative to the length of exposure to the tracer. In such a continuum, longer exposures will result in longer MRTs as the components have a greater time to approach steady state.

The validity of the hydrolysis–incubation method was tested by Paul et al. (1999) by incorporating the analytically derived-pools and fluxes, as described in Table 2, into the DAYCENT model (Parton et al., 1998). Crop residue inputs and abiotic data required by the model were obtained from the KBS–LTER web site (http://lter.kbs.msu.edu/; verified 10 Feb. 2006). Model output was related to CO_2 values measured in the field at the KBS–LTER site (Fig. 8). As previously noted, the high CO_2 evolution rates of subsurface soils in the laboratory, together with the decrease in NHC of the subsurface horizons of this site, resulted in an increased size of the C_s and C_a pools, and a decrease in MRT of both pools with depth. Paul et al. (1999) reduced subsurface decomposition rate constants, determined by laboratory incubation, by 20% to account for the possibility that the subsurface soils may have given artificially high CO_2 values in the laboratory. This could have been because of changes in temperature or breakdown of aggregates and mixing during sampling. The 20% decrease in decomposition rates was based on the observation that burial of cellulose strips at depth resulted in 20% lower decomposition than at the surface (Coleman et al., 1980).
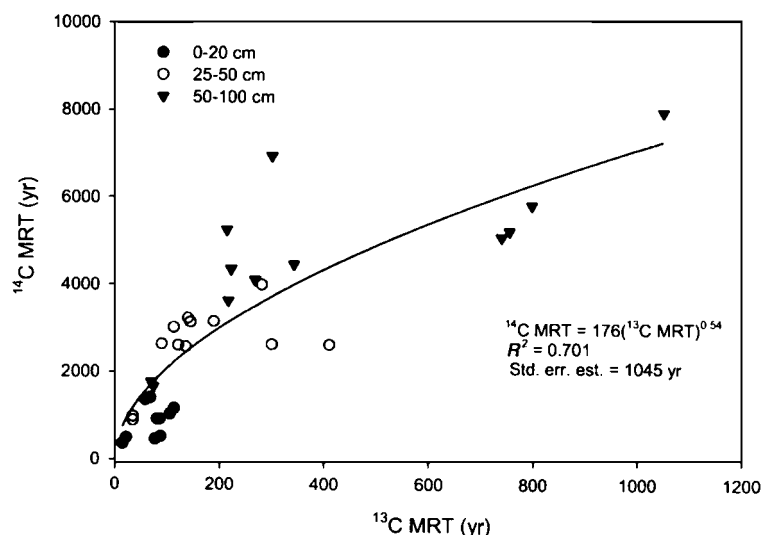

Fig. 7. Mean residence time (MRT) of total soil organic C by ^{14}C-dating vs. ^{13}C after a $C_3 \leftrightarrow C_4$ plant switch to continuous corn for soils from three depths (adopted from Paul et al., 2001b).
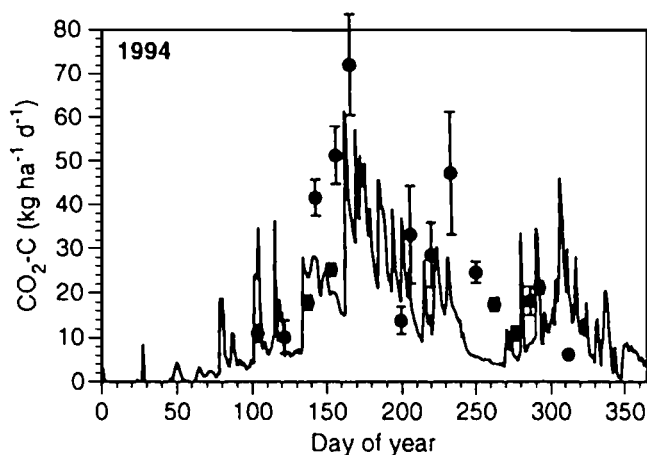
Fig. 8. CO_2 fluxes generated using DAYCENT modeling parameterized with hydrolysis–incubation data (solid line) compared with CO_2 fluxes measured in the field (dots represent means with standard error) for the conventional tillage plots at the Kellogg Biological Station–Long Term Ecological Research site (from Paul et al., 1999).

A possible difference in field temperature for the subsurface horizons was not incorporated into the model, but may already be partially incorporated into the 20% lower decomposition rates observed at depth by Coleman et al. (1980).

The predictions of CO_2 evolution provided by the model were approximately one third lower than those measured (Fig. 8). Paul et al. (1999) attributed this to the fact that root respiration, which has often been found to be approximately one third of total belowground respiration (Law et al., 2001; Warembourg and Paul, 1973), was measured in the field, but not included in the model. The close fit between measured and modeled data, shown in Fig. 8, was said to validate the use of the analytically derived inputs measured by hydrolysis–incubation as well as the applicability of the model used. There were differences between the modeled data and the field data in the fall. Paul et al. (1999) attributed this to the observation that plant residues, when applied to soil, require a time of physical breakdown and incorporation before decomposition. This factor is not now considered in DAYCENT (Parton et al., 1998).

DISCUSSION

Our review and analysis of the literature show that NHC measurements are simple and repeatable, have a high signal to noise ratio, and isolate a fraction that is much older than whole-soil C. The rapid changes in the size of this fraction in response to changes in management do not fit with the concepts of a passive fraction in many present SOC models. This also is not consistent with the generally held concept that resistant fractions should only undergo slow changes during SOC loss or accrual. Several possible explanations that we propose for the differences between the measurable NHC and a conceptual resistant pool are (i) acid hydrolysis does not remove all the modern plant residues, particularly lignin; (ii) more than just biochemical complexity is involved in

forming resistant C; (iii) NHC, although much older than the SOC, does not adequately represent the resistant C pool; (iv) our understanding of SOM dynamics is incomplete; or (v) the models are not correct. The possibility of contamination by recent plant residue inputs is minimized in ^{14}C-dating techniques by removal of all plant residues by flotation and hand picking under a microscope (Martel and Paul, 1974a). This operation is not possible in the many analyses often associated with fractionation of the large number of samples in soil-management studies. However, the differences in %NHC of isolated silt and clay fractions observed by Plante et al. (2006), as shown in Fig. 4, should not be affected by recent plant residues, indicating that plant residue contamination is not a major factor in the interpretation of hydrolysis results.

It is difficult to determine the amount of SOC that is biochemically recalcitrant relative to that which is difficult to decompose because of physical protection and association with silt and clay minerals (Paul and Van Veen, 1978). López-Ulloa et al. (2005) found that SOC stabilized in Andisols was associated with metal–humate complexes and the mineral allophone. Long-term stabilization in Inceptisols was associated with sorption to clay minerals and noncrystalline (hydr-) oxides. The loss of soil C on tillage can be due to the altered physical protection in soils that may allow resistant C to be released. Land-use change can also increase rates of erosion which will remove soil C (Lal et al., 1998). Changes in the composition of the crop residues, rooting type, and depth also play a role, as do the effects of the incorporation of plant residues. The results with no tillage show enhanced buildup of SOC and the proportion of NHC with no changes in the type of crop grown or the amount of residue added (Lal et al., 1998). A two-stage hydrolysis procedure with a weak acid pretreatment has been used in the past to ensure that soil sugars do not caramelize during the 6 M HCl exposure to high temperatures (Martel and Paul, 1974a; Persson, 1967). This should especially be considered where the NHC is to be used for further chemical characterization.

A number of authors (Schmidt et al., 1999; Skjemstad et al., 1996, 2002) have suggested that materials identified as charcoal, black carbon, or char could account for a substantial portion of recalcitrant SOC in agricultural soils. These materials were most often measured by UV irradiation (Skjemstad et al., 1993) or preferential extraction (Kuhlbusch et al., 1995). In Ponomarenko and Anderson's (2001) examination of North American soils similar to those reviewed in this study (Collins et al., 2000; Follett et al., 1997), they concluded that char was found in agricultural soils formed under both grassland and forested vegetation and on different parent materials. Char was present in all particle-size fractions, but was especially prevalent in the silt-sized fraction where it often had clay coatings or inclusions. The resistance to UV irradiation and the strong aromatic peak at 130 ppm in CP mass ^{13}C NMR lead them to conclude that up to 60% of the SOC of these soils could be char. The presence of char could help explain some of the NHC results in this review. The high MRT and the high

proportion of NHC in silt-sized fractions are consistent with the presence of char. Char cannot be related to the 30% increase in NHC in agricultural soils where oak leaf–cattle manure compost was added for 6 yr (Fortuna et al., 2003). It also cannot explain the doubling of both SOC and NHC in the afforested soils during a 50-yr period with no fires (Morris, 2006, unpublished data) that formed a significant part of this literature analysis. As is so often the case with SOC studies, the interaction of multitude processes and controls makes it difficult to assign one explanation such as char to SOC recalcitrance.

The rapid increase in NHC during short periods of afforestation or return of land to grassland vegetation is difficult to interpret. Morris (2006, unpublished data) showed that changes in NHC were highly correlated to two-fold gains or losses of SOC after approximately 50 yr of afforestation. This suggests that the NHC is a dynamic pool sensitive to management changes, and should not be considered strictly a passive pool. The increased age that comes with depth is likely a consequence of decreased inputs, lower temperature, and possibly lower microbial activity resulting in reduced degradation. The possibility of geological C (e.g., that brought in with the parent materials) must also be considered on some sites.

Earlier reviews of SOC dynamics (Paul and Van Veen, 1978) recognized that persistence in soil is not due to chemical recalcitrance or biochemical complexity alone, but is also attributable to an interaction of biochemical complexity with soil particles and aggregates. The model of Van Veen and Paul (1981) utilized a physical protection factor to account for the decomposition rate changes attributable to aggregation and interaction of SOC with soil silt and clay. The high correlations between different methodologies based on tracers and analytical methods that give different MRTs can best be explained by the hypothesis that young SOC does not differ greatly in composition from older SOC. This leads to the corollary that SOC does not necessarily change with age once it is formed. Biochemical resistance (as measured by acid hydrolysis), physical protection within aggregates (especially microaggregates), and silt and clay interactions all play an important role in SOC stabilization. Incubation that allows the microorganisms through their enzymes to fractionate SOC is not a direct measure, but does reflect these controlling factors.

The use of three SOC pools with first order reaction kinetics has no specific scientific validation, but does give meaningful estimates that cannot be obtained when dynamics are analyzed on the basis of one or two pools. Use of a two-pool unconstrained model that does not consider the size of the resistant C pool leads to pool sizes that differ considerably from the hydrolysis–incubation data. Paul et al. (2001a) showed that the size of C_s was underestimated by 75% and the MRT by 29 yr when a two-pool unconstrained model was used.

The temperature sensitivity of different pools of SOC is very important in predicting the effects of global warming on SOC dynamics and sequestration. Zogg et al. (1997) found that the size of the C pool available for rapid decomposition (Ca) varied with incubation temperature. Knor et al. (2005) suggest that nonlabile C is more sensitive to temperature changes than labile C. Fang et al. (2005) found that temperature sensitivity of the resistant organic matter was not different from that of labile pools. Reanalysis of a large number of soils between 1978 and 2003 across England and Wales (Bellamy et al., 2005) showed a very large loss during that period. The loss, irrespective of land use, was said to suggest a link to climate change. The possibility of loss from different pools, however, was not investigated.

The hydrolysis–incubation methodology relies on the assumption that differences in estimates of C_a, whether induced by incubation conditions or by climate or management in the field, will be reflected in the values for k_a, C_s, and k_s. Comparisons between soils or field treatments will be valid, and C_a variation induced by incubation will not be a problem for parallel incubations with uniform conditions. However, more information is required as to whether different pools of SOC have different temperature responses (e.g., Q_{10} values). At present, models tend to assign the same Q_{10} coefficients to all fractions (Jenkinson and Rayner, 1977; Parton et al., 1987).

CONCLUSIONS

This review and analysis of the literature indicates that the answer to the question, "do acid hydrolysis and incubation measure meaningful soil organic matter pools?" is a qualified yes. The hydrolysis–incubation method measures reproducible, sensitive pools. It can analytically determine the pool sizes and turnover rates of the continuum of related SOC that ranges from days to months to centuries and even to millennia. This was validated by comparing results determined using ^{13}C and ^{14}C, and by comparison of modeled data, using analytically determined pools and fluxes, with CO_2 evolution rates measured under field conditions.

The method works because SOC pools are best measured by techniques that take into account the various controls involved in SOC dynamics. Long-term laboratory incubation is a biological fractionation that allows soil enzymes and microbes to fractionate SOC into meaningful pools while implicitly accounting for differing degrees of substrate availability due to various protection mechanisms. Incubation data are, however, insufficient for estimating the size and turnover rate of resistant C because of their short time spans relative to the long MRTs of resistant C. Therefore, complementary information is required to estimate the size of the resistant pool.

There are a number of points that must be considered in utilizing and interpreting results using the hydrolysis–incubation method. In ^{14}C dating, NHC is on average 1200 to 1400 yr older than total SOC in surface soils, and even more so at depth. It is the oldest fraction determined in analysis of humates and SOC associated with silt and clay (Paul and Van Veen, 1978). In spite of this age, NHC is correlated with total SOC contents and can be rapidly lost or gained after changes in soil management, such as afforestation or cultivation. It must be

interpreted then that persistence in soil is not due to biochemical recalcitrance alone.

There is a strong correlation between pools and fluxes measured by hydrolysis–incubation and those measured by ^{13}C incorporation due to a $C_3 \leftrightarrow C_4$ vegetation change, as well as by ^{14}C dating. Large differences in the age of SOC determined with different methods suggest that results must be related to the length of the experiment. Tracer and even hydrolysis–incubation data need to be reported relative to the time of the experiment and tracer exposure.

The use of the combined hydrolysis–incubation method highlights that SOC is a continuum of closely related materials that are affected by multiple controls and can change rapidly under changing environments or management. All components of SOC (the active, slow, and resistant pools) are essential to the formation and stabilization of SOC. It should not be surprising that SOC cannot be formed or sequestered without the participation of all pools. The resistant fraction as defined by acid hydrolysis is a useful component of the hydrolysis–incubation method. The fact that it does not necessarily infer recalcitrance as defined in models indicates that the models may have to be updated to recognize the possibilities for fairly rapid changes in all components of the continuum of SOM on changes in management.

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