# Formation and Loss of Humic Substances During Decomposition in a Pine Forest Floor

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## ABSTRACT

**Since twice as much C is sequestered in soils as is contained in the atmosphere, the factors controlling the decomposition rate of soil C are important to the assessment of the effects of climatic change. The formation of chemically resistant humic substances might be an important process controlling recycling of $CO_2$ to the atmosphere. Our objectives were to measure the rate of formation and loss of humic substances during 13 yr of litter decomposition. We placed nets on the floor of a white pine (*Pinus strobus*) forest to separate each annual layer of litter for 13 yr and measured humic substance concentration using NaOH extraction followed by chromatographic fractionation. The humic acid fraction increased from 2.1% of the C in litterfall to 15.7% after 1 yr. On a grams per square meter (g $m^{-2}$) basis the humic substance fraction increased during the first year and then declined, with a half decay time ($t_{1/2}$) of 5.1 yr, which was significantly slower than the bulk litter ($t_{1/2}$ = 3.9 yr). The carboxylic C concentration estimated from $^{13}C$ nuclear magnetic resonance (NMR) increased in the litter over time, though total mass of carboxylic acid C in the forest floor also declined over the 13-yr period ($t_{1/2}$ = 4.6 yr). While humic substances in the forest floor decomposed at a somewhat slower rate than bulk litter during Years 1 to 13, they decomposed much faster than has been calculated from $^{14}C$ dating of the refractory fraction of organic matter in the mineral soil.**

Approximately twice as much C is stored in the soil as in the atmosphere (Lal et al., 1995). Consequently, the factors that control the storage of C in the soil are among those that play an important role in regulating the concentration of $CO_2$ in the atmosphere. The first-order decomposition rate constant of lignin, one of the slowest decomposing substances present in original plant material has been reported as 0.18 $yr^{-1}$ a half decay time ($t_{1/2}$) of 3.8 yr in a temperate soil (Lynch, 1991). However, the observed residence time of C is on the order of hundreds or even thousands of years in some mineral soils (Campbell et al., 1967). The factors that retard the mineralization of C originating from plant compounds are largely responsible for the storage of C in soil and the chemical transformation of C into humic substances may help explain this decrease in biodegradability. Humic substances are regarded as more resistant to decomposition than compounds directly synthesized by plants (Stevenson, 1994). Other hypotheses that might explain the observed persistence of C in soil include: physical protection from microbes within interstices too small for access by microbial cells, and formation of organomineral complexes that tend to protect against enzymatic attack. These hypotheses are not mutually exclusive. For example, adsorption of humic substances to Fe oxyhydroxides (Stevenson, 1994) and subsequent accretion of layers of the complexes might represent a combination of chemical and physical protection from enzymes.

Humic substances are operationally defined, but have been demonstrated to have characteristics distinct from compounds synthesized directly by plants (Stevenson, 1994). There are several theories of how humic substances are formed, but most studies agree that they are comprised of large (>700 atomic mass units [AMU]) molecules, with carboxylic acid, phenolic hydroxyl, and aromatic functional groups (Stevenson, 1994). Operationally, humic substances have been defined as substances extractable in NaOH. With the recognition that there might be NaOH soluble substances that are not humic, subsequent steps involving precipitation of humic acids at pH 1 and adsorption of fulvic acids to a hydrophobic resin have been added (Swift, 1996). Other steps have been added by Leenheer (1981) to further separate aquatic dissolved organic matter into humic substances, hydrophylic acids, hydrophobic neutral substances, hydrophilic bases, and hydrophilic neutral substances.

It is generally believed that during humification the carboxylic acid functional group concentration of organic matter increases (Stevenson, 1994). The carboxylic acid functional groups in humic substances are particularly important in controlling acidity, cation exchange, and translocation of metals in soils. Several studies have compared the humic substance concentration or functional group concentration (using cross-polarization, magic angle spinning [CP/MAS] $^{13}C$ NMR) of various soil horizons that reflect increasing degrees of decomposition (Kögel-Knabner et al., 1991; Zech et al., 1992; deMontigny et al., 1993). Other studies have reconstructed long-term rates of decomposition from chronosequence studies (Harmon et al., 2000). The actual rate of development and loss of humic substances or functional group characteristics in forest soils have not, to our knowledge, been measured directly over the long term (e.g., 13 yr).

Our objectives were: (i) to measure the rate of formation and loss of humic substances in fallen litter as a function of time, and (ii) to estimate the rate of formation and loss of carboxylic functional groups in a forest soil organic matter as a function of time. To accomplish these objectives, we laid nets on the floor of a *Pinus strobus* forest for 13 yr to keep annual layers separate, measured total C loss, analyzed the concentration of humic substances by NaOH extraction, and chromatographic fractionation, and estimated the change in concentration of carboxyl C by CP/MAS $^{13}C$ NMR.

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Published in Soil Sci. Soc. Am. J. 67:899–909 (2003).

**Abbreviations:** AMU, atomic mass unit; $^{13}C$ NMR, carbon-13 nuclear magnetic resonance; CP/MAS, cross polarization, magic angle spinning; DOC, dissolved organic carbon; DP/MAS, direct polarization, magic angle spinning; $t_{1/2}$, half decay time.

## MATERIALS AND METHODS

### Separation of Annual Litter Layers

The research site was a pine plantation established in 1956 on the north-facing Watershed 17 at the Coweeta Hydrologic Laboratory, in the Nantahala Mountains of North Carolina, a long-term ecological research site of the National Science Foundation. Mean annual precipitation is 194 cm and mean annual air temperature is 12.6°C (Swift et al., 1988). The forest floor was a mor type. The soil was an Evard-Cowee gravely loam, a fine-loamy, parasesquic or oxic, mesic Typic Hapludult. Texture in the 0- to 20-cm depth was 59% sand, 22% silt, and 19% clay (McGinty, 1976). The organic matter concentration was 5.9% in the 0 to 30 cm, and 5.0% in the 30- to 60-cm mineral soil depths (McGinty, 1976). The pH was 4.2 in the Oa horizon (this study) and 4.7 in the 0- to 10-cm depth of the A horizon. In 1970-1971 litterfall was measured as 388 g $m^{-2}$ $yr^{-1}$, 318 g $m^{-2}$ $yr^{-1}$ of which was foliar (Cromack and Monk, 1974).

Beginning in August, 1984, we laid a 91 by 91 cm square of nylon mesh, with openings measuring 2 by 4 mm, on five plots each year until 1997 (the technique of Jorgenson et al., 1980). Consequently, the autumn 1984 litterfall was the first layer delineated. A 15-cm high "fence" of screen surrounded each plot to prevent movement of litter by wind. The pliant nylon mesh was chosen because it allowed litter to lie in a more natural manner than stiffer fiberglass window screen. Litterfall was measured every 3 (autumn) to 6 wk beside each of the five plots in the 1985-1986 litterfall period and again in the 1997-1998 period. Litterfall was used as the standing stock for Time 0. Measurement of decomposition rate by this method requires a litterfall rate that is either known for each year or that it remains constant over the period (Jorgenson et al., 1980). Foliar litterfall in 1997-1998 was 218 g $m^{-2}$ $yr^{-1}$ C $\pm$ 25 (sd). In 1985-1986 foliar litterfall was 204 $\pm$ 37 g $m^{-2}$ $yr^{-1}$ C, and was not significantly different than in 1997-1998 ($P \leq 0.05$). Consequently, it was assumed that litter inputs did not exhibit any long-term trend throughout the 13-yr period, and thus the decline in mass in the annual layers could be used as a measure of decomposition.

All annual layers were separated and collected in August 1998, giving 13 yr of separated, annual layers (the 1984 layer was not used). When litter was harvested, we noted that even in the advanced stages of decay, the particles were bound together by a network of either living or dead mycelia. Litter was carefully separated from nets in the laboratory and air dried at about 24°C to a constant mass and weighed. Woody material and cone debris were separated from foliar litter but in this paper we have only analyzed the foliar component of the litter. Subsamples were ground in a small ball mill for analysis. Subsamples were also dried at 70°C to determine moisture content, and then combusted to determine ash-free dry mass. Carbon and N concentration were measured using a Perkin-Elmer 6400 C-H-N analyzer (Perkin-Elmer, Norwalk, CT).

We calculated the loss of C from the first year of decomposition during the first year as the litterfall (in g $m^{-2}$) minus the mass of C (in g $m^{-2}$) laying on the 1997 net that was approximately 1 yr old. The decomposition occurring from previous Years $t$ to Year $t - 1$ was calculated as the C mass in Layer $t$ minus C mass in the Layer $t - 1$.

### Flux of Fine Particulate Carbon

Measurement of decomposition rates using this technique depends on the assumption that the migration of fine particles through the nets and annual layers was small. We measured the flux of fine particulate C through the net by placing 56-mm Whatman glass fiber filter discs (Whatman International Ltd, Maidstone, UK) just below the undersurface of the nets in 1997. The discs were placed under layers of 1-, 2-, 3-, 4-, 5-, 6-, 9-, 11-, and 13 yr-old litter in a nonoverlapping arrangement so that they would represent the cumulative flux from all annual layers above each filter disc. One set of filter discs was removed after 14 d to serve as a control for the chance that the disturbance in placing the filters would cause deposition of particles on the surface. A second set of filters was left in place for 1 yr. For downward-moving fine particles to be deposited in the matrix of the filter it was important that the filters not repel water. In the laboratory, we observed the ability of the moist filters to absorb droplets of water clinging to particles of litter touching the filter and they appeared not to repel droplets of water.

The filters were collected carefully and wrapped in aluminum foil. The filters were dried at 105°C, weighed to the nearest 0.1 mg, combusted at 450°C, and the loss of weight was measured. The organic C deposited on the filter was estimated as 50% of the loss of mass on combustion. The C deposited on the control filters was subtracted. To calculate the annual flux of fine particulate C from Layer $x$ to Layer $x - 1$, we assumed that most of the flux into Layer $x - 1$ originated from Layer $x$, and not those laying several layers above. We based this assumption on the finding that the flux of fine particulate C actually declined in the older layers as a percentage of the mass in the layer above the filter.

### Extraction and Fractionation

Two methodological tests were performed using the NaOH extraction procedure. First we extracted three replicate 0.6-g samples of a composite of the 13-yr old litter with seven sequential extractions with 30 mL of 0.1 *M* NaOH under $N_2$ for 16 h each. We measured the cumulative mass of C extracted by each sequential NaOH extraction under either oxic or anoxic conditions to determine the optimal number of extractions.

We also determined whether unaltered lignin might be dissolved during the NaOH extraction, thus being misinterpreted as humic substances, and whether it might be removed by precipitation of the NaOH extract at pH 7 and adsorption on XAD-8 resin. We used a commercial lignin preparation (Organosolv Lignin, Aldrich Chemical Co., Milwaukee, WI) with a number-average molecular weight of 800 AMU and a weight-average molecular weight of 3500 AMU (Aldrich Chemical Co., product information circular, Milwaukee, WI). We extracted it using 0.1 *M* NaOH under either ambient oxic or anoxic, N purged conditions. Then we neutralized the extract rapidly to pH 7 with HCl, allowed it to sit 24 h, centrifuged the extract, and pumped the supernatant through XAD-8 resin and eluted the adsorbed substances with 0.1 *M* NaOH.

Polyphenol concentrations of the litter samples were determined by the Folin-Ciocalteau method using three sequential extractions with 50% water and 50% methanol (Waterman and Mole, 1994).

Subsamples of the new litterfall, and of 1-, 3-, 5-, 7-, 9-, 11-, and 13-yr-old litter were extracted by the procedure outlined in Fig. 1. This procedure represents a slightly modified version of the procedure for analysis of humic substances of the International Humic Substances Society (Swift, 1996) supplemented by Leenheer's (1981) more detailed fractionation procedure. Samples consisting of the equivalent of 0.3 g C were extracted with 30 mL of 0.1 *M* NaOH using three sequential 16-h extractions under anoxic conditions in a $N_2$ purged bag.

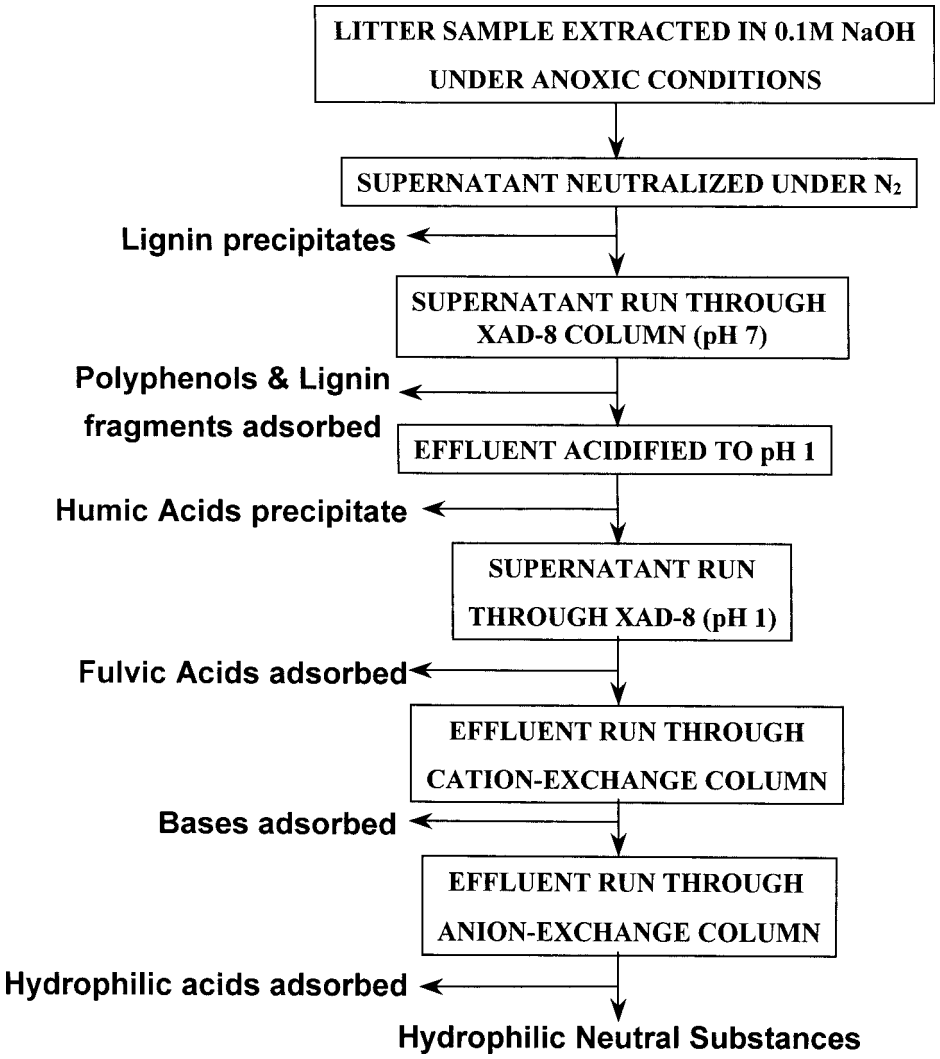


**Fig. 1. Extraction and fractionation procedure.**

After centrifugation, the solutions were quickly neutralized to pH 7, allowed to sit for 24 h and then centrifuged again. The three supernatants were combined, diluted five-fold, and a 250-mL sample was then pumped through 3.75 mL XAD-8 resin. The weak hydrophobic acid fraction (phenolic fraction) was eluted with 0.1 *M* NaOH. The effluent was acidified to pH 1, allowed to sit 24 h without further pH adjustment, and then centrifuged. Because some particles were not removed by centrifugation, the supernatant was filtered through a 0.45-μm membrane filter to remove the humic acid fraction. The supernatant was again pumped through XAD-8 resin at about pH 1 and the fulvic acid fraction was then eluted with 0.1 *M* NaOH. The effluent was then pumped through cation- and anion-exchange columns as described by Leenheer (1981). The hydrophilic acid fraction was calculated as the fraction of the total C removed by the anion exchange column. Subsamples were taken for dissolved organic C (DOC) analysis after each step of the procedure. The sum of the humic acid (C precipitated at pH 1) and the fulvic acid fractions were interpreted as the humic substance fraction.

To estimate the loss of humic substances via leaching, we performed 13 sequential extractions with water of a composited sample of the new litterfall and a composited sample of the 1-yr-old litter from both the pine watershed and a nearby deciduous watershed. We used the method outlined in Qualls (2000) to estimate the water soluble organic C from the sequential water extractions. The water contained 20 mg $L^{-1}$ $HgCl_2$ to suppress decomposition during extraction. This water extract was also subjected to the fractionation procedure in Fig. 1 without the NaOH-extraction step.

## $^{13}$C NMR Analysis

The carboxylic acid C concentration has been used as an index of humification of soil organic matter. We used area of the peak in direct polarization, magic angle spinning (DP/MAS) or CP/MAS $^{13}$C NMR spectra from 165 to 190 ppm as a measure of carboxylic acid and amide functional groups.

Solid-state CP/MAS $^{13}$C NMR spectra were obtained for the litterfall, 1-, 9-, and 13-yr-old litter. In addition, spectra were obtained for the humic acid and humin fractions of the litterfall and the 9- and 13-yr-old litter. A single spectrum was acquired for each sample, where the 'sample' was a composite of 2 g from each of the five replicate plots. Solid-state CP/MAS $^{13}$C spectra were measured at 50.298 Mhz on a 200 Mhz Chemagnetics CMX spectrometer (Varian Inc., Palo Alto, CA) with a 7.5 mm-diam. probe. The spinning rate was 5000 Hz. The acquisition parameters for the solid samples were a contact time of 1 ms, pulse delay of 1 s, and a pulse width of 4.5 μs for the 90° pulse (Wershaw et al., 2000). The number

of free induction decay signal acquisitions ranged from 1552 for the litterfall to 5229 for the 13-yr-old litter. A line broadening of 100 Hz was applied in the Fourier transformation of the free induction decay data. In addition, DP/MAS $^{13}C$ spectra were measured on the new litterfall and 13-yr-old litter samples. The DP/MAS $^{13}C$ spectra were obtained using a 37° pulse and a 48-s pulse delay, based on optimization experiments reported in Wershaw et al. (2000). The number of free induction decay signal acquisitions was 468 for litterfall and 3020 for 13-yr-old litter.

The NMR spectra were divided into chemical shift regions as follows: aliphatic C, 0 to 45 ppm; ether C, 45 to 62 ppm; aliphatic-OH C, 62 to 90 ppm; anomeric C, 90 to 110 ppm; aromatic C, 110 to 165 ppm; carboxyl, amide, and ester C, 165 to 190 ppm; ketone C, 190 to 230 ppm. The total area under the spectrum and areas within each chemical shift region were measured by cutting and weighing. The area between 165 to 190 ppm is attributed to carboxylic acid C, amide C and ester C. We assumed that the ester C contribution was small compared with the carboxyl C. Antweiler (1991) found about 0.6% of the mass of Suwannee fulvic acid was hydrolyzable ester C.

### Carboxylic Functional Group Concentration

Because the carbonyl C of the carboxyl and amide functional groups is unprotonated, reduced cross-polarization efficiency causes an underestimation of the signal because of carbonyl C relative to other C atoms (Mao et al., 2000). The DP/MAS $^{13}C$ spectrum is not subject to that underestimation (Kinchesh et al., 1995). We calculated the ratios of the relative peak areas between 165 and 190 ppm for the DP/MAS spectra divided by the corresponding CP/MAS spectra of the new litterfall and the 13-yr-old litter. This ratio was 1.2 for new litterfall and 1.4 for the 13-yr-old litter. To correct the CP/MAS relative peak areas for the other samples, we multiplied by a factor of 1.3 to give a corrected measure of the carboxyl plus amide C concentration.

Using the DP/MAS peak areas between 165 and 190 ppm directly, or using the relative peak area that had been corrected for its underestimation in the CP/MAS spectra, we calculated the relative area due to carboxyl C alone, without the amide C, as follows. We estimated the proportion of amide C independently using elemental analysis of C and N. Using $^{15}N$ NMR, most N in humic acids has been attributed to amide N. We assumed 90% of the N was amide N and terminal amine N based on a study of NaOH soil extracts (Knicker et al., 1993). Thus we assumed that:

$$C_{\text{amide}} = 0.9 \times N_{\text{total}}, \quad [1]$$

where $C_{\text{amide}}$ is the moles of amide C and $N_{\text{total}}$ is the moles of total N. The relative peak area for amide C ($A_{\text{amide C}}/A_{\text{total}}$) can thus be estimated by:

$$A_{\text{amide C}}/A_{\text{total}} = 0.90\ (N/C) \quad [2]$$

where $N/C$ is the molar ratio of total N/total C, based on elemental analysis of the sample. Now the relative peak area of the 165- to 190-ppm range can be divided into:

$$(A_{\text{COOH}} + A_{\text{amide}})/(A_{\text{total}}) = (A_{\text{COOH}}/A_{\text{total}}) + (A_{\text{amide}}/A_{\text{total}}) \quad [3]$$

where $A_{\text{COOH}}/A_{\text{total}}$ is the relative area attributed to carboxyl C.

Substituting Eq. [2] into Eq. [3] and solving for $A_{\text{COOH}}/A_{\text{total}}$

$$(A_{\text{COOH}}/A_{\text{total}}) = [(A_{\text{COOH}} + A_{\text{amide}})/A_{\text{total}}] - [0.9 \times (N/C)] \quad [4]$$

Note that applying this calculation to separate the carboxyl and amide relative peak areas without having corrected for an underestimation in the CP/MAS spectra would result in a further underestimation in the carboxyl concentration.

Finally the ratio of carboxyl C/total C was multiplied by the mass of C remaining in each of the five replicate plots of litterfall, (Year 0), and litter of ages 1, 9, and 13 yr to obtain an estimate of the rate of loss of carboxyl C from forest floor during decomposition.

## RESULTS AND DISCUSSION

### Methodological Tests

The amount of C extracted anoxically from the 13-yr-old litter as a function of the number of sequential extractions is shown in Table 1. Three sequential extractions removed 90.2% as much C as seven extractions so we chose three extractions as a balance between complete extraction and possible additional hydrolysis of otherwise insoluble lignin or other components.

Over 99% of the C in the lignin preparation was soluble in 0.1 *M* NaOH in a single extraction (Table 2). We believed that hydrolyzed fragments of the lignin polymer might be soluble in NaOH because of the presence of ionizable phenolic hydroxyl groups but that these might become hydrophobic at pH 7. Extraction of the lignin and neutralization of the extract under anoxic conditions resulted in precipitation of a total of 68% of the C extracted. Subsequently, the XAD-8 extraction at pH 7 removed a total of 82.9% of the extracted lignin. In the lignin extracted in air, only 18% of the C in the extract was removed by precipitation and XAD-8 adsorption. We believe that under oxic conditions, some hydrolyzed phenolic hydroxyl groups were oxidized to carboxylic acid groups that remained charged at pH 7. With a litter sample after a single extraction, only 6% of the C in the original solid sample was precipitated at pH 7 under oxic conditions. About 3.8% was precipitated under anoxic conditions but less C, 44.4 vs. 48.1%, dissolved in 0.1 *M* NaOH. For three anoxic extractions of new litter, 7.8% of the C subsequently precipitated at pH 7 (Table 2). Compared with the lignin preparation, the potential error of extracting lignin was much less in the extracted litter sample and the difference between the oxic and anoxic extractions was small even though the lignin concentration of the pine litter was 31% (Cromack and Monk, 1974). Because humic substances are operationally defined, it is possible that some relatively unaltered lignin is routinely included in humic substance extractions of soil. We speculated that the depolymerization necessary to produce the commercial lignin preparation we used might also have tended to increase its solubility in NaOH. Nevertheless, all extractions were

**Table 1. Cumulative C extracted by a series of sequential 0.1 *M* NaOH extractions of 0.5-g litter samples. Other initial weights of 0.1, 0.27, and 1.0 g yielded similar results. Standard errors of three replicate extractions are indicated.**

| Extraction | Cumulative C extracted |
|---|---|
| | % of total C concentration |
| 1 | 47.5 ± 1.8 |
| 2 | 61.2 ± 2.0 |
| 3 | 66.9 ± 2.1 |
| 4 | 69.3 ± 2.2 |
| 5 | 71.5 ± 2.2 |
| 6 | 72.8 ± 2.2 |
| 7 | 74.2 ± 2.2 |

**Table 2. Solubility in 0.1 *M* NaOH and fractionation of the neutralized NaOH extract for samples of lignin and 5-yr-old litter using a single extraction exposed to air or under anoxic conditions. Standard errors of three replicates are indicated.**

| | Lignin | | Litter | |
|---|---|---|---|---|
| | oxic | anoxic | oxic | anoxic |
| | % of C in solid sample | | | |
| Dissolved in 0.1 *M* NaOH | 99.7 ± 1.8 | 99.7 ± 1.7 | 48.1 ± 1.9 | 44.4 ± 1.8 |
| Precipitate at pH 7 | 1.5 ± 0.6 | 68.0 ± 0.9 | 6.0 ± 0.1 | 3.8 ± 0.1 |
| Eluted from XAD-8 after pH 7 | 22.2 ± 1.5 | 14.5 ± 3.0 | 4.1 ± 1.4 | 3.6 ± 1.9 |
| XAD-8 effluent at pH 7 | 81.8 ± 3.1 | 17.1 ± 0.7 | 40.4 ± 6.2 | 33.3 ± 5.9 |
| Hydrophobic neutral fraction | 0 ± 0.8 | 0.5 ± 0.3 | 2.0 ± 0.9 | 3.7 ± 1.2 |

done anoxically and the pH 7 precipitation and XAD-8 treatment steps were utilized.

The fluxes of fine particulate C through the nets above the annual layers were small compared with the mass in each layer, and were almost undetectable in layers of age 5 to 13 yr (Table 3). No fluxes exceeded 1.8% of the mass of C lying above the layer except for 1-yr-old litter. Because the fluxes declined with the age of the layer, we assume that most of the small flux of fine particles originated from one or two layers above. We attribute the small magnitude of the fine particle fluxes to the tendency of the litter fragments to be bound together by mycelia, which we observed with a microscope. Consequently, we regarded the "contamination" of the old layers by fine particles from younger layers as minor.

## Carbon and Humic Substances Remaining Versus Age

The C remaining in the forest floor declined abruptly in the first year by about 37%, consistent with observations made by Cromack and Monk (1974) on the same watershed (Fig. 2). Thereafter, the C in layers aged 1 to 13 yr declined at a rate corresponding to a half-decay time of 3.9 yr with a reasonably good fit to an exponential decay model. The decomposition rate of the *Pinus strobus* litter was much slower than was observed for deciduous species on nearby watersheds, probably because of a high lignin concentration of 31% (Cromack and Monk, 1974).

The standing stock of the humic substance fraction (the humic acid fraction plus the hydrophobic acid fraction) increased significantly (*t* test, $P < 0.01$) during the first year, then declined slowly with a half-decay time corresponding to 5.1 yr. The rate of loss, excluding the increase during the first year, fit an exponential decay model well ($R^2 = 0.78$). The decay constant for humic substances was significantly different than that for total forest floor C, as tested by an analysis of covariance comparing the slopes of the ln (y) transformations of the two curves.

**Table 3. Flux of fine particles deposited on glass fiber filters lying below nets separating annual layers of litter. Standard errors for five replicate plots are indicated.**

| Age of layer | Flux from net above | Flux as a % of C on all nets above layer |
|---|---|---|
| yr | g C $(m^2\ yr^{-1})^{-1}$ | % |
| 1 | 1.2 ± 0.8 | 3.2 ± 2.4 |
| 2 | 2.5 ± 1.8 | 0.5 ± 0.4 |
| 3 | 6.7 ± 4.1 | 1.3 ± 0.7 |
| 4 | 6.8 ± 4.1 | 1.8 ± 1.4 |
| 5 | 0.6 ± 2.1 | 0.1 ± 0.2 |
| 6 | 1.9 ± 2.2 | 0.3 ± 0.2 |
| 9 | 1.6 ± 2.9 | 0.1 ± 0.3 |
| 11 | 1.0 ± 0.9 | 0.04 ± 0.04 |
| 13 | 0.3 ± 0.2 | 0.02 ± 0.02 |

The changes in the standing stock of humic substances over time were a product of a decline in the mass of each annual layer and (excepting the first year) an increase in the percentage of the total C in the humic substance fraction (Table 4). The humic acid fraction of total C increased significantly ($P < 0.01$) from 2.1% in new autumn litter to 15.7% in 1-yr-old litter and then increased to 26.3% after 13 yr. The fulvic acid (hydrophobic acid) fraction varied from about 7.5% in new litter to 9.9% in 13-yr-old litter but there was no significant trend with time.

The hydrophilic acid fraction, in procedures for fractionating aquatic DOC, is regarded as being comprised of organic acids with a higher ratio of charge to molecular size, compared with fulvic acids. (Leenheer, 1981). These organic acids averaged only about 3% of the total C and did not increase as a percentage over time. However, the hydrophilic acid fraction has been found to be an important fraction of the DOC in soil solution (Qualls and Haines, 1991).

In our procedure, the phenolic fraction was that which failed to precipitate at pH 7, but was hydrophobic enough to adsorb to XAD-8 resin at pH 7 and desorb at alkaline pH (Fig. 1). Polyphenols occur in this fraction (Leenheer, 1981; Qualls and Haines, 1991), but phenolics originating from lignin lysis might also occur in this fraction (see Table 2). The polyphenols in this fraction might be considered as part of the humic substances and they would likely be included in the fulvic or humic acid fraction of the less detailed procedure outlined by Swift (1996). We simply report this fraction separately because of the possibility that it may contain lysed lignin fragments and because polyphenols are produced as part of the original plant tissue. This phenolic fraction (analyzed by chromatography) ranged from 6.0% of the total C in freshly fallen foliar litter to 2.8% in 11-yr-old material, and there was a significant decrease with age of the litter (Table 4). The analysis of the polyphenol concentration of litter samples using the Folin-Ciocalteau method was similar to that of the phenolic fraction isolated by chromatography for new litter. However, in older litter there was little correspondence with the phenolic fraction. The Folin Ciocalteau method measures reducing equivalents (Waterman and Mole, 1994) rather than pH-dependent ionization. The partial oxida-

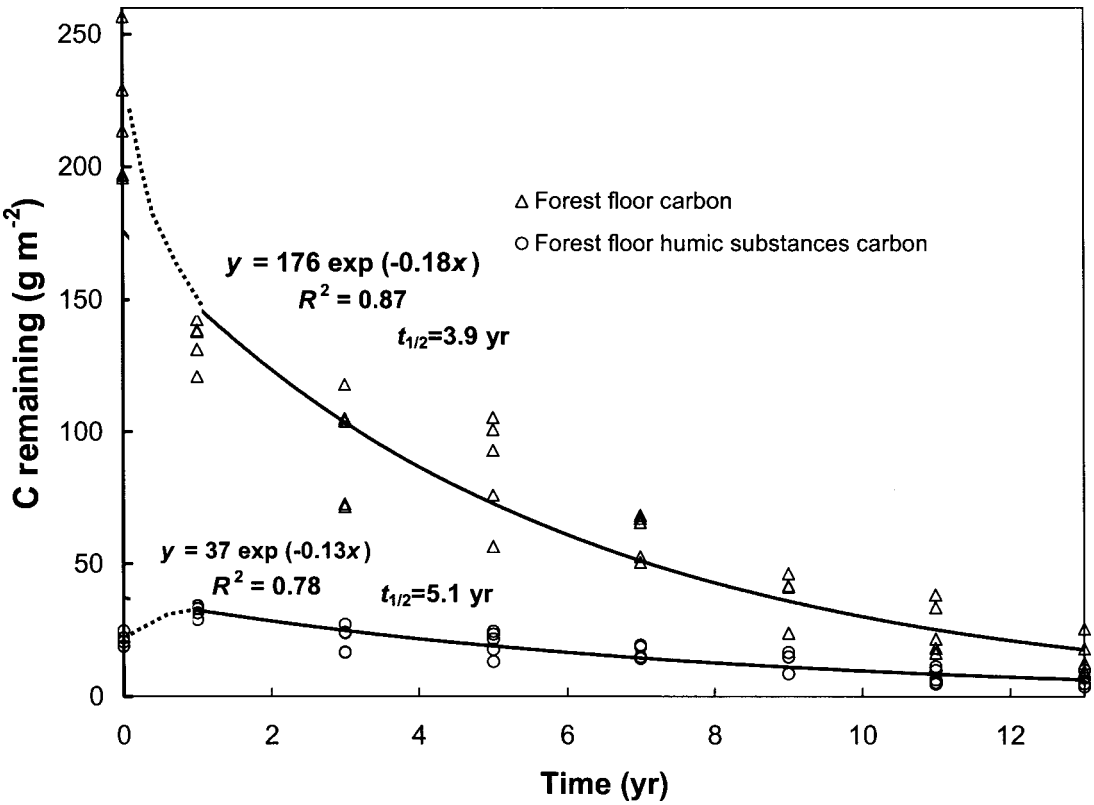


**Fig. 2. Loss of C and humic substances from the forest floor as a function of time. Each point represents one of five replicate plots. Data for the first year is connected by a dashed line. Data for Years 1 to 13 are represented by a solid exponential regression line and the regression statistics refer to data from 1 to 13 yr. As a statistical rationale for excluding the first year decomposition from the regression, we found that neither the 95% nor the 99% confidence intervals for the data at x = 0 yr included the intercept predicted from the regressions in Fig. 2.**

tion of polyphenols over time might help explain this lack of correspondence.

## Carboxyl Carbon Remaining in Litter Versus Age

Carboxyl C remaining in the litter declined exponentially with age of the litter with a half decay time of about 4.6 yr (Fig. 3), slightly faster than the humic substance fraction. This rate of loss was significantly different than that of the total forest floor C (Fig. 2), as tested by analysis of covariance of the slopes of the ln($y$) versus time curves. In contrast to the loss of total C (Fig. 2), the loss of carboxyl C was not disproportionately great during the first year, so we included the data for fresh litterfall in the regression analysis. The regression model using exponential decay (Carboxyl C = 12.6 exp [−0.15$t$]) was highly significant ($P < 0.001$) and the predicted value for the 9-yr-old litter was within the 99% confidence interval for the mean of the data at 9 yr. Nevertheless, the model must be used with caution because only four times are represented. The loss of carboxyl C in grams per square meter per year ($m^{-2}$ $yr^{-1}$) was a product of the loss of mass from the litter and an increase in the carboxyl C fraction from 5.7 to 10.0% over 13 yr (Table 5, Fig. 4). In fact, the carboxyl C concentration of both the whole litter and the humic acid fractions increased sharply during the first year just as did the percentage of C in the percentage of humic acid fraction using the fractionation procedure (Tables 2 and 5). The increase in carboxyl concentration during the first year of decomposition, from 5.8 to 7.7%, was because of an increase in the carboxyl content of the humic acid fraction from 6.5 to 11.5% during the first year (Table 5).

**Table 4. Percentage of the total C concentration of dried litter samples in various fractions of the 0.1 *M* NaOH extract. Standard errors for five replicates are indicated.**

| Fraction | Age of litter, yr | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 3 | 5 | 7 | 9 | 11 | 13 |
| | % | | | | | | | |
| Precipitate at pH 7 | 7.8 ± 0.7 | 9.1 ± 1.2 | 7.2 ± 0.8 | 6.1 ± 1.0 | 8.7 ± 3.3 | 10.8 ± 3.1 | – | 10.4 ± 3.5 |
| Phenolic fraction† | 6.0 ± 0.9 | 3.9 ± 0.2 | 3.9 ± 0.6 | 3.2 ± 0.2 | 2.4 ± 0.5 | 2.6 ± 0.3 | 2.8 ± 0.5 | 3.3 ± 0.6 |
| Polyphenol by Folin‡ | 7.7 ± 0.3 | 1.3 ± 0.2 | – | 0.5 ± 0.02 | – | 0.5 ± 0.02 | – | 0.3 ± 0.02 |
| Humic acid † | 2.1 ± 0.3 | 15.7 ± 2.8 | 15.1 ± 2.5 | 13.5 ± 1.1 | 18.8 ± 3.0 | 22.6 ± 2.3 | 20.9 ± 4.0 | 26.3 ± 3.9 |
| Fulvic acid † | 7.5 ± 1.0 | 6.4 ± 2.4 | 6.1 ± 1.8 | 7.9 ± 3.7 | 7.3 ± 2.2 | 10.4 ± 3.6 | 6.0 ± 1.6 | 9.9 ± 2.5 |
| Hydrophilic acid | 3.5 ± 0.3 | 2.3 ± 0.4 | 3.4 ± 1.2 | 2.8 ± 0.3 | 2.9 ± 1.5 | 2.4 ± 0.8 | 3.8 ± 0.9 | 2.9 ± 0.7 |

† Significant ($P \leq 0.05$) tendency to increase or decrease with age, excluding Year 0.
‡ Using the Folin-Ciocalteau method on 50% methanol extracts.

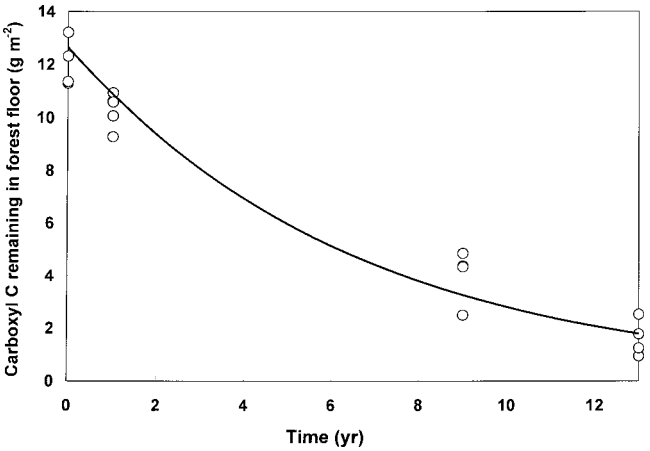


**Fig. 3. Loss of carboxylic C functional groups from the forest floor. Each data point represents one of five replicate plots.**

As expected, the humic acid fraction contained more carboxyl C than the unextracted litter (Table 5). The residual humin (litter insoluble in 0.1 *M* NaOH) contained less carboxyl C than the unextracted litter. Most of the carboxyl C was extracted by NaOH (about 82% in fresh litterfall and 77% in 13-yr-old litter) but a significant portion remained unextracted. This portion might represent some humic substances that were insoluble in NaOH, or they might represent carboxyl functional groups in other insoluble macromolecules.

The high concentration of carboxyl functional groups is one of the most important properties of humic substances. They are responsible for many of the important ecological roles of humic substances such as cation exchange, the acidity of organic soil horizons, and the formation of organometallic complexes (Stevenson, 1994). Our data illustrate that while the concentration of carboxyl C increases as part of the humification process, there is a net loss of carboxyl C because of the decreasing mass of organic matter over time. It is likely that this loss represents a combination of the loss of carboxyl C originally present in new litter and production of new carboxyl groups during oxidative degradation or microbial synthesis. Microscopic observation of the various layers revealed abundant remains of fungal mycelia binding particles together.

We examined the correlation between concentration of carboxyl C (Table 5) and four characteristics believed to reflect the extent of decomposition: (i) the percentage C remaining ($R^2 = 0.97$, $P < 0.05$), (ii) the C/N ratio ($R^2 = 0.98$, $P < 0.05$) (iii) the aliphatic C/aliphatic-OH C ratio (Baldock et al., 1997) ($R^2 = 0.74$, $P < 0.05$) and (iv) the percentage of humic substance C concentration ($R^2 = 0.95$, $P < 0.05$). Thus the content of carboxyl C is well correlated with other traditional measures of the extent of decomposition.

## Comparison of Carboxyl Carbon Concentrations with Other Studies

The vast majority of solid state $^{13}C$ NMR analyses of litter and soil organic matter have been performed using the CP/MAS technique, particularly because of the very long signal acquisition times necessary for solid state $^{13}C$ DP/MAS measurements. We found that it underestimated the peak area from 165 to 190 ppm by 20 to 40%. A study by Mao et al. (2000) also reported that the CP/MAS $^{13}C$ NMR of peat and humic acid samples significantly underestimated carboxyl C concentration relative to DP/MAS measurements.

Other studies that have measured the relative peak area in the carbonyl region (about 165–190 ppm) of different layers of coniferous forest floors have found results very similar to our CP/MAS data. Baldock and Preston (1995), using CP/MAS $^{13}C$ NMR to examine forest floor layers in a Red pine (*Pinus resinosa*) planta-

**Table 5. Concentration of major functional groups using solid-state direct polarization, magic angle spinning (DP/MAS) and cross polarization, magic angle spinning (CP/MAS) $^{13}C$ nuclear magnetic resonance ($^{13}C$ NMR) spectra estimated from the relative peak area between the ppm shift regions indicated in Column 1. Carbon and N concentration from elemental analysis are also shown. Each column represents a single spectrum (see Fig. 4) run on a composited sample from all five plots. The 13-yr-old humic acid sample was omitted because of problems with Fe interference with the spectrum.**

| | | DP/MAS | | CP/MAS | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Fraction | | | | | | | | | | |
| | | Litter | | Litter | | | | Humic acid | | Humin | | |
| | | Age, yr | | | | | | | | | | |
| ppm | Function group | 0 | 13 | 0 | 1 | 9 | 13 | 0 | 1 | 0 | 1 | 13 |
| | | % | | | | | | | | | | |
| 0–45 | % aliphatic | 22.7 | 25.6 | 15.6 | 16.4 | 18.3 | 23.9 | 31.0 | 29.3 | 11.9 | 14.2 | 23.4 |
| 45–62 | % ether, secondary amide | 8.6 | 9.5 | 7.4 | 11.4 | 10.7 | 9.3 | 9.9 | 10.4 | 12.1 | 11.9 | 12.7 |
| 62–90 | % aliphatic-OH | 27.0 | 19.3 | 35.7 | 33.6 | 26.4 | 26.4 | 15.1 | 14.8 | 43.7 | 42.1 | 27.9 |
| 90–110 | % anomeric | 9.1 | 7.6 | 12.12 | 13.3 | 11.3 | 9.2 | 11.3 | 8.5 | 14.4 | 13.5 | 11.3 |
| 110–165 | % aromatic | 22.8 | 21.4 | 22.0 | 17.9 | 21.2 | 17.7 | 26.4 | 26.1 | 13.6 | 13.5 | 17.7 |
| 165–190 | % carboxyl + amide | 7.0 | 13.1 | 5.7 | 7.1 | 10.2 | 9.2 | 5.9 | 10.3 | 4.0 | 4.4 | 6.6 |
| N.A.† | % carboxyl‡ | 5.8 | 10.0 | 5.8 | 7.7 | 10.5 | 10.0 | 6.5 | 11.5 | 4.4 | 4.4 | 5.8 |
| 190–230 | % ketone | 2.7 | 3.5 | 1.8 | 0.3 | 1.9 | 4.4 | 0.5 | 0.6 | 0.3 | 0.4 | 0.4 |
| N.A.† | C concentration | 50.9 | 27.4 | 50.9 | 49.7 | 39.2 | 27.4 | 54.7 | 50.2 | 46.1 | 46.2 | 27.4 |
| N.A.† | N concentration | 0.8 | 1.1 | 0.8 | 1.2 | 1.6 | 1.1 | 1.1 | 1.5 | 0.5 | 0.9 | 1.1 |

† N.A., not applicable.
‡ Calculated using ratio of DP/MAS divided by CP/MAS peak area to correct for the lower efficiency of CP/MAS in detecting non-protonated C and Eq. [1] through [3] (in text) to correct for amide concentration.

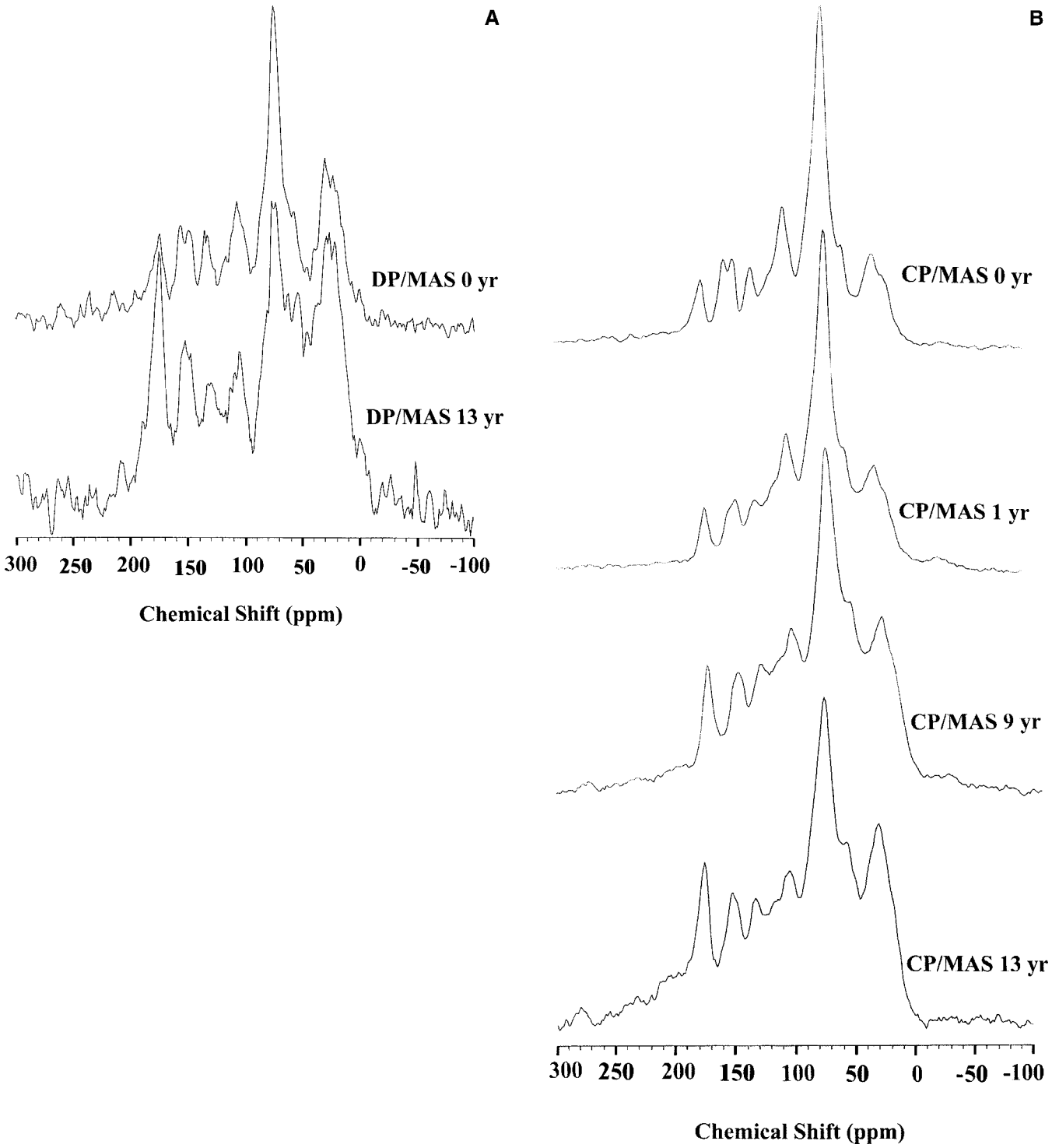


**Fig. 4. (A) Carbon-13 direct polarization magic angle spinning ($^{13}C$ DP/MAS) and (B) $^{13}C$ cross polarization magic angle spinning (CP/MAS) and spectra for litter samples. Spectra for the humic acid and humin fractions are not shown.**

tion, found carbonyl C increased from 5% in fresh litter to 8% in the Oa horizon. In a comparison of the forest floor of six different forests with Spruce, Beech, and Pine vegetation, Zech et al. (1992) found an average of 7% carbonyl C in the Oi horizon and 9.7% in the Oa horizon. Again, these values are similar to those found in our study. In three of the same sites, Kögel-Knabner et al. (1991) found that the relative peak area from 160 to 220 ppm in the humic acid fractions increased from 6% in the Oi horizon, to 11% in the Oe horizon, and 16% in the Oa horizon. These results were consistent with our conclusion that most of the increase in carboxyl C with age occurred in the NaOH-extractable fraction. In coniferous forests, deMontigny et al. (1993) found that the relative peak area between 159 and 185 ppm increased from 6% in litter to 8% in the Oa horizon in

one forest type, and from 4 to 9% in another forest type. Lorenz et al. (2000) found only a small and inconsistent difference in relative peak areas in the 165- to 190-ppm range for Black spruce and Norway spruce needles during 10 to 12 mo. of decomposition. However, only 20 to 25% of the mass was lost over this relatively short period.

## Changes in the Concentration of Other Functional Groups with Age

Comparing the DP/MAS with the CP/MAS spectra for the 0- and 13-yr-old litter, the percentage peak area for aliphatic C, (0–45 ppm), ether plus secondary amide C (45–62 ppm), aromatic C (110–165 ppm), carboxyl plus amide C (165–190), and ketone C (190–230 ppm) were higher, while the percentages for aliphatic-OH C (62–90 ppm) and anomeric C, (90–110 ppm) were correspondingly lower.

The percentage concentration of aromatic C changed little as the litter aged (Table 5, Fig. 4), suggesting that the aromatic C was being decomposed at about the same rate as the bulk litter. The aromatic C present at any time likely represents: (i) plant derived lignin and polyphenols, (ii) aromatic C in humic substances modified from lignin or polyphenols, or (iii) aromatic C synthesized during decomposition and stabilized in the humic fraction. It is possible that the lack of a large change in aromatic C percentage represents a balance between loss of lignin C and formation of humic-associated aromatic C. Some studies have noted an increase of aromatic C in residues of increasing age, presumably as carbohydrates are lost faster than aromatic compounds (deMontigny et al., 1993). Baldock et al. (1997) found an increase in aromatic C percentage from 20% in Red Pine fresh litter to 25% in the Oa and A horizons. In contrast, Zech et al. (1992) found little change in aromatic C in forest floor layers of increasing degree of decomposition.

In our study, the anomeric C percentage, associated with carbohydrates, decreased with age of the litter, but the decrease was small, meaning that the loss of anomeric C was only slightly faster than the loss of C in the litter as it decomposed (Table 5). *Pinus strobus* litter contains 31% lignin (Cromack and Monk, 1974), a relatively high percentage that may physically protect cellulose from decomposition.

In comparing the DP/MAS and CP/MAS relative peak areas for aliphatic C (0–45 ppm), the peak area for DP/MAS is considerably larger in the new litterfall and slightly larger in the 13-yr-old litter (Fig. 4). This suggests that even in the air-dried litter, some of the aliphatic C, such as waxes, resins, and lipids, may be in a liquid-like state, or exhibited a high degree of molecular motion, because the CP/MAS technique would not detect liquid-state C. The percentage of aliphatic C increased with age in litter from 15.6 to 23.9% using CP/MAS and from 22.7% to 25.7% using DP/MAS (Table 5). Every study we reviewed has observed a comparable increase in the percentage of alkyl C in forest floor layers of increasing degree of decomposition (Kögel-Knabner et al., 1991; Zech et al., 1992; deMontigny et al.; 1993; Baldock et al., 1997). For example, using CP/MAS, Baldock and Preston (1995) found an increase of from 16% alkyl C in Red pine fresh litter to 19% in the Oa horizon and 21% in the A horizon.

Another commonly observed characteristic of litter decomposition is a decrease in the C/N ratio (Jorgensen et al., 1980). In our study, the C/N ratio (mass ratio) decreased from 63 in litterfall to about 25 after both 9 and 13 yr of decomposition (Table 5). The C/N ratio for the entire O horizon was 42 (this study). The C/N ratio of the 13-yr-old litter was similar to that of the 0- to 10-cm depth of the A horizon (McGinty, 1976). By 13 yr, the C/N ratio was about the same in the bulk litter as in the humin fraction, meaning that N was not more concentrated in NaOH-soluble fractions. The incorporation of N into humic substances is another characteristic of the humification process (Stevenson, 1994).

## Fate of Carbon Lost from the Forest Floor Layers

Carbon lost from the forest floor layers delineated by nets could occur by: (i) mineralization, (ii) leaching of DOC, (iii) downward movement of fine particles, or (iv) export by invertebrates. The downward movement of particles has been shown to be a minor form of export. We also assume the export by invertebrates was minor because earthworm (Oligochaeta) casts were rarely found and only one earthworm was found in 48 soil pits dug in a neighboring watershed. Furthermore, downward movement of frass and invertebrate remains would have been measured as fine particulate matter export. Respiration of assimilated C by microbes and invertebrates would have been included as "decomposition" or mineralization. Invertebrates were not excluded from the layers since they were exposed from the surface during the first year, the mesh was 2 by 4 mm which is larger than most litter invertebrates, and millipedes (Diplopoda) were frequently found in the internal litter layers.

The leaching of DOC from the forest floor was measured as 40.5 g $m^{-2}$ $yr^{-1}$ on a nearby watershed dominated by deciduous species in 1986-1987 (Qualls et al., 1991). This flux is equivalent to 23.7% of foliar litterfall. Essentially all of this leaching originated from the Oi horizon of the forest floor. We made an estimate of the leaching of DOC from the white pine forest floor by assuming that the leaching of DOC from both the pine and deciduous forest floors was proportional to the amount of water soluble C in litterfall. Our measures of the water soluble C in new pine and deciduous litter were 20.4 and 21.1%, respectively, of the total C in litter.

$$\text{DOC flux from pine litter} = \text{DOC flux from deciduous litter} \times (\%\ \text{water soluble C in pine litter} \times \text{pine litterfall}/\%\ \text{water soluble C in deciduous litter} \times \text{deciduous litterfall}) \quad [5]$$

Using this equation, we estimated a net flux of 49.7 g $m^2$ $yr^{-1}$ DOC from the pine forest floor. This annual

flux is equivalent to 22.8% of foliar litterfall. Our water extractions indicated that 10.4% of the C in 1-yr-old litter was water-soluble. Although some of this water-soluble C may have been produced during decomposition, it appears that not all water-soluble C was lost during the first year of decomposition, probably because of desorption equilibria (Qualls, 2000). Consequently, a portion of the large amount of C lost in the first year (Fig. 2) was probably because of leaching in addition to mineralization.

Using three assumptions: (i) that the forest floor was in steady state, (ii) that fine particulate export was minor, and (iii) that net export by invertebrates was minor; then we estimate that 22.8% of the C in the 13-yr-old litter was leached, 69.7% was mineralized, and 7.5% remained after 13 yr.

A fractionation of the water-soluble extracts of litterfall indicated that about 38% was humic substance C, mostly fulvic acid, and another 5% was the phenolic fraction. In the NaOH extracts, 7.5% of the C in the freshly fallen litter was in the fulvic acid fraction. The fact that the fulvic acid fraction in the NaOH extracts was more or less constant over time (Table 4) meant that the mass of fulvic acid (in g $m^{-2}$) declined at about the same rate as litter C, in contrast to the humic acid fraction. The leaching of the fulvic acid fraction probably contributed to the loss of the mass of carboxyl C, particularly during the first year, because fulvic acids in forest floor solution are much higher in carboxyl C concentration than litter (Guggenburger et al., 1994). In another study, freshly fallen litter was found to have a substantial concentration of fulvic acid and the authors concluded a portion of the process of humification began during leaf senescence (Qualls et al., 1991).

### Formation and Loss of Humic Substances and the Long-Term Storage of C

In this study, much of the humic substance fraction, especially the humic acid fraction, was formed in the first year of decomposition. In contrast, the fulvic acid fraction was already present in newly senesced litter. Thus, most of the process occurred during the first year of decomposition. Our measurements of the net process of accumulation and loss were likely the result of a continuing process of formation and the mineralization of humic substances, and leaching of fulvic acids.

After the first year, humic substances decomposed with a half-decay time of 5.1 yr, somewhat slower than the bulk litter ($t_{1/2}$ = 3.9 yr). Humic substances are relatively resistant to decomposition (Stevenson, 1994). Evidence for their resistance to decomposition includes their tendency to become more concentrated in soil, and the observation that $^{14}C$ dates of humic substances in soils are older than bulk soil organic matter (Campbell et al., 1967). Campbell et al. (1967) found a mean C residence time of 250 yr in a forest Alfisol. Raich and Schlesinger (1992) used the ratio of soil respiration to soil C pools to estimate a considerably lower mean residence time of 29 yr for temperate forests. The order of magnitude differences in the residence time of soil C suggested by these estimates, of course, may reflect site-specific differences but also method related differences. Short-term decomposition studies may not detect very small fractions that decompose at very slow rates. In our unusually long decomposition study, only about 7.5% of the C remained after 13 yr in the oldest layer, and the rate of loss after the first year fit an exponential model relatively well. Nevertheless, some very small fraction could have been far more resistant to decomposition, eventually yielding a residue with a much longer-term residence time.

## CONCLUSION

Our study of pine litter decomposition extended over 13 yr, over which time about 93% of the C was lost. The mass per unit area of humic substances increased during the first year, because of the formation of the humic acid fraction, and then decreased from Years 1 to 13. From Years 1 to 13, the loss of humic substances corresponded to a $t_{1/2}$ of 5.1 yr, slower than that of the total C with a $t_{1/2}$ of 3.9 yr. The concentration of humic substances and carboxyl C in litter increased from 0 to 13 yr but the mass of carboxyl C decreased. The loss of mass of carboxyl C corresponded to a $t_{1/2}$ of 4.6 yr. The lower rates of loss of humic substances and carboxyl C than total C correspond with what might be expected during humification, but the differences are not so great as to explain the long residence times of soil measured by $^{14}C$ dating and C budgets. Continued long-term studies of decomposition processes may shed light on these differences.

### ACKNOWLEDGMENTS

Research supported in part by Nevada Agricultural Experiment Station, publication #5202369. We greatly appreciate the cooperation of the personnel of the Coweeta Hydrologic Laboratory, support from the NSF Long-term Ecological Research program, and field assistance from Todd Ackerman.

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