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EARLY STAGE HUMIFICATION DURING AMENDMENT DECOMPOSITION AND ITS INFLUENCE ON Cu-BINDING CAPACITY OF DISSOLVED ORGANIC CARBON

By

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B.A. Carleton College, 1989

A THESIS

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

(in Plant, Soil, and Environmental Sciences)

The Graduate School

The University of Maine

May, 2002

Advisory Committee:

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Thesis Advisor: Dr. M. Susan Erich

An Abstract of the Thesis Presented
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This thesis monitored the progression of early-stage humification during the decomposition of four soil amendments and analyzed the effect of humification on the copper (Cu) binding capacity of amendment-derived dissolved organic carbon (DOC). Amendments chosen for the 8-week incubation were: wheat straw (*Triticum aestivium* L.), crimson clover (*Trifolium incarnatum* L.), a primary papermill residue (PPR), and a primary papermill residue mixed with secondary wastewater treatment sludge (PPR+SS). Specific attention was given to the <1 kDa, low molecular weight (LMW) fraction of amendment DOC as the high solubility and charge characteristics of this fraction likely influence soil processes. Amendments were incubated at 22 °C and extracted 6 times over 8 weeks with deionized-distilled water. The LMW fraction was separated by pressure filtration through a 1000 MWCO membrane. Molar

absorptivity was determined at 285 nm, phenolic acid content by the Folin-Ciocalteu reagent, and charge density by titration. Fluorescence spectra were determined after standardizing carbon concentration to 3 mM. Copper complexation capacity was determined on extracts of wheat straw and crimson clover at day 0 and 7 using the equilibrium ion exchange method.

For wheat straw and crimson clover, early-stage humification progressed through an increase in molar absorptivity (A_{285}), phenolic and total charge density, and averaged molecular size, and through the polymerization of breakdown products as determined through transitions in fluorescence peak locations. The most significant humification transitions occurred during the first week of the incubation. A shift in fluorescence peak location also occurred within the first week for the LMW fraction of amendment DOC. This transition within even the LMW pool demonstrates the scale invariance of the humification process. Monitoring humification in papermill residues proved more difficult as these materials lacked a significant soluble C pool.

For wheat straw and crimson clover, DOC extracted both initially and following a 7 day incubation desorbed and complexed resin-bound Cu. Interpretation of weak, outer-sphere binding was complicated by poor replicability at high solution Cu concentrations. Strong, inner-sphere binding was responsible for 0.11-0.55 mmol Cu g^{-1} C with the higher values corresponding to LMW extracts. For bulk extracts, there was no consistent pattern of increasing binding capacity with increasing degree of early-stage humification. For the LMW fraction, the maximum strong Cu binding capacity increased with increasing degree of humification. One plausible explanation for this difference is that LMW humic materials likely contain a greater relative

percentage of surface-exposed acidic functional groups and experience less geometric or steric hinderance to metal binding. Enhanced mobility of Cu due to its ability to complex with the LMW fraction of soluble organic matter may result in increased toxicity and runoff or leaching potential.

DEDICATION

In honor and memory of Allie and Lew Walton

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Chapter I

LITERATURE REVIEW

Dissolved Organic Carbon (DOC)

The aerobic degradation of plant materials occurs through the enzymatic oxidation and de-polymerization of tissue components, resulting in the initial formation of progressively smaller, more water-soluble molecules (Wershaw et al., 1996) (Figure 1). Soluble, monomeric products may be taken up by soil microbes for maintenance functions, utilized for energy production and respired, adsorb unaltered to soil mineral constituents, and/or undergo enzymatically mediated transformations that may enhance their solution phase stability. Recognizing this range of potential pathways, Guggenberger and Zech (1994a) defined dissolved organic carbon (DOC) as the complete pool of soluble compounds ranging from little modified plant oligosaccharides through recalcitrant lignin-derived materials to fulvic acid-like microbial resynthesis products. DOC may also be identified in terms of the practical aspects of collection or isolation. Herbert and Bertsch (1995) operationally defined dissolved organic matter (DOM) as organic matter passing a 0.45 μm filter, though acknowledging that this fraction likely included some colloidal material.

DOC is further characterized by a high concentration of acidic functional groups included in both aliphatic and aromatic organic acids (Mathur et al., 1993). The composition of such groups influences soil pH, nutrient mineralization rates, metal complexation, pollutant transport, and both microbial and phyto-toxicity (Mathur et al., 1993; Moore and Matos, 1999; Ohno and First, 1997). Cronan et al. (1992) determined that not only did acid functional group chemistry affect the above-

mentioned factors, but that changes in environmental conditions and land management practices altered functional group density and acid strength itself. Such alterations may in turn amplify or ameliorate further changes in pH, mineralization rates, and both metal complexation and pollutant transfer capacity. Other studies (Liang et al., 1996) have suggested that variations in DOC molecular weight affect the likelihood of DOC adsorption to soil mineral phases.

A growing body of work has focused on the initial degradation pathways of various organic materials. Through ^{13}C NMR analysis, Krosshavn et al. (1992) concluded that both vegetative source and degree of humification affect OM functional group distribution. Eijsackers and Zehnder (1990) and Gressel et al. (1995) focused on the decompositional fate of integral plant cellular components (i.e. lignin, cellulose, hemicellulose, polysaccharides, lipids, proteins, and hydrocarbons) and concluded that there was a significant compositional and degradation rate difference between monocotyledonous and dicotyledonous stem and leaf tissues. Sikora and McCoy (1990) conceptualized organic matter degradation as the microbial- and leaching-facilitated decomposition of three discrete fractions: (1) a soluble fraction composed of low molecular weight (LMW) acids, sugars and cytoplasmic and membrane constituents that provides ready microbial substrate, (2) a degradable fraction that is stabilized to some degree by protection mechanisms or substrate complexity, and (3) a highly stable, recalcitrant fraction that contributes directly to SOM. Eiksackers and Zehnder (1990) further noted that while soil fauna generally dominate the OM degradation process, direct microbial action was a key factor in

agro-ecosystems. This heightened microbial role was attributed to the generally lower recalcitrance of crop residues.

Kuiters and Sarink (1986) observed that the organic-rich leachate produced through the degradation of leaf and needle litter was a significant source of the dissolved organic matter (DOM) found in soil solution and natural waters. Leenheer (1994) agreed on this likely source, but observed that < 20% of DOM in natural waters consisted of identifiable compounds. The remainder was defined as a complex mixture of humified environmental residues. While the exact chemical transformations involved in humification are poorly understood, the process involves the complexation or polymerization of precursor products including lignin, microbially synthesized phenols, N-containing amino acids, and plant derived secondary metabolites. The humified fraction of soil organic matter contains materials of varying solubilities, including the recalcitrant but still soluble fulvic acids (FA). Harper et al. (2000) defined FA and HA as composites of smaller molecular units including aliphatic and aromatic groups, oils, amino acids, phenols, phenolic acids, phenolic esters, fatty acids, alkanes, tannins, and mono-and polysaccharides. Phenolic acids are chemically defined by the presence of aromatic rings bearing one or more hydroxyl substituents (Kuiters, 1990). A model fulvic acid includes 3 linked regions (i.e. altered carbohydrates, lignin residues, and lipid residues) with side-chains attaching to the lignin-derived core through aromatic ketonic linkages (Figure 2). This structure is a product of both hydrolytic and biotic and abiotic oxidation reactions.

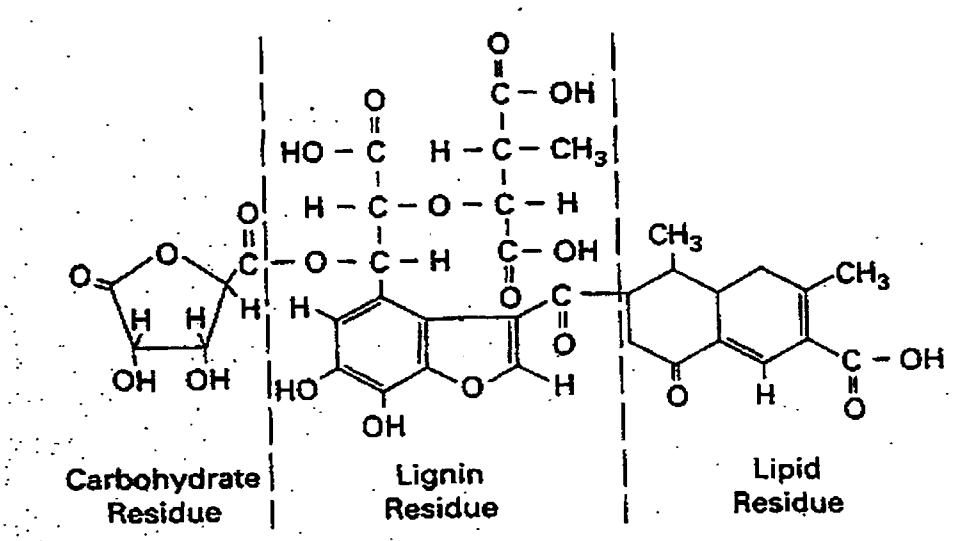

Figure 2. A model fulvic acid. [Wershaw et al., (1985)]

In an evaluation of the composting process, Sanchez-Monedero et al. (1999) focused specifically on the recognizable products released during litter decomposition that may serve as humification precursors. Within a range of composted materials, a significant ($p < 0.05$) correlation was found between the decreasing solution concentration of water-soluble phenolic monomers and increasing humification as defined by multiple indices (i.e., humic acid carbon as a percentage of total organic carbon (HAC/TOC), total extractable carbon (HAC/EXC), and relative to fulvic acid carbon (HAC/FAC)). The plant tissue concentration of water soluble LMW phenolic acids is affected by vegetation age, type, and growth rate, soil moisture and nutrient

content, season, plant stress including the extent of herbivory and nutrient availability, the presence or absence of soil microsymbionts, and soil management and cultivation history (Horner et al., 1988; Kuiters, 1990; Northup et al., 1995; Siquiera et al., 1991). In living plant tissue, monomeric phenols are considered as toxins or mobile defenses, while the polymeric phenols behave as digestibility-reducing compounds or immobile defenses (Horner et al., 1988).

While LMW phenols are water soluble, the majority of phenolic compounds are released through the oxidative breakdown of cell wall sugar conjugates during litter decomposition. Such compounds may subsequently form recalcitrant 'tanning complexes' with plant cellular proteins, a process potentially limiting the availability of both phenols and an otherwise labile source of nitrogen. Blum and Shafer (1988) observed that the half-life of soluble phenolics and phenolic monomers was less than 10 days, demonstrating their degradability by soil microbes. Martens (2000) incorporated 7 plant residues of known phenolic acid concentration into a loam soil and, following an 84 day incubation, observed that between 16% (soybean) and 69% (oat) of the initially present phenolic acids had been mineralized. Sugai and Schimel (1993) analyzed the microbial utilization of radio-labeled salicylic (SAL) and *p*-hydroxybenzoic (PHY) acids and observed that a greater percentage of PHY was incorporated into SOM (41% versus 17% of SAL) while a greater percentage of SAL was respired (77% versus 41% of PHY). Siquiera et al. (1991) concluded that as LMW phenolics continuously undergo polymerization, conjugation and degradation reactions they should not be thought of as soil solution end products.

While Sanchez-Monedero et al. (1999) found no significant correlation between water soluble carbohydrates and humification indices, soluble sugars are the litter fraction most readily decomposed by soil microbes and may significantly affect patterns and rates of nutrient release and immobilization (Palm and Rowland, 1997). Martens (2000) measured CO_2 respired during the decomposition of organic amendments and found that for a 57 day incubation, CO_2 evolved was significantly correlated to residue carbohydrate content ($r = 0.93$). Sugai and Schimel (1993) concluded from a study of microbial response to ^{14}C labeled glucose and salicylic (SAL) and p-hydroxybenzoic (PHY) acids, that microbes selectively incorporated glucose into cellular structural materials while metabolizing and respiring the aromatic acids. Qualls and Haines (1992) found a strong correlation ($r = 0.83$) between percent DOC lost during a 134 day incubation and the hydrophilic neutrals component of throughfall, litter leachate, stream water and soil O, A, and B horizon DOC. The hydrophilic neutral fraction comprised 10-36% of total DOC for all samples and was characterized as $\cong$ 54% soluble carbohydrate. Herbert and Bertsch (1995) noted that the remainder of this fraction was comprised, in significant part, of LMW aliphatic acids.

Low Molecular Weight DOC

While approximately 80% of the compounds that comprise DOM cannot be definitively identified, operational separations, such as between fulvic versus humic acids or hydrophilic versus hydrophobic acids and bases, allow the examination of discrete DOC fractions and their interactions with both soil solution and solid phase components. One fraction of DOC receiving increased attention is the low molecular

weight (≤ 1500 daltons) fraction that includes both organic acids (aliphatic, aromatic and amino acids plus siderophores) and simple mono- and oligosaccharides (Homann and Grigal, 1992; Harter and Naidu, 1995). Though LMW organic acids (OA) comprise ≤ 10% of total soil solution DOC, their high solubility and metal complexation capacity disproportionately affect soil processes (Fox et al., 1990; Bergelin et al., 2000). LMW OA play a key role in mineral weathering and soil genesis (Huang and Schnitzer, 1986), plant solubilization and uptake of both macro- and micronutrients (Nigam et al., 2001), and the alleviation of metal-induced plant root and aquatic organism toxicity (Hue et al., 1986). LMW OA are synthesized by plants, animals and microbes and are likely building blocks of the structurally heterogeneous fulvic and humic acids that have been widely studied in plant extracts, soils and natural waters (Stevenson, 1982). LMW OA concentration in the soil may range from μM to mM and abundance normally follows the sequence aliphatic > aromatic > amino acids. Distribution in the soil is influenced by the type and abundance of vegetation, soil characteristics including aeration, moisture and clay content and the presence and activity of soil microbes (Harter and Naidu, 1995). In agricultural soils, OA concentration is also affected by the adoption of management practices such as reduced tillage and green manure or compost application (Bolan et al., 1994).

LMW OA range from short chain aliphatic acids with weights of 50-60 daltons to long chain fatty acids with molecular weights approaching 400 daltons. The majority of the common LMW OA have molecular weights under 300 daltons and may contain both acidic and neutral functional groups (Fox, 1995). Carboxylic

acids are the most important functional group as they contribute significantly to overall acidity and solubility (Fox, 1995). LMW OAs may influence soil chemical processes through their ability to complex metals, react with mineral surface exchange sites, and/or extend domains of congruent dissolution (Vulava et al., 1997). Homann and Grigal (1992) compared LMW fulvic acids with high molecular weight (HMW) humic acids and concluded that LMW acids were both less easily flocculated and more effective at binding soil metals into soluble complexes.

Fox (1995) studied LMW OA release in forest soils and concluded that the major OA contributors were root exudates, soil fungi, the leaching of forest floor litter, the combined effect of rain and throughfall, and the decomposition of soil organic matter. Plant roots exude organic acids in response to nutrient deficiencies and bulk organic acid concentrations may reach 15-20 g kg^{-1} root dry weight in the soil rhizosphere (Fox, 1995). There are over a dozen OA commonly extracted from leaf litter leachates (Fox, 1995), and the soil under such litter is frequently enriched in the aromatic constituents of the LMW OA pool. In terms of organic matter decomposition, plants and soils contain fulvic, humic and long-chain fatty acids, polysaccharides, proteins and lignin, all of which may release LMW OAs upon breakdown (Homann and Grigal, 1992). Lagier et al. (2000) sequentially ultrafiltered a landfill DOC extract and concluded that 47% of the extract weighed $\leq$ 3000 daltons with 43% of that fraction weighing $\leq$ 1000 daltons.

Harter and Naidu (1995) noted that while significant research has focused on the interaction between fulvic/humic acids and soil metals, less information exists regarding organo-metallic interactions involving LMW OA. The metal complexation

capacity of an OA is a function of the specific acid present (and thus the relative placement of its acidic functional groups), its concentration in solution and the nature of the metal-ligand complex formed. Complexes involving 5- and/or 6- member ring structures, such as those involving aliphatic di-carboxylic acids, are highly stable (Harter and Naidu, 1995). Taga et al. (1991) observed that the strong Cu binding sites on a soil solution humic acid were carboxyl sites with weak metal binding occurring at both amino and phenolic sites. Lagier et al. (2000) reached a similar conclusion in studying Cu chelation by a landfill leachate humic acid. Vulava et al. (1997) examined the effect of the LMW chelator DTPA on Cu solubility and concluded that Cu-DTPA ligand formation was affected by absolute and relative concentrations of Cu and DTPA, soil solution pH and ionic strength and the order of Cu-DTPA application (i.e. whether testing the ability of DTPA to solubilize soil-bound Cu or to chelate soil solution Cu). Romkens et al. (1999a) fractionated O horizon DOC into HMW and LMW components and observed that at a high Cu/C ratio the maximum binding capacity was greater for the LMW fraction than the HMW fraction (0.45 mmol Cu g^{-1} C versus 0.25 mmol Cu g^{-1} C, respectively). Fox et al. (1990) studied the effect of LMW OA addition on Al release from a spodic horizon and hypothesized that reactions at Al oxide exchange sites would increase the solution concentration of Al-ligand complexes. The researchers concluded that, for the sixteen naturally occurring organic acids studied, the number of carboxylate groups per OA molecule increased from one to three as the OA-Al stability constants (log K_{Al}) increased from 0.80 to 7.80.

Nigam et al. (2001) studied the influence of LMW plant root exudates on metal mobilization by modeling the interaction between Cd and a suite of common, root derived carboxylic and amino acids. In terms of plant uptake, the Cd content of maize roots was almost twice as high under carboxylic acid treatments as under amino acid treatments. The researchers concluded that carboxylic acids were more effective than amino acids at complexing Cd and that within the carboxylic acids tested citric acid complexed more Cd than malic acid. Mench et al. (1988) studied the metal binding capacity of LMW maize root exudates. The researchers concluded that the LMW fraction formed stable complexes with Cu, Pb, and Zn and that Cu could form both mono- and bis-complexes (i.e. metal + 2 ligands). Young et al. (1982) studied Cu chelation by a polymaleic acid (PMA) (MW 1200) and concluded that there was 1 Cu ion complexed per PMA molecule and that the complex was of the form CuL_2 (i.e. Cu + 2 ligand sites). Evangelou and Marsi (2001) noted that infrared spectroscopic analysis of Cu-humic acid complexes revealed bidentate chelation involving either two adjacent carboxyl or adjacent carboxyl and phenolic groups.

Evangelou and Marsi (2001) studied the metal complexation capacity of three distinct molecular size fractions of decomposing corn leaves and stalks and observed that as fraction molecular size decreased both carboxyl acidity and carboxyl acidity as a percentage of total acidity increased. Cadmium (Cd), calcium (Ca) and copper (Cu) binding capacities were measured for each size fraction and the researchers concluded that complexation strength followed the order Cu > Cd > Ca. This was explained by the fact that Cu and, to a lesser extent Cd, formed strong inner-sphere complexes with soluble humic materials while Ca formed weak outer-sphere complexes. In terms of

molecular size profiles, the researchers concluded that for all three metals, at all tested concentrations, stability constants increased as fraction molecular size decreased. As practical utility of their research they noted that land management practices encouraging the application or build-up of organic residues may influence soil metal mobility.

Copper

While metal-rich waters and soils may occur naturally, the anthropogenic input of metals creates potentially hazardous metal burdens in diverse environments. Copper (Cu) is a metal of interest affecting soil and aquatic organisms and plant root development (Jarausch-Wehrheim et al., 1996; Parker et al., 1998). Copper exists in the soil in multiple forms as it may be adsorbed onto the charged surface of clay minerals, bound with amorphous oxyhydroxides of iron or manganese, present in the lattice of primary and/or secondary minerals, or complexed with soil organic matter (Ponizovsky et al., 1999). Karathanasis (1999) analyzed the subsurface migration of Cu and Zn in agricultural soils and observed that both metals demonstrated increased solubility under slightly to moderately acid soil conditions (pH 5-6.5). The researchers concluded that this was due, in part, to complexation with DOM. Temminghoff et al. (1997) reached a similar conclusion in column leaching experiments designed to measure the effect of DOC on Cu mobility in contaminated soils. Romkens et al. (1999b) concluded that not only were DOC-Cu complexes stable, but that DOC effectively mobilized Cu that was adsorbed to both soil organic matter and mineral phases.

The mean U.S. soil copper burden is 50 mg Cu kg^{-1} soil, and may reach, in some locations, an order of magnitude higher (Holmgren et al., 1991). Soil copper loading may result from industrial practices, field spreading of sewage sludge or agricultural application of fungicides or herbicides. Holmgren et al. (1991) noted that in locations with above average soil Cu concentrations Cu had been commonly utilized as a fungicide. Cu-based fungicides, sold as Bordeaux mix (Bm), constitute $CuSO_4$ and lime-based sprays. Bm has traditionally been applied as a mildew treatment in vineyards, orange groves, and coffee plantations and is now increasingly applied to agricultural crops such as potatoes and hops. Treatment is by foliar spray and excess Cu is transferred from leaves and fruit to the soil. Further transfer occurs when leaves, stems or stalks saturated with fungicide decay on the soil surface. Cu burdens as high as 1500 mg kg^{-1} soil and 500 kg ha^{-1} have been measured in French vineyard and Kenyan coffee plantation soils, respectively (Flores-Velez et al., 1996).

Moolenaar and Beltrami (1998) examined Italian viticultural methods and observed a mean Bm application rate of 26 kg Cu ha^{-1} yr^{-1} in "organic" vineyards versus a 10 kg Cu ha^{-1} yr^{-1} rate in "conventional production" vineyards. They noted as explanation that organic growers, in shunning organophosphate-based pesticides, often rely exclusively on Bm to combat leaf and fruit mildew. To model soil Cu accumulation the researchers applied a dynamic balance model that accounted for metal input, adsorption, and leaching rates. They extrapolated Bm application rates to the European soil saturation limit (100 mg Cu kg^{-1} soil) under the assumption that adsorption reactions controlled the model. Extrapolation for organic vineyards suggested that if Cu adsorption significantly exceeded Cu leaching, soil saturation

could be reached within 13 years. Extrapolation for conventional production vineyards suggested a 'time to soil saturation' of 30 years. In terms of the averaged permissible groundwater limit of 100 mg Cu $(m^3)^{-1}$ the 'time to saturation' for organic versus conventional methods was 139 versus >300 years, respectively. Flores-Verez et al. (1996) observed that Bm has been used as a fungicide and bactericide in French vineyards since the 1850s and the 'time to saturation' may already have been exceeded. Furthermore, the utilization of Bm is increasing across Europe as EU directives both encourage the adoption of organic production practices and allow higher Bm-derived soil Cu burdens than may occur through the land application of sewage sludge.

Guggenberger et al. (1994b) studied the hydrophobic and hydrophilic fractions of soil solution DOC. They concluded that the hydrophilic fraction had a higher Cu complexation capacity than the hydrophobic fraction (0.03-0.14 mmol Cu mol^{-1} DOC versus 0.01-0.04 mmol Cu mol^{-1} DOC, respectively) and that due to its relative soil mobility, the hydrophilic fraction likely played a significant role in metal leaching. The authors concluded that as DOM-metal complexation was, in part, a metal-H^+ ion exchange process, differences in binding capacity were related to differences in hydrophilic and hydrophobic exchange acidities. Calculated exchange acidities were 10.6-14.3 versus 8.5-11.8 $mmol_c\ g^{-1}$ C for the hydrophilic and hydrophobic fractions, respectively.

Kuiters and Mulder (1993) utilized a Sephadex-25 column (fractionation range 0.1-5 kDa) to measure differences in Cu binding potential between forest floor litter and the forest humus horizon. While the Cu binding capacity of the humus

horizon DOC was greater than the capacity for litter layer DOC (2.6-6.0 mmol Cu g^{-1} DOC versus 0.7-0.9 mmol Cu g^{-1} DOC, respectively), Cu binding by the litter leachate was still significant. They concluded that as litter layer DOC was dominated by LMW compounds, differences in binding capacity were attributed, in part, to the relatively larger percentage of non-complexing C compounds (e.g. carbohydrates and amino sugars) in fresh litter leachates. Elution profiles were further analyzed to specifically correlate molecular size fractions with Cu-binding capacity. The authors concluded that the LMW fraction of litter layer and humus horizon DOC extracts was responsible for 50-99% versus 30-60% of Cu-binding capacity, respectively.

Romkens and Dolfing (1998) operationally defined fulvic acid (FA) as a LMW acid (< 3 kDa) with a higher proton binding capacity and greater solubility than humic acid (HA) (3-100 kDa). To study molecular weight differences between FA and HA they tested whether the addition of a $CaCl_2$ flocculant would affect both the molecular size profile of soil DOC and DOC-Cu complexation. Upon addition of $CaCl_2$ 50% of soil system DOC flocculated, suggesting that the HMW HAs were precipitating from solution. They concluded that while HMW DOC had a higher Cu binding affinity, the LMW DOC had a higher binding capacity (0.45 mmol Cu g^{-1} DOC versus 0.25 mmol Cu g^{-1} DOC for the HMW fraction). If results were extrapolated to a field scale with a realistic agricultural soil Cu burden of between 5-150 mg Cu kg^{-1} soil, they believed that a significant concentration of Cu remained in solution even after flocculation-based attempts to precipitate soluble metal complexes. Romkens et al. (1999a) cautioned that researchers should not overlook the significant role that LMW acids play in the complexation and transport of soil copper.

Chapter II

DOC ANALYTICAL TECHNIQUES

Molecular Size and Weight: Ultrafiltration

The availability of membrane filters with defined pore sizes has allowed the development of rapid fractionation and concentration techniques for aquatic humic substances, soil solutions, and natural waters. A range of filtration membranes exist with nominal molecular weight cut-offs (MWCO) between 50 and 10^6 daltons. While cut-off values are defined in terms of molecular weight, fractionation should be considered more precisely in terms of molecular size. Swift (1985) observed that a given membrane generally retained ≥ 90% of spherical, uncharged solute molecules greater than its MWCO. Factors influencing retention include molecular configuration and both solute-solute and solute-membrane charge interactions. As aqueous extracts are filtered under pressure, extract concentration and pressure gradients must also be considered (Wershaw and Aiken, 1985). Wershaw and Aiken (1985) concluded that the potential for larger-molecular-size breakthrough increased concomitant with the increase in retentate concentration and that permeate volume should never exceed 90% of initial solution volume.

Ultrafiltration has been increasingly utilized in the study of the low molecular weight components of both plant and soil derived DOC. Mench et al. (1988) used a 1000 MWCO ultrafiltration membrane to fractionate and characterize maize plant root exudates. They concluded that LMW compounds comprised 58% of the soluble exudate organic C and that the LMW fraction consisted primarily of reducing sugars (54%) and organic acids (42%) with small concentrations of amino acids (3.0%),

proteins (0.6%), and phenolic compounds (0.5%). Brunner et al. (1996) fractionated an aqueous chestnut leaf litter extract and examined the relative phytotoxicities of the <1000 MWCO (LMW) and >10,000 MWCO (HMW) fractions. The researchers concluded that the LMW fraction neither suppressed barley root growth nor affected root tip morphology while the HMW fraction both suppressed root growth and produced root tip deformity at DOC concentrations as low as 5 mg kg^{-1} soil. Dell'Amico et al. (1994) performed a similar fractionation on humic substances extracted from composted municipal sewage sludge and concluded, conversely, that the LMW fraction (<1000 MWCO) had a suppressive effect on both root and shoot dry weight while the HMW (>1000 MWCO) and the "bulk" (i.e. non-ultrafiltered) fractions stimulated both root and shoot growth. Meyer et al. (1987) ultrafiltered DOC from a blackwater river and examined relative biodegradability of LMW (<1000 MWCO) and HMW (>10000 MWCO) fractions. They concluded that while <5% of the HMW fraction was microbially degraded, 86% of the LMW fraction was utilized as microbial substrate.

UV-Vis Spectrophotometry + Organic Reagents

Molar Absorptivity

Spectrophotometric absorbance at 272-285nm has been used to non-destructively analyze marine, riverine, and terrestrial aquatic samples. This wavelength range has been explored on the grounds that π-π^* electron transitions occur within this portion of the UV spectrum for both simple (i.e. phenolic acids) and complex (i.e. PAHs) aromatic compounds. As some of these compounds may exist as either breakdown products of more complex organic substances or as polymerized

precursors to humic substances, a measure of 270-285nm absorbance per mole of organic carbon in the analyte provides information regarding aromaticity, degree of humification and/or sample provenance. A further benefit of this technique is that nitrate does not absorb at these wavelengths and thus no spectral interference occurs due to its presence (Chin et al., 1994).

Chin et al. (1994) analyzed the molar absorptivity of sedimentary porewaters, aquatic fulvic acids, tannin rich surface waters, and a commercial humic acid. Calculated molar absorptivity ranged between approximately 60 and 900 L mol OC^{-1} cm^{-1} (i.e. spectrophotometric absorbance per cm cell / molar organic C concentration per liter) with TOC concentration constrained between 2.5-4.5 mM C. Lake Michigan porewater displayed the lowest, and the humic acid displayed the highest, molar absorptivity. The researchers further observed that aquatic fulvic acids originating from water bodies devoid of higher plant life displayed lower absorptivity than aquatic fulvic acids originating from water bodies rich in the organic residues of higher plants (150 L mol OC^{-1} cm^{-1} versus 250-500 L mol OC^{-1} cm^{-1}, respectively). For the fulvic acid samples, regression of molar absorptivity (A_{285}) against aromaticity (α) as determined by percent C in the 110-160 ppm shift region of ^{13}C CPMAS NMR scans revealed a strong correlation ($\alpha = 0.05(A_{285}) + 6.74$; $r^2 = 0.90$). Traina et al. (1990) measured molar absorptivity of stock humic acids at 272 nm and found a similar strong correlation ($\alpha = 0.707(A_{272}) + 12.626$; $r^2 = 0.88$). Chin et al. (1994) recorded a second correlation between molar absorptivity and the averaged molecular weight of the fulvic acid samples as determined by size exclusion chromatography ($M_w = 3.99(A_{285}) + 490$; $r^2 = 0.97$). This strong correlation suggests a

further relationship between aromaticity and molecular polymerization. Both Chin et al. (1994) and Traina et al. (1990) concluded that these relationships could be put to predictive use as both aromaticity and polymerization of humic materials correlate with an affinity for metals and organic pollutants.

Rostan and Cellot (1995) utilized the molar absorptivity ratio to detect both seasonal and provenance variation in Rhone River DOC. Such use of this ratio was initially proposed by Buffle and Deladoey (1982) and successfully applied to the differentiation of terrestrial versus aquatic DOC in eutrophic lakes, ponds and "peaty waters." They believed that this parameter was independent of DOC concentration but sensitive to structure and concluded that an A_{285} value ≤ 10 L (g OC)$^{-1}$ cm^{-1} suggested the presence "aquagenic" aliphatic compounds while a value ≥ 20 L (g OC)$^{-1}$ cm^{-1} suggested the presence of "pedogenic" refractory compounds. Rostan and Cellot (1995) reached a similar conclusion, determining that the higher A_{285} value for the upper river and feeder streams was due to the dominance of allocthonous DOC, while lower values for the lower river main axis was attributed to the dominance of autocthonous DOC.

Mathur et al. (1993) utilized A_{280} as an optical means of testing compost maturity. Four farmyard manures were composted for 60 days with DOC extracts collected every 7-9 days for photometric analysis. Measured DOC averaged 44 mg DOC g^{-1}C dry weight initially, increased during the initial decomposition process and then decreased once the thermophilic phase had passed. While absorption for all 4 manures demonstrated a rapid increase followed by a generally slow decline, the authors noted that, though DOC content had decreased, day-60 A_{280} values were not

significantly different than day-0 values. They concluded that while the bonds absorbing at this wavelength were still clearly present, the compounds involved had shifted from simple aromatics toward those with more complex, humified structures.

Absorbance at 625 nm: Anthrone-H_2SO_4

Brink et al. (1960) applied the colorimetric anthrone reagent to soil organic matter to test its reliability as an indicator of soil polysaccharide content. In the presence of hexose sugars, anthrone dissolved in concentrated H_2SO_4 produces a blue color with an absorption maximum at 625nm. Brink et al. (1960) noted both that anthrone could react with non-carbohydrate compounds and that interference from aromatic proteins had been recorded. They believed, however, that protein interference from soil extracts was generally insignificant. Application of the reagent to soils ranging from a prairie silt loam to a New England podzol revealed that anthrone-reactive sugars comprised between 3.4 and 8.1% of total soil DOC. Katz et al. (1983) concluded that the presence of NO_3^- at concentrations as low as 20 ppm could interfere with absorbance. Martens and Frankenberger (1990) analyzed the potential for ionic strength-related interference and observed that when soil solutions were tested with or without prior ion exchange treatment significantly different anthrone-reactive hexose concentrations were found. In a study designed to test the effects of ion interference on A_{625}, Grandy et al. (2000) applied the anthrone-sulfuric acid treatment to both field derived and simulated soil-water extracts spiked with known amounts of glucose standard. The researchers concluded both that fractional recovery of the glucose spikes was high (78-100%) and that no significant correlation was found between spike recovery and solution ionic composition.

Gallet and LeBreton (1995) measured anthrone-reactive carbohydrates in water extracts of fresh spruce needles and bilberry leaves and determined that soluble carbohydrates comprised 11.6-12.0 % and 7.0-10.8 % of spruce needle and bilberry leaf dry matter, respectively. DeLuca and Keeney (1993) analyzed soluble anthrone-reactive carbon (ARC) in soybean straw and sorghum residue incubations as a measure of microbially available carbon. ARC at the beginning of the incubation was approximately 15 $\mu g\ g^{-1}$ soil for both soybean straw (C:N 66:1) and sorghum (C:N 8:1) residue. This high concentration was attributed to soluble sugars released during initial plant material decomposition. With only one exception ARC remained above the background level (4.1 $ug\ g^{-1}$ soil) for the duration of the experiment in those incubations containing either low C:N residue or high C:N residue plus glycine. An experiment adding cellulose to the test soil revealed that when applied with no additional source of N, ARC remained near the background level. When cellulose was added with NH_4, ARC peaked at approximately 30 $ug\ g^{-1}$ soil at day 20 before falling to the background level by end of the experiment. While initial free sugar content was low in both treatments the increasing concentration seen in the +N incubations was attributed to the microbially-facilitated breakdown of cellulose into anthrone-reactive monosaccharides. In comparing the decomposition of both amendments and cellulose the authors believed that initial ARC, when present, flushed quickly from plant materials and represented an ephemeral pool of microbial substrate. This view was corroborated by Collins et al. (1990) who concluded from a study of wheat straw decomposition that nonstructural carbohydrates disappeared within the first 33 days of incubation.

Absorbance at 750 nm: Folin-Ciocalteu Reactive Phenolic Acids

The Folin-Ciocalteu reagent contains phosphomolybdic and phosphotungstic acids that are reduced in alkali solution. The reaction produces a heteropoly blue color with an absorption maximum at 750nm. Reduction occurs in the presence of phenolic hydroxyl groups, cyclic compounds containing either NH or aldehyde groups, and the purine and pyrimidine bases. F-C reagent reduction by the latter two groups, however, was deemed insignificant in comparison to reduction in the presence of phenolic acids (Box, 1983). Reduction may also occur with inorganic substances and interference has been observed in the presence of Fe (at > 2 mg/L), Mn (>0.3 mg/L), and S^{2-} (> 30 μg/L). Box (1983) further concluded that while the F-C reagent allowed for good relative measure of the water soluble humic substances in leaf and natural water extracts, results varied with the standard chosen. The F-C reagent technique has been applied successfully in contexts where total phenolic acid concentration is more significant than the identification of particular acids. Researchers have used it to measure the phenolic acid content of agricultural soils (Ohno and First, 1998), forest soil pore waters (Gallet and Keller, 1999), lake and stream water (Box, 1983), leaves and needles of both deciduous and coniferous trees (Kuiters and Sarink, 1986), tropical legumes (Palm and Sanchez, 1990), leaf litter and humus from mountain sites (Gallet and LeBreton, 1995) and composted organic wastes (Sanchez-Monedero et al., 1999).

Kuiters and Sarink (1986) analyzed the water soluble phenolic acid (WSPA) concentration of leaves and needles collected from seven tree species both during autumnal leaf fall and after 4 months of decomposition. WSPA content ranged from

2.5 mg TAE (tannic acid equivalents) g^{-1} dry wt for hornbeam leaves to 0.045 mg TAE g^{-1} dry wt for spruce needles. When samples were collected 4 months later the WSPA concentration had fallen to 0.195 mg TAE g^{-1} dry wt for hornbeam leaves and was undetectable in spruce needles. Palm and Sanchez (1990) analyzed initial WSPA content of leaves collected from three tropical woody legumes and calculated TAE polyphenol concentrations of between 34.35 mg TAE g^{-1} dry wt (*Inga edulis*) and 10.42 mg TAE g^{-1} dry wt (*Erythrina* sp). Polyphenol concentration was significantly correlated with initial lignin concentration ($p < 0.05$). Tian et al. (1992) analyzed WSPA concentration of woody perennial (both N_2-fixing and non N_2-fixing) and herbaceous (N_2-fixing) cover crop leaves. WSPA concentration (TAE) ranged between 15 mg g^{-1} dry wt (herbaceous N_2-fixer) and 53 mg g^{-1} dry wt (woody non N_2-fixer) and was not correlated with initial lignin concentration. Sanchez-Monedero et al. (1999) analyzed the WSPA concentration of 6 compost mixtures on the grounds that WSPA concentration would be sensitive to the chemical transformations that occur during composting. Samples were collected over a 5 month interval and analyzed for WSPA using p-coumaric acid as the standard. Initial WSPA concentrations ranged between 2.3 mg g^{-1} dry wt. (municipal solid waste + sorghum bagasse) and 4.4 mg g^{-1}dry wt (sorghum bagasse + pine bark + urea) and fell throughout the incubation, ultimately equaling <1.0 mg g^{-1} dry wt. for all compost mixtures.

Blum et al. (1991) analyzed the phenolic acid content of a soybean cropped sandy loam soil under different management practices (wheat-no till, wheat-conventional till, and fallow-conventional till). Total WSPA content was determined

using the F-C reagent (ferulic acid as the standard) and regressed against HPLC determination of 7 common plant tissue phenolic acids. The researchers calculated F-C reagent WSPA concentrations of between 50-250 $\mu g\ g^{-1}$ soil and when regressed against HPLC data, concluded that the F-C reagent overestimated WSPA by a factor of 16 ($WSPA_{HPLC} = -0.3536 + 0.0795 WSPA_{FC}$; $r^2 = 0.87$). The researchers noted that a similar overestimation had been found between the two methodologies in a sandy loam soil cover cropped with crimson clover, hairy vetch, rye, and subterranean clover. Cilliers et al. (1990) analyzed the chlorogenic and caffeic acid concentrations of apples and cider and reported an average F-C overestimation factor (relative to HPLC) of 13 ($r^2 = 0.99$). Whitehead et al. (1982) used HPLC to measure the concentration of common phenolic acids extracted from herbaceous root tissues of 14 plant species. WSPA concentrations ranged between 45 $\mu g\ g^{-1}$ soil organic C (*Equisetum arvense*) and 450 $\mu g\ g^{-1}$ soil organic C (*Ranunculus repens*) (with 1.7 % and 2.1 % soil organic C, respectively). Whitehead et al. (1983) further measured the phenolic acids extracted from woody and herbaceous plant roots plus beech leaf litter. WSPA concentrations for the root samples ranged from 50 $\mu g\ g^{-1}$ dry matter (unspecified pasture grass) to 370 $\mu g\ g^{-1}$ dry matter (*Agrostis stolonifera*) with a concentration for fallen beech leaves of 74 $\mu g\ g^{-1}$ dry matter.

Fluorescence Spectroscopy

Fluorescence spectroscopy is a powerful tool for probing the chemical structure of both humified and simple organic materials. The technique requires little sample preparation, minimizing the potential for sample alteration through extraction

or stabilization procedures, and is sufficiently sensitive to permit examination at environmentally relevant concentrations (Miano et al., 1988). The fluorescence phenomenon is the result of the immediate radiative dissipation of energy following molecular excitation. Radiation typically occurs at a slightly longer wavelength than the initial absorption as some energy is inevitably lost through vibrational absorption (Senesi, 1990). The wavelength of both absorbed energy and its re-radiation are specific to individual molecular energies and this specificity provides information regarding the source and structure of the excited molecule.

Fluorescence interference may result from the colloidally-mediated scatter of incident light (Tyndall scatter), immediate (10^{-15} s) re-emission of absorbed light due to incomplete electronic transition (Rayleigh scatter), and/or vibrational effects seen in conjunction with Rayleigh scatter when working with dilute solutions (the Raman effect) (Senesi, 1990). Compounds that fluoresce with measurable intensity are either unsaturated aliphatics capable of a high degree of resonance or aromatics or other closed-ring compounds (Visser, 1983; Miano et al., 1988). Fluorescence intensity may be altered by functional group substitutions, by complexation reactions with paramagnetic metals that quench fluorescence, by changes to molecular structure brought about by changes in pH, ionic strength, or temperature of the medium, or through solute-solvent interactions (Senesi, 1990).

Ghosh and Schnitzer (1980) analyzed the effect of altering pH and ionic strength on fluorescence spectra garnered from soil humic acids. They observed that as solution ionic strength increased from 1 mM to 100 mM, relative fluorescence intensity decreased. They concluded that at low solution ionic strength fluorescence

intensity is enhanced because molecules present are flexible and uncoiled. When the researchers incrementally increased pH from 2.0 to 9.5, fluorescence intensity also rose steadily. Below pH 7.0 the authors believed that increased intensity resulted from the ionization of available electron withdrawing functional groups. Above neutrality the ionization of semi-quinone type structures increased solution free radical concentration and enhanced fluorescence intensity. Blaser and Sposito (1987) analyzed the effect of pH on the fluorescence intensity of a chestnut leaf litter extract and concluded that relative intensity was highest at pH 5.0. Tam and Sposito (1993) observed the same relationship in their analysis of both the gentisic acid standard and an aqueous pine litter extract. They correlated this maximum with an increased deprotonation of phenolic acids, stating that this occurs at a lower pH (between pH 3-5) in the molecularly excited state than in the ground state. Senesi et al. (1991) concluded that due to the complex, heterogeneous nature of humic substances the observed fluorescence fingerprint should not be used as a diagnostic tool for identifying specific, individual humification products, but rather to aid in differentiating source and molecular characteristics by probing cumulative structure.

Fluorescence spectra may be generated in various modes: (1) emission spectra collected by fixing the excitation wavelength and observing emission peaks over a wavelength range, (2) excitation spectra collected by varying the excitation energy and observing emissions at a fixed wavelength, (3) synchronous scan spectra obtained by optimizing the distance between the emission and excitation wavelengths (thus minimizing the peak interference that occurs within complex mixtures), and scanning over a wide wavelength range, and (4) excitation-emission matrix (EEM) spectra that

generate 3 dimensional contour plots by simultaneously scanning all excitation and emission pair possibilities within a proscribed wavelength range. EEM spectra offer maximum information in that they contain all possible transect lines drawn using modes 1-3 (Yang et al., 1994).

Visser (1983) studied humification by examining molecular weight and functional group related changes in fluorescence spectra. He examined fulvic and humic acids of aquatic origin and compared them with humic materials of bacterial origin. He determined that lower molecular weight fractions displayed greater fluorescence intensities than higher molecular weight fractions and absorbed at shorter, more energetic wavelengths. He further determined that fluorescence intensity appeared more correlated with fundamental aromatic structure then with the presence of particular functional groups. Major excitation peaks (λ_{ex}) were found within two designated ranges: **I** with an average wavelength of 334 ±7 nm and **II** with an average wavelength of 415 ± 20 nm. Only one major emission peak (λ_{em}) was detected, a broad, poorly defined peak located between 370 and 550 nm. With respect to molecular size fractions, he observed that while excitation wavelengths varied by as little as 2-4 nm between size fractions, higher molecular weight emission wavelengths were 25 nm and 100 nm longer than lower molecular weight emission wavelengths for microbial material and aquatic fulvic/humic acids, respectively.

Miano et al. (1988) analyzed nine fulvic or humic acids extracted from both terrestrial and aquatic sources. They concluded that while the excitation spectra of fulvic acids varied significantly by source, spectra of the humic acids displayed little variation. They further observed that humic acid emission spectra were of a lower

intensity and at a longer wavelength, resulting, they believed, from the relatively more condensed ring structure of humic acids. At λ_{ex} = 320 nm they observed an average fulvic acid λ_{em} of 447 ±10 nm versus an average humic acid λ_{em} of 500 ± 17 nm. Senesi et al. (1991) analyzed 50 samples of fulvic and humic acids isolated from soils, composted organic materials, sewage sludges, and fungal synthates. They observed the same relatively lower intensity and longer λ_{em} of the humic acid samples and concluded these features were associated with the increase of linearly-condensed aromatic ring structures bearing carbonyl or carboxyl groups. The shorter λ_{em} and higher intensities observed for the composts and fulvic acids were associated with the presence of structurally simpler components with lower levels of aromatic condensation. The presence of hydroxyl, methoxyl or amino substituents was also thought to enhance fluorescence intensity.

Erich and Trusty (1997) extracted DOC from soil organic horizons collected from 9 forested sites and generated EEM spectra for each sample. All spectra displayed a peak with λ_{ex} between 320-348 nm and λ_{em} between 436-458 nm. This peak corresponds to the fulvic acid peaks described by Senesi (1990) and Miano et al. (1988). Two secondary peaks were also observed, one with $\lambda_{ex}/\lambda_{em}$ = 250-260/436-458 nm and the second with $\lambda_{ex}/\lambda_{em}$ = 280/338-345 nm. Coble et al. (1990) observed a peak at $\lambda_{ex}/\lambda_{em}$ = 280/325-45 in an extract of marine DOC and concluded that it displayed tryptophan-like fluorescence. Tryptophan, an aromatic amino acid, exhibits intensity maxima at $\lambda_{ex}/\lambda_{em}$ = 287/348 and is responsible for 90% of total protein-related fluorescence (Wolfbeis, 1985). Ohno and Cronan (1997) found a similar peak ($\lambda_{ex}/\lambda_{em}$ = 271-277/336-345) in their examination of corn residue DOC and also

attributed it to tryptophan. Brunner et al. (1996) studied the low molecular weight fraction (<1000 daltons) of an aqueous chestnut leaf litter extract and observed fluorescence peaks at both $\lambda_{ex}/\lambda_{em}$ = 275/325 and $\lambda_{ex}/\lambda_{em}$ = 330/440. They attribute the first peak to either tyrosine or tryptophan and the second to simple phenolic compounds (phenols, coumarins or flavonoids). In their study of corn residue DOC, Ohno and Cronan (1997) further noted the presence of a fluorescence peak at $\lambda_{ex}/\lambda_{em}$ = 313-316/435-444. Yang et al. (1994) observed a similar peak in their study of ponderosa pine needles. Ohno and Cronan (1997) defined peaks of this approximate wavelength as being of fulvic-like material. They noted that while DOC extracted from fresh residues was not truly fulvic (not having gone through the degradation and microbial reworking that constituted humification), both its spectra and response to XAD resin treatment suggested a fulvic-like nature.

Zsolnay et al. (1999) proposed a humification index (HIX) (i.e. HIX = $\Sigma I_{(435-480)}/\Sigma I_{(300-345)}$) based on the relative fluorescence of two well-defined regions analyzed at λ_{ex} = 254 nm. Region 1, defined by emission wavelengths in the 300-345 nm range, corresponds to the shoulder of fluorophore A. Region 2, defined by emission wavelengths in the 435-480 nm range, corresponds to the shoulder of the unidentified fluorophore B. The authors compared soil extracts with extracts prepared from both soil FA and microbial cell lysis products. A graphical presentation of their results highlighted three distinct pools with HIX values ranging between 2-7 for lysis products, 7-12 for soil DOC, and 13-16 for FA. This distinction corresponded to a decreasing relative intensity of the aromatic amino fluorophore concomitant with the increasing relative intensity of the unidentified fluorophore. Cox et al. (2000) utilized

HIX to examine DOC extracted from organic amendments and soils. Humification index values ranged from approximately 1 for liquid- to 20 for solid- olive-mill waste sludge extracts. Humification index values for solid urban waste, sewage sludge, and soil DOC extracts ranged from 5-8. The authors concluded that the low HIX value for liquid mill waste resulted from the relatively high percentage of non-humified components. As the effect of pH on HIX proved the most significant for the solid mill waste, the authors concluded that the high carboxyl charge density of this material was a product of its humified state.

Ohno (2002), redefined this equation as $HIX = \Sigma I_{(435-480)} / \Sigma I_{(300-345)+(435-480)}$, recognizing that this rendered the equation less sensitive to changes in the intensity of the amino fluorophore. As this peak is the product of a labile component, its degradation has the potential to overwhelm what smaller intensity transition occurs with the other index fluorophore. This correction generates HIX values in the range of 0-1, and while dampening the magnitude of progression observed using the traditional formula, more specifically addresses the changing nature of both index fluorophores. Ohno subsequently examined aqueous extracts of field corn residue, soil DOM and a purified soil fulvic acid (FA). HIX values using the tradition formula evolved from 2.1 to 6.5 to 28.5 for corn, DOM and soil FA, respectively. When re-calculated according to the redefined equation, results evolved over the range 0.57 to 0.84 to 0.94. Fluorescence scans generated at an excitation wavelength of 254 nm showed that (1) aromatic amino N was only clearly identifiable for the corn residue extract and (2) the undefined peak was distinctly visible only in DOM and FA extracts.

DOC-Metal Complexation

Many techniques exist for studying the metal complexation capacity of natural waters, plant leachates, soil solutions, and composted wastes. Each technique has both its strengths and limitations. Researchers have used potentiometric titrations coupled with ion selective electrodes (Stevenson et al., 1993), the relative quenching of fluorescence chromophores (Yang, 1994), metal-saturated Sephadex gels (Kuiters and Mulder, 1993), rhizotoxicity and plant tissue analysis (Nigam et al., 2001), ultrafiltration (Mulligan et al., 1999), equilibrium dialysis (Berggren, 1989), and batch complexation studies designed to measure both conditional stability constants (^{c}K) and metal binding capacities (L_t) (Luster et al., 1996). Problems for various techniques may arise involving the detection limitations of the electrode, fluorescence interference resulting from solution ionic strength or DOC concentration, hydrogen bonding to the chromatographic gel, or inadequate system equilibration time.

Luster et al. (1994) modified the equilibrium ion exchange method (EIM) to measure copper complexation by chestnut leaf litter DOC. This technique uses a strong cation exchange resin to examine the competing equilibrium between resin-metal and DOC-metal complexes. The authors demonstrated that at low solution ionic strength the technique could be successfully employed over a wider range of experimental parameters by including the nonlinear portions of adsorption isotherms in their analyses. They concluded that if the concentration of solution-bound metal (i.e. in DOC-metal complexes) relative to solution free metal (i.e. in aquoion or hydroxo complexes) was plotted versus solution bound metal [(M_b/M_f) versus (M_b)]

the shape of the plot both suggested the nature of the metal-ligand complex formed and allowed the calculation of ^{c}K and L_t.

Various resins have been used for EIM and system equilibration times vary with resin type. Luster et al. (1994 and 1996) believed that 24 and 60 hours were required for leaf litter DOC to equilibrate with Cu bound to Serdolit CS2 and BioRad AG 50W-X8 resins, respectively. Taga et al. (1991) studied Cu equilibration between a peat humic acid and Sephadex C-25 sulphopropyl resin and concluded that it could be reached in less than 1 hour. Romkens et al. (1998) addressed potential competition for $[Cu]_{free}$ between the resin and the experimental stock solution and concluded that at pH 6.0 less than 1% of $[Cu]_{total}$ would occur in inorganic complexes (i.e. as $CuNO_3^+$ or $CuCO_3$). Further factors to consider include the potential for DOC sorption to the resin and poor equilibration due to ionic strength effects. While Werner (1987) concluded that solution ionic strength did not significantly influence L_t, he did observe that analytical precision increased as solution ionic strength decreased from 1.0 M to 10 mM. Buffle et al. (1980) concluded that with pH $\geq$ 6.0 and a total interfering cation concentration of < 0.05M, competition for ligand binding sites was negligible. Luster et al. (1994) addressed ionic interference by setting the background solution ionic strength to 0.01 M and desalting the leaf litter extract on an H^+-saturated cation exchange resin prior to EIM analysis. In examining the potential for leachate sorption they measured DOC concentration both prior to and following H^+ resin treatment and concluded that resin DOC retention was < 5%. Other studies of resin-DOC interactions have calculated sorption concentrations of 3% in a forest soil solution (Guggenberger et al., 1994b), 5% in a forest soil O

horizon and 15% in a corn residue extract (Ohno and Cronan, 1997). The results of Ohno and Cronan (1997) suggest that a desalting pre-treatment for fresh crop residues could alter bulk DOC composition and thereby affect experimental results.

Chapter III

OBJECTIVES

1. To seek evidence for the occurrence of early-stage humification during the decomposition of select soil amendments.
2. To determine whether amendment-derived DOC is able to desorb and complex resin-bound copper (Cu).
3. To determine what affect early-stage humification has on the Cu desorption and complexation potential of amendment-derived DOC.

Chapter IV

MATERIALS AND METHODS

Amendments

An 8-week incubation was conducted using 4 potential soil amendments: wheat straw (*Triticum aestivium* L.), crimson clover (*Trifolium incarnatum* L.), a primary papermill residue (PPR), and a primary papermill residue mixed with secondary wastewater treatment sludge (PPR+SS). Wheat straw was collected from a Kansas farm in the autumn following grain harvest. All aboveground components were collected, dried at 60° C, ground to pass a 1mm sieve, and stored in a sealed container at room temperature until use. Crimson clover was grown at Rogers Farm, Stillwater, ME and was prepared and stored following the same protocol. Papermill residues were stored at 5° C in air-tight containers and were dried and ground under the same protocol prior to use. The papermill residues were both classified as 'low metal and low dioxin' and were produced in two New England mills (Andrew Carpenter, personal communication). Wheat straw, crimson clover and papermill residues were chemically analyzed for C and N using a Leco CN2000 Analyzer. Cations (Al, Ca, Fe, K, Mg) were measured with an ICP-AES (Model 975 Plasma AtomCorp or TJA-IRIS 1000) following dry ashing and HCl dissolution of the ash (Miller, 1998). Lignin, cellulose, and ash content were determined by the method of Goering and Van Soest (1970) (Table 1).

Because initial C:N ratios for the 4 amendments ranged between 12:1 (crimson clover) and 300:1 (primary papermill residue), NH_4NO_3 was utilized to

Table 1. Selected chemical characteristics of amendments[a]

Amendment	C:N	carbon	nitrogen	lignin	cellulose	ash
				(%)		
Crimson Clover	12	43.7 (0.02)	3.65 (0.02)	4.19 (0.13)	17.5 (0.21)	0.15 (0.07)
Wheat Straw	100	44.0 (0.04)	0.44 (0.00)	5.13 (0.07)	33.5 (0.42)	2.75 (0.16)
PPR+SS[b]	63	34.5 (0.11)	0.55 (0.00)	8.37 (0.98)	43.7 (0.28)	10.43 (1.12)
PPR[c]	303	33.3 (0.04)	0.11 (0.00)	4.33 (1.20)	63.7 (0.49)	14.03 (1.12)

	Ca	Mg	K	P	Al
Amendment			mg kg^{-1}		
Crimson Clover	12850 (71)	3210 (14)	18500 (0)	1885 (0)	243 (12)
Wheat Straw	1900 (57)	994 (37)	9380 (540)	249 (11)	50 (2)
PPR+SS[b]	57450 (490)	1600 (0)	731 (27)	2025 (7)	23250 (500)
PPR[c]	1035 (21)	968 (3)	307 (13)	247 (3)	34300 (140)

[a]values presented are mean values for two replicates ± (one standard deviation)

[b]Primary Papermill Residue + Secondary Sludge

[c]Primary Papermill Residue

adjust the C:N ratio to 40:1 for wheat straw and both papermill residues. To address the initial disparity in amendment P and K content, both the wheat straw and the primary papermill residue received KH_2PO_4. This nutrient solution equalized P concentrations (10.1 ± 1.4 mg P g^{-1} substrate C) across all amendments and roughly equalized the K concentration between wheat straw and clover (69.3 ± 22.4 mg K g^{-1} substrate C) and between the primary and secondary papermill residues (8.9 ± 6.6 mg K g^{-1} substrate C). N, P and K were monitored throughout the incubation, and both the primary and secondary papermill residues received 1.77 mL of 0.4 M NH_4NO_3 + 0.2 M KH_2PO_4 solution during the 5th week of the incubation.

Decomposition Incubations

Incubations were carried out in 250 mL polyethylene screw-top bottles. Acid washed silica sand (80 g) was mixed with 2.55 g of soil collected at the Rogers experimental farm plus amendment at a concentration of 2.0 g C incubation $bottle^{-1}$. Mixed sand, amendment and soil were moistened to 20% by weight with either D.I. H_20 or, when necessary, D.I. H_20 mixed with the appropriate nutrient solution. Bottles were capped, shaken to ensure thorough mixing and maintained loosely covered in a 22 °C incubator for the duration of the experiment. Bottles were removed from the incubator, weighed every 2-3 days, and remoistened if necessary. There were 10 bottles, i.e. 2 replicates of 4 amendment plus two blanks (sand plus soil with no amendment), with the initial extraction (time 0) involving 10 separately prepared bottles that were destructively sampled. This method allowed for the quantification of initially available DOC without necessitating its removal from the bottles used in the 8 week incubation.

Sampling

At days 7, 14, 28, 42, and 56, bottles were removed from the incubator and prepared for extraction by the addition of 125 mL D.I. H_2O. Bottles were shaken for 30 minutes on a wrist action shaker, allowed to stand for 30 minutes, and centrifuged for 30 minutes at 3600 min^{-1}. Supernatant was vacuum filtered (90 kPa) through Nylaflo 0.2 μm nylon membrane filters. Material collected on the filters was removed and returned to the incubation bottles. To return the bottles to proper weight/moisture conditions following sampling, they were left uncapped in the incubator for 12-16 hours. If post-sampling moisture content was greater than 15% (by weight) above pre-sampling bottle weight, bottles were placed in a 35 °C convection oven to speed the drying process. Time in the oven never exceeded 4 hours and was restricted mainly to the primary paper mill residue. Bottles for the time 0 extraction were prepared as for the 8 week incubation, allowed to stand for 30 minutes and extracted following the same protocol.

Supernatant Analysis

Supernatant was refrigerated immediately upon collection and all analyses were conducted within one week of extraction. Immediate analyses included measuring pH (Ross combination electrode), electrical conductivity (VWR Scientific Model 2052 conductivity meter), and TOC (Shimadzu TOC-500). Carbon analysis was conducted using potassium hydrogen phthalate as the standard. Standard concentrations were 10 and 100 ppm C and standards were run following each extraction interval prior to sample analysis. The mean coefficient of variation for analytical standards over the 6 sampling intervals was 1.1% (± 0.6%) for 100 ppm C

and 16.0% (± 8.9%) for 10 ppm C. A 5 mL aliquot of each aqueous extract was diluted to 3 mM C and absorbance at 285 nm was measured on a Bausch and Lomb Spectronic 2000 spectrophotometer. Deionized-distilled water was used to both calibrate the instrument and to monitor analytical consistency during sample analysis. Instrument drift during analysis was negligible. Cations (Al, Ca, Fe, K, Mg) and P were measured with an ICP-AES (Model 975 Plasma AtomCorp or TJA IRIS 1000), and NH_4^+ and NO_3^- with a Lachat Auto-Analyzer (QC values in Appendix A).

Five 10 mL aliquots of each aqueous extract were pressure filtered (using N_2 gas at 350 kPa) through a 10 mL Diaflo stirred cell fitted with a YM1 1000 MWCO ultrafiltration membrane. Filtration efficiency can be compromised by the over-concentration of the solution retentate, and filtration was deemed operationally complete when 60% of each 10 mL aliquot had passed through the cell. A 10 mL aliquot of both ultrafiltration permeate and the bulk (i.e., not ultrafiltered) extract were subsequently analyzed fluorimetrically (Hitachi F-4500 fluorimeter) to examine aromatic moeities. Analytical preparation involved standardizing each sample in terms of carbon concentration (3 mM), ionic strength (10 mM using KCl) and pH (5.5). Samples containing less than 3 mM C were not analyzed. Excitation-emission matrices (EEM) were generated for each aliquot to allow examination of aromatic peak location and variations both over time and between amendment in peak intensities. Instrumentation parameters were: EX and EM slits, 5 nm; response time, 8 s; and scan speed, 1200 nm min^{-1}.

A 10 mL aliquot of the bulk sample and the ultrafiltration permeate was analyzed for both soluble phenolic acids and soluble hexose sugars. Soluble phenolics

were analyzed using the Folin-Ciocalteu (F-C) reagent with ferulic acid as the standard (Blum et al., 1992). Five mL of each aliquot plus 0.75 mL of 1.9 M Na_2CO_3 and 0.25 mL of the F-C reagent were pipetted into a test tube, shaken, allowed to develop in darkness for 1 hour and analyzed at 750 nm using a Bausch and Lomb Spectronic 2000 spectrophotometer. Soluble sugars were analyzed using the anthrone-sulfuric acid procedure with glucose as the standard (Brink et al., 1960). The anthrone reagent was mixed with reagent grade H_2SO_4 at a concentration of 0.2% (w/v) and allowed to stand in darkness for 1 hour. Ten mL aliquots of this mixture were pipetted into test tubes containing 5 mL aliquots of aqueous extract, shaken on a vortex shaker, allowed to develop in darkness for 30 minutes and analyzed at 625nm on a Bausch and Lomb Spectronic 2000 spectrophotometer. The linear range of the standard curves for both ferulic acid and glucose were between 3 mM and 7 mM C.

Microbial Respiration

In a set-up identical to that used in the decomposition study, 250 mL polyethylene bottles were prepared containing acid-washed sand, amendment, soil, and 20% by weight D.I. H_20 (± nutrient solution as required). Two blanks (sand plus soil plus water without amendment or nutrient solution) were included in the experiment to allow correction for native soil organic matter induced respiration. All polyethylene bottles were placed uncapped within ½ gallon Mason jars and jar tops were covered with Parafilm prior to securing lids to improve sealing quality. Glass vials containing 10 mL of 2M NaOH were suspended within the Mason jars and removed every 3-4 days to determine CO_2 evolved. Remaining OH^- was titrated with 0.5 N HCl in the presence of 1.5 M $BaCl_2$ using phenolphthalein as the indicator

(Zibilske, 1994). The 0.5 N HCl was standardized with Na_2CO_3 (Skoog and West, 1969). During each titration period the polyethylene bottles were weighed, moisture contents were corrected and the bottles were left open to allow O_2 replenishment. Following weekly DOC extraction at the same time intervals used for incubation sampling, bottles were returned to proper weight using the method outlined for the decomposition study. The bottles spent a maximum of 16 hours per week outside of the Mason jar (i.e. with no CO_2 being trapped).

Metal Complexation Capacity and Charge Density

Sample Preparation

Based on an assessment of DOC concentration and extract properties, wheat straw and crimson clover were chosen for an examination of Cu desorption potential of the organic ligands present in the plant residues, both initially and after a 7 day incubation. Initial C:N ratios were corrected in the same manner as during the 8 week incubation, with NH_4NO_3 utilized to lower the C:N ratio for wheat straw to 40:1. Phosphorous and K concentrations were not equalized between amendments due to the short length of the incubation. Dissolved organic carbon (DOC) was collected from both amendments at time 0 following the methodology already outlined. Seven day incubations were carried out in 250 mL polyethylene screw-top bottles following the already defined protocol and DOC was collected from each incubation bottle as described. Aliquots of the supernatant collected at both time 0 and following the 7 day incubation were pressure filtered as described to collect the low molecular weight (<1000 MWCO) fraction. Both the bulk extract and the ultrafiltration permeate were analyzed for cations (K, Mg, Ca, Na), P, and inorganic N as described previously.

Acid-Base Chemistry

Potentiometric titrations were conducted using the method outlined by Ohno and Cronan (1997). Titrations were conducted in a Plexiglas capped, glass reaction vessel maintained at a constant 25.0 $\pm$ 0.1 °C. A Ross combination electrode was standardized at pH 4.0 and 10.0 using buffers held at the same constant temperature. Extracts were diluted to either 10 or 20 mM C in 50 mL total solution volume using CO_2-free D.I. H_2O. Extract ionic strength was standardized at 10 mM using 1.0 M KCl. To further minimize CO_2 contamination extracts were bubbled with a steady stream of N_2. Prior to titration, solutions were brought to pH 3.0 using HCl and allowed to equilibrate for 15 minutes under N_2. Titrations were undertaken with 0.048 M standardized NaOH dispensed in either 0.1 or 0.05 mL aliquots depending on solution C concentration. A 50 mL solution of 0.01M KCl adjusted to pH 3.0 was titrated over the experimental pH range (3.0-11.0) and used as a blank correction. All titrations were conducted in triplicate.

Extracts were titrated between pH 3.0-11.0 to assess total charge density. Carboxyl charge density was defined operationally as that fraction of titratable acidity below pH $\leq$ 7.0. This operational definition is due to the difficulties inherent in recognizing equivalence points for DOC extracts. An average ionization constant was determined for each extract using the method of Albert and Serjeant (1989) and explicitly correcting for titration outside the range of pH 4.0-10.0. A further correction was employed to address the dissociation of H^+ from phosphate over the pH range studied.

Metal Binding Capacity

The copper binding capacity (B_{max}) and pH-dependent conditional stability constant (K_{Cu}) for Cu-DOC were calculated for both bulk extracts and the extract LMW fraction using the equilibrium ion exchange (EIM) method of Luster et al. (1994). A BioRad 50W X-8 200-mesh strong cation exchange resin (H^+ form) was used as per Luster et al. (1996) as a soil proxy. The resin was prepared by slurry packing into a chromatographic column and cleaning with 2 column volumes of 1.0 N HCl. Resin was subsequently rinsed with D.I. H_2O until the addition of $AgNO_3$ no longer precipitated excess Cl^-. Resin was converted to Na^+ form by rinsing with two column volumes of 1.0 N NaOH followed by D.I. H_2O. Prepared resin was flushed from the column with D.I. H_2O and refrigerated in a sealed container. Copper solutions were prepared at 6 concentrations ranging from 3.0 μM to 1.0 mM. Dissolved organic carbon concentration was standardized at 25 ppm C and background solution ionic strength was standardized at 0.01 M using $NaNO_3$.

The experiment was conducted using 60 mL polyethylene screw-top bottles. Sixty mg of Na^+-form resin, appropriately measured aliquots of Cu solution and $NaNO_3$ were weighed into bottles and equilibrated for 1 hour at 21 °C on a table top shaker. Aliquots of DOC were added to bring total solution volume to 30 mL and total C concentration to 25 ppm. Solution pH was standardized at pH 6.0 using either NaOH or HNO_3 and bottles were re-equilibrated on a shaker table in a 4 °C incubator for 24 hours. Following incubation, solutions were filtered (0.2 μm) to separate resin from the filtrate. The supernatant was analyzed for Cu using an ICP-AES (TJA IRIS 1000). A reference isotherm was generated for bottles brought to 30 mL total volume

without the addition of DOC (i.e. resin + Cu solution + $NaNO_3$). This allowed determination of a Cu distribution coefficient (^cD_M) between the resin $(Cu)_R$ and solution $[Cu]_f$ that was employed in calculating the distribution of Cu in the presence of DOC. For the reference isotherm, solution Cu (i.e. $[Cu]_f$) was defined as either the aquoion (Cu^{2+}) or hydroxocomplex ($Cu(OH)_n^{(2-n)}$). For the generation of sample isotherms (i.e., in the presence of DOC), Cu complexation was modeled as a desorption-mediated transfer of Cu from the resin surface to the DOC complex. As it was thus assumed that the concentration of free Cu ($[Cu]_f$) did not change significantly between the reference and the sample isotherms, the concentration of DOC-bound Cu ($[Cu]_b$) was calculated by difference from the ICP-AES data.

Scatchard plots generated for each extract were curvilinear, demonstrating the participation of multiple binding site classes (see Appendix B). Data were subsequently fit to various models to explore both the presence of, and potential interactions between, ligand classes. The best fit was obtained by modeling metal binding as a non-competitive interaction between 2 binding site classes, each exhibiting 1:1 binding geometry. The requirement of non-competition dictates that both the geometry of metal binding and the specific ligand functional group involvement are products of metal concentration. Use of this model was validated by Luster et al. (1996) for Cu binding with DOC extracted from both juniper and chestnut leaf litters. The specification of 1:1 geometry was supported by both Cabaniss et al. (1988) and Buffle et al. (1980). Cabaniss et al. (1988) observed that at pH 5-8 with approximately 1 mM DOC and pCu_{total} = 4.0-7.0, less than 10% of Cu

binding involved multi-site complexes. Buffle et al. (1980) concluded that if DOC concentration was < 3 mM C, CuL_2 (Cu + 2 ligand) binding geometry did not occur.

Statistical Analysis

All values except potentiometric titration data are presented as mean values for two replicates and appear graphically ± 1 standard deviation. Potentiometric titration data are presented as mean values for three replicates ± 1 standard deviation. Statistical tests of significant difference were conducted at $\alpha = 0.05$ and analyzed using the SAS statistical program. Fluorescence EEM matrices were created using Sigma Plot and presented as smoothed mean value plots. Least squares linear regression analysis was used to test for correlation between independent factors. Non-linear curve fitting was used to generate Cu-DOC binding parameters following the model: $\mathbf{Y= (B_{max1}* X)/(K_{d1}+X) + (B_{max2}*X)/(K_{d2}+X)}$. This concentration-dependent binding model was deemed robust when data fitting involved > 10 points. For all extracts except crimson clover $_{\text{bulk-time 0}}$ and wheat straw $_{\text{LMW-week 1}}$, a strong fit was generated ($r^2 > 0.99$) using all 12 data points (i.e. 6 Cu concentrations x 2 replicates). For these two extracts, a strong fit ($r^2 > 0.98$) was generated using the mean value at each Cu concentration. The use of mean values was necessitated by the degree of inter-replicate variance for solution Cu at the highest applied Cu concentration (1 mM Cu). While this concentration was chosen to guarantee weak binding site saturation (i.e., to determine B_{max2}), results suggest that interpretation of B_{max2} may be subject to greater error than the strong binding site saturation (i.e., B_{max1}) data that were determined at a significantly lower Cu concentration.

Chapter V

RESULTS AND DISCUSSION

Electrical conductivity, pH and elemental ion concentrations were measured weekly for each amendment extract (Figure 3, Table 2). Extract pH increased for all amendments between time 0 and week 1 and continued rising to a week 2 incubation maxima for crimson clover, wheat straw and PPR. Crimson clover pH increased as high as 8.7 concomitant with the increase in NH_4^+ concentration (Figure 4). The initially high NH_4^+ and NO_3^- concentrations measured in wheat straw, PPR and PPR+SS extracts resulted from the nutrient addition designed to lower the initial C:N ratios to 40:1. The increase in solution N concentration in both PPR and PPR+SS extracts at week 5 resulted from a further addition of NH_4NO_3. This increase is also evident as a week 5 spike in PPR and PPR+SS extract electrical conductivity. Electrical conductivity in all cases dropped significantly during the first week of incubation. In the crimson clover incubation electrical conductivity did not fall further until after the second week. This plateau may relate to the elevated NH_4^+ concentration.

The dissolved organic carbon (DOC) concentration of each amendment was measured at time 0 (Figure 5). This soluble C pool accounted for 13.5%, 32.7%, 0.5%, and 0.1%, respectively, of wheat straw, crimson clover, PPR, and PPR+SS total substrate carbon. Caine (1982) measured carbon loss from leaves, mosses and sedges and concluded that roughly 30% of total substrate carbon leached from these materials within the first 24 hours of decomposition. Kuiters and Sarink (1986)

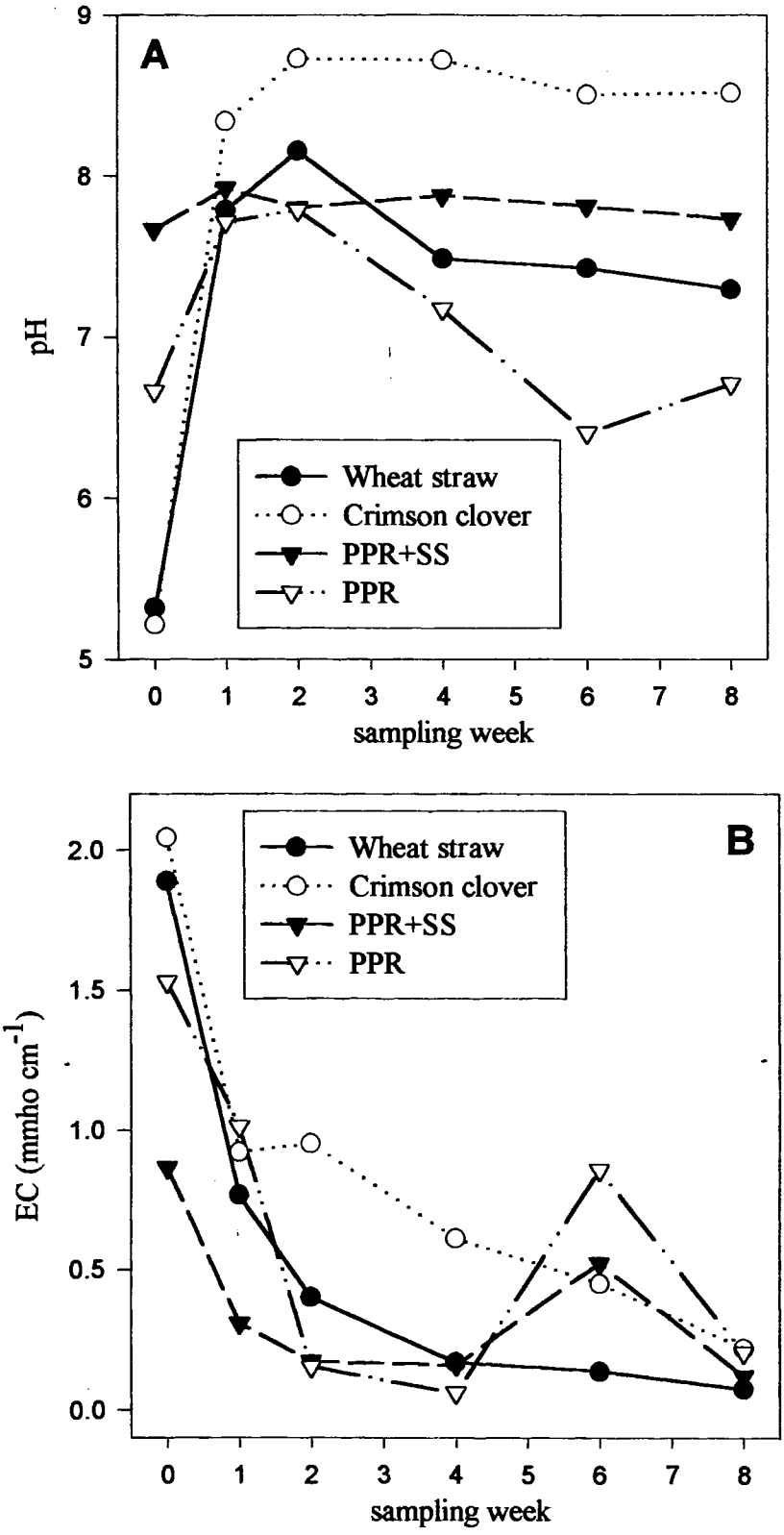

Figure 3. Amendment extract pH (A) and electrical conductivity (B) over an 8 week incubation. All values presented are mean values for two replicates. PPR = Primary Papermill Residue; PPR+SS = Primary Papermill Residue + Secondary Sludge

Table 2. Selected chemical characteristics of amendment DOC extracted over an 8 week incubation[a]

Amendment	Week	Ca	Mg	K	Al	Fe	P	NO_3^--N	NH_4^+-N
		mg/L							
Wheat Straw	0	35.7 (0.99)	24.3 (0.57)	349 (7.07)	3.15 (0.11)	0.72 (0.01)	60.4 (1.63)	103 (0.0)	99.0 (0.85)
	1	12.8 (0.0)	8.06 (0.19)	268 (3.53)	0.68 (0.06)	0.81 (0.03)	13.9 (0.35)	23.6 (4.95)	0.61 (0.06)
	2	4.04 (0.21)	2.23 (0.11)	106 (2.83)	<0.15[b]	0.47 (0.08)	18.9 (0.42)	<0.05[b]	13.3 (2.05)
	4	1.50 (0.09)	0.80 (0.04)	47.5 (0.57)	<0.15	0.40 (0.04)	8.62 (1.26)	<0.05	0.95 (1.34)
	6	0.91 (0.06)	5.90 (2.17)	<1.0[b]	<0.15	0.24 (0.0)	<0.1[b]	<0.05	1.99 (2.82)
	8	0.13 (0.13)	2.42 (0.62)	<1.0	<0.15	0.15 (0.0)	<0.1	<0.05	0.88 (0.62)
Crimson Clover	0	194 (1.41)	76.0 (0.71)	534 (7.07)	3.75 (0.02)	0.33 (0.0)	43.4 (0.64)	1.95 (0.15)	12.7 (0.14)
	1	6.20 (0.16)	12.0 (0.28)	222 (7.07)	0.89 (0.12)	0.92 (0.02)	0.38 (0.11)	<0.05	35.7 (6.36)
	2	4.00 (0.51)	6.51 (0.79)	153 (5.66)	0.54 (0.12)	1.58 (0.11)	3.98 (0.15)	<0.05	77.1 (8.13)
	4	2.27 (0.33)	3.55 (0.28)	61.9 (4.53)	0.25 (0.03)	1.18 (0.02)	5.12 (0.23)	<0.05	67.2 (11.74)
	6	3.16 (0.04)	3.78 (0.03)	<1.0	0.30 (0.02)	0.81 (0.27)	0.50 (0.03)	<0.05	56.1 (9.12)
	8	2.49 (0.45)	1.77 (0.53)	<1.0	0.20 (0.02)	0.25 (0.14)	0.10 (0.07)	<0.05	21.6 (9.40)
PPR[c]	0	8.59 (0.11)	7.03 (0.02)	75.3 (0.42)	<0.15	<0.15[b]	47.6 (0.0)	152 (0.71)	148 (1.41)
	1	1.78 (0.57)	2.90 (0.81)	65.3 (0.71)	<0.15	<0.15	22.7 (1.70)	78.8 (9.97)	97.8 (0.78)
	2	<0.1[b]	<0.1[b]	13.5 (0.71)	<0.15	<0.15	10.7 (0.49)	<0.1	11.9 (2.12)
	4	<0.1	0.61 (0.28)	6.28 (0.13)	<0.15	<0.15	3.35 (0.55)	<0.1	<0.1[b]
	6[e]	3.40 (0.04)	45.6 (1.06)	<1.0	<0.15	<0.15	<0.1	73.9 (0.57)	67.6 (0.28)
	8	0.79 (0.04)	12.2 (0.42)	<1.0	<0.15	<0.15	<0.1	13.7 (0.64)	12.6 (0.28)
PPR+SS[d]	0	50.6 (4.17)	7.33 (0.66)	2.25 (0.28)	<0.15	<0.15	0.12 (0.01)	60.5 (0.14)	58.3 (0.92)
	1	36.2 (8.41)	5.57 (0.78)	<1.0	<0.15	<0.15	<0.1	0.17 (0.07)	3.29 (4.64)
	2	24.1 (0.21)	1.53 (0.13)	<1.0	<0.15	<0.15	<0.1	<0.1	<0.1
	4	21.5 (1.27)	1.26 (0.13)	<1.0	<0.15	<0.15	<0.1	<0.1	<0.1
	6[d]	8.78 (2.72)	11.7 (0.14)	<1.0	<0.15	<0.15	<0.1	3.40 (0.04)	32.2 (2.97)
	8	9.95 (1.20)	2.77 (0.22)	<1.0	<0.15	<0.15	<0.1	0.79 (0.04)	<0.1

[a]values reported ± (one standard deviation)

[b]instrumental detection limit

[c]primary papermill residue

[d]primary papermill residue + secondary sludge

[e]PPR and PPR+SS received KH_2PO_4 + NH_4NO_3 solution at week 5

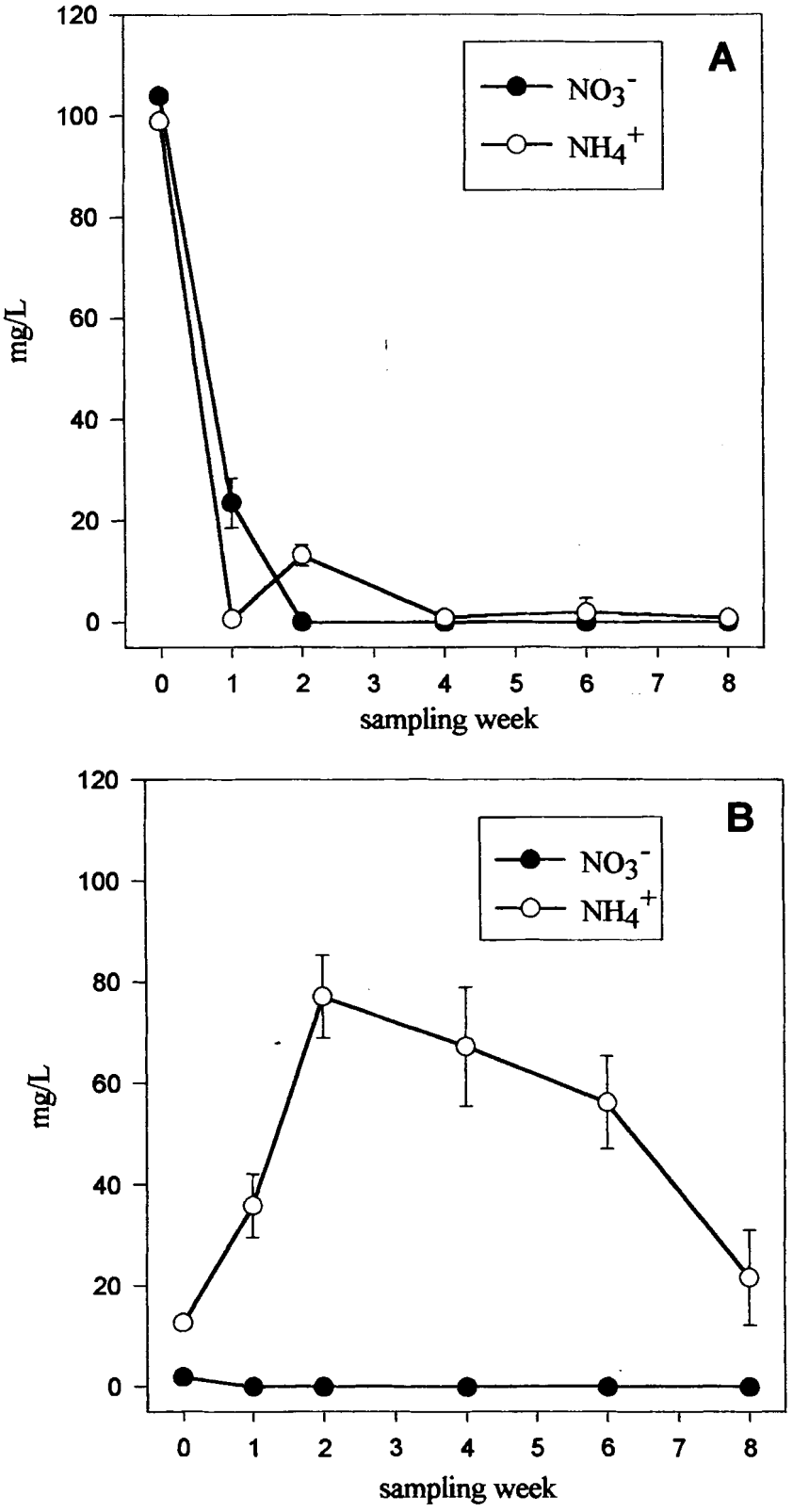

Figure 4. Inorganic N measured over an 8 week incubation. Values presented are mean values for two replicates. Error bars represent one standard deviation. A) Wheat straw; B) Crimson clover

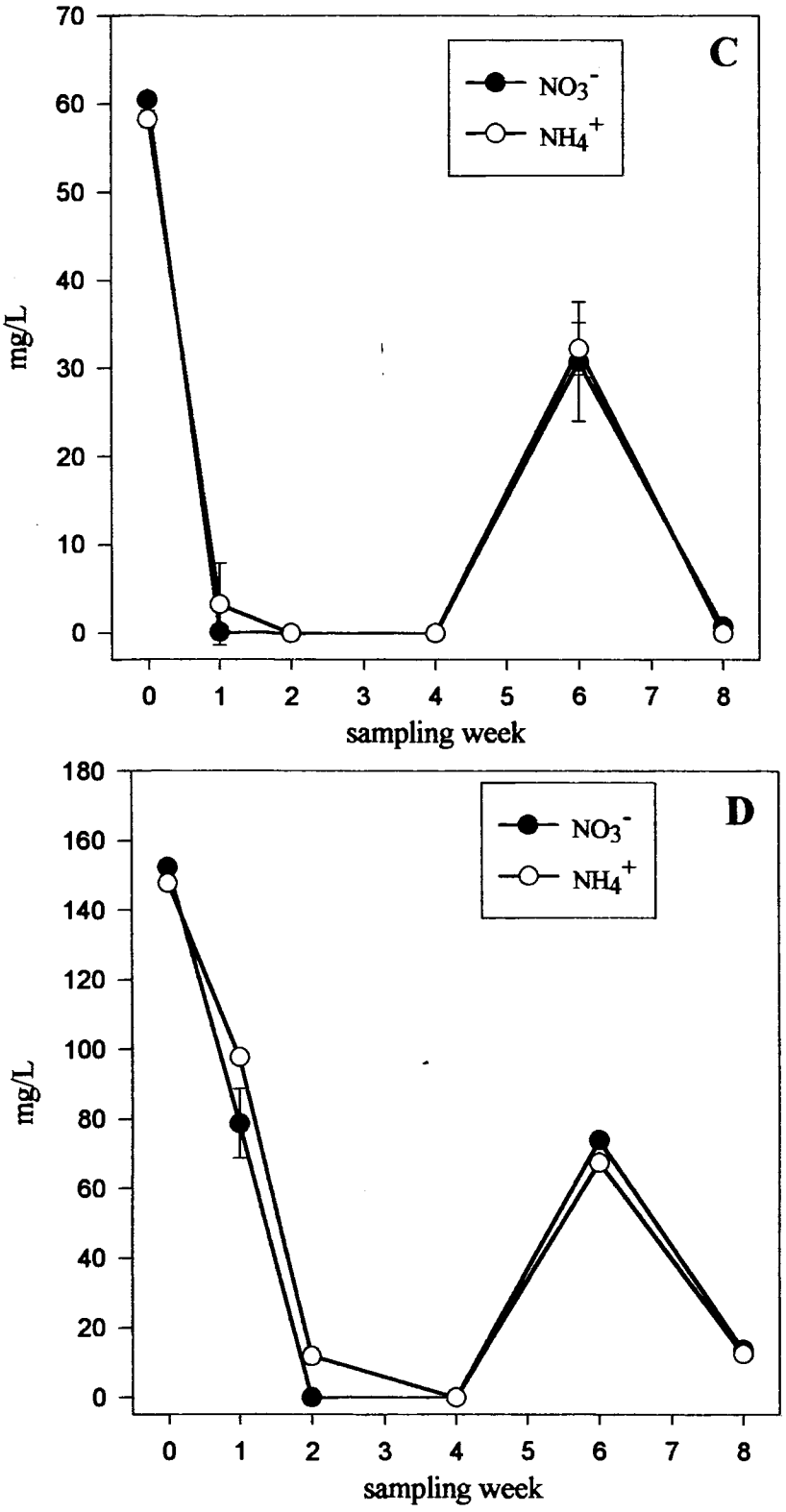

Figure 4 (cont.). Inorganic N measured over an 8 week incubation. Values presented are mean values for two replicates. Error bars represent one standard deviation. C) Primary Papermill Residue + Secondary Sludge; D) Primary Papermill Residue

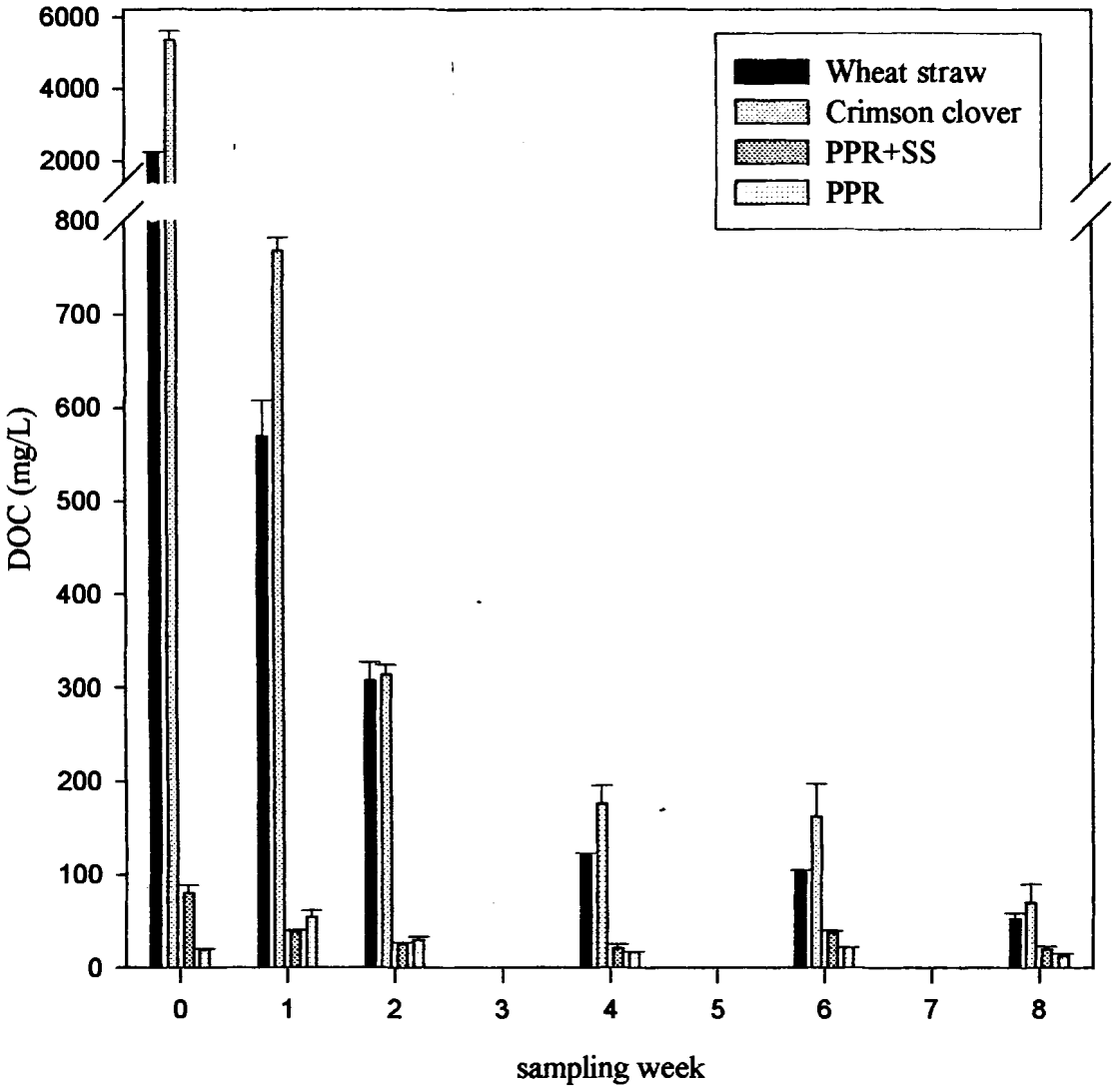

Figure 5. DOC concentration of amendment extracts over an 8 week incubation. Error bars represent one standard deviation. PPR = Primary Papermill Residue; PPR+SS = Primary Papermill Residue + Secondary Sludge

observed that up to 40% dry weight of leaves and needles was leached within 4 months of litter fall. This soluble carbon pool represents the materials that are readily available for microbial utilization, and its composition was investigated through the use of colorimetric reagents. The Folin-Ciocalteu-reactive soluble phenolic acid (FSP) concentration was determined using ferulic acid as the reference. If total ferulic acid equivalents (FAE) are expressed in terms of ferulic acid C, their contribution to total extract DOC can be calculated. While this approach makes correlation imprecise between experiments using different standards, it allows for an internally consistent assessment of FSP. Calculated in this manner, initial time 0 FSP-C ranged between 1.4% and 6.8% of DOC for PPR and crimson clover, respectively (Figure 6). These values are generally consistent with published values for a range of plant materials. Martens (2000) employed chromatographic techniques to analyze the phenolic acid concentration of agricultural crop residues and found that the total phenolic acid concentrations ranged from 0.08% for red clover to 3.8% for corn. Sakala et al. (2000), using the Folin method with tannic acid as the standard, calculated total polyphenol contents of 0.5% and 1.5% for maize stover and green pigeonpea leaves, respectively.

Anthrone-reactive soluble hexose sugars (ARS) were determined using glucose as the reference. Calculated as a percentage of DOC, ARS accounted for 26.8% and 17.4% of time 0 wheat straw and crimson clover extracts, respectively (Figure 7). When calculated in terms of total amendment C, ARS represented 3.0% of total wheat straw and 4.7% of total crimson clover substrate. McDowell (1985) concluded that 12.7% of a collected leaf litter leachate was comprised of monomeric

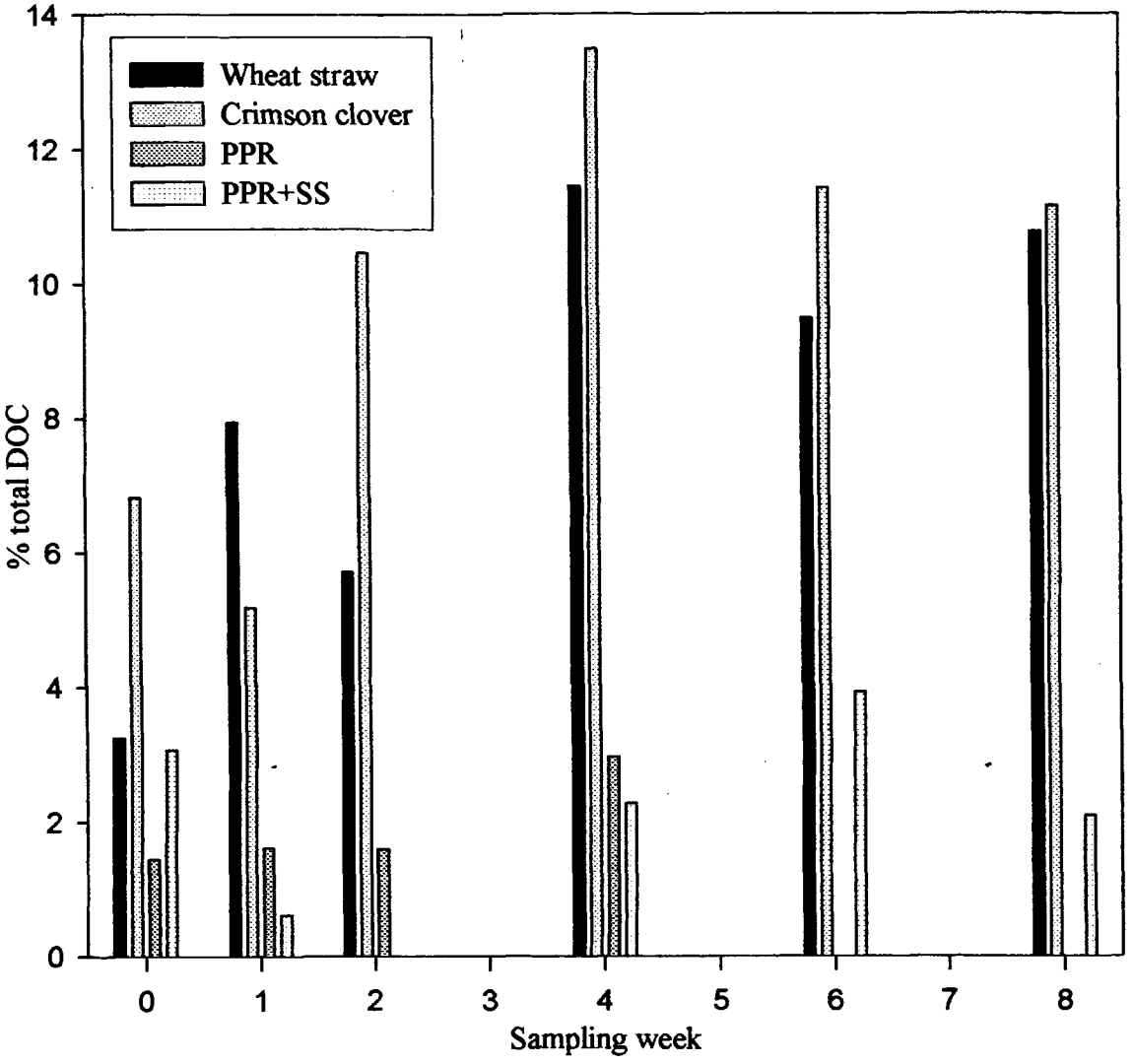

Figure 6. Folin-Ciocalteu reactive soluble phenolic acids expressed as a percentage of DOC. PPR= Primary Papermill Residue; PPR+SS = Primary Papermill Residue + Secondary Sludge

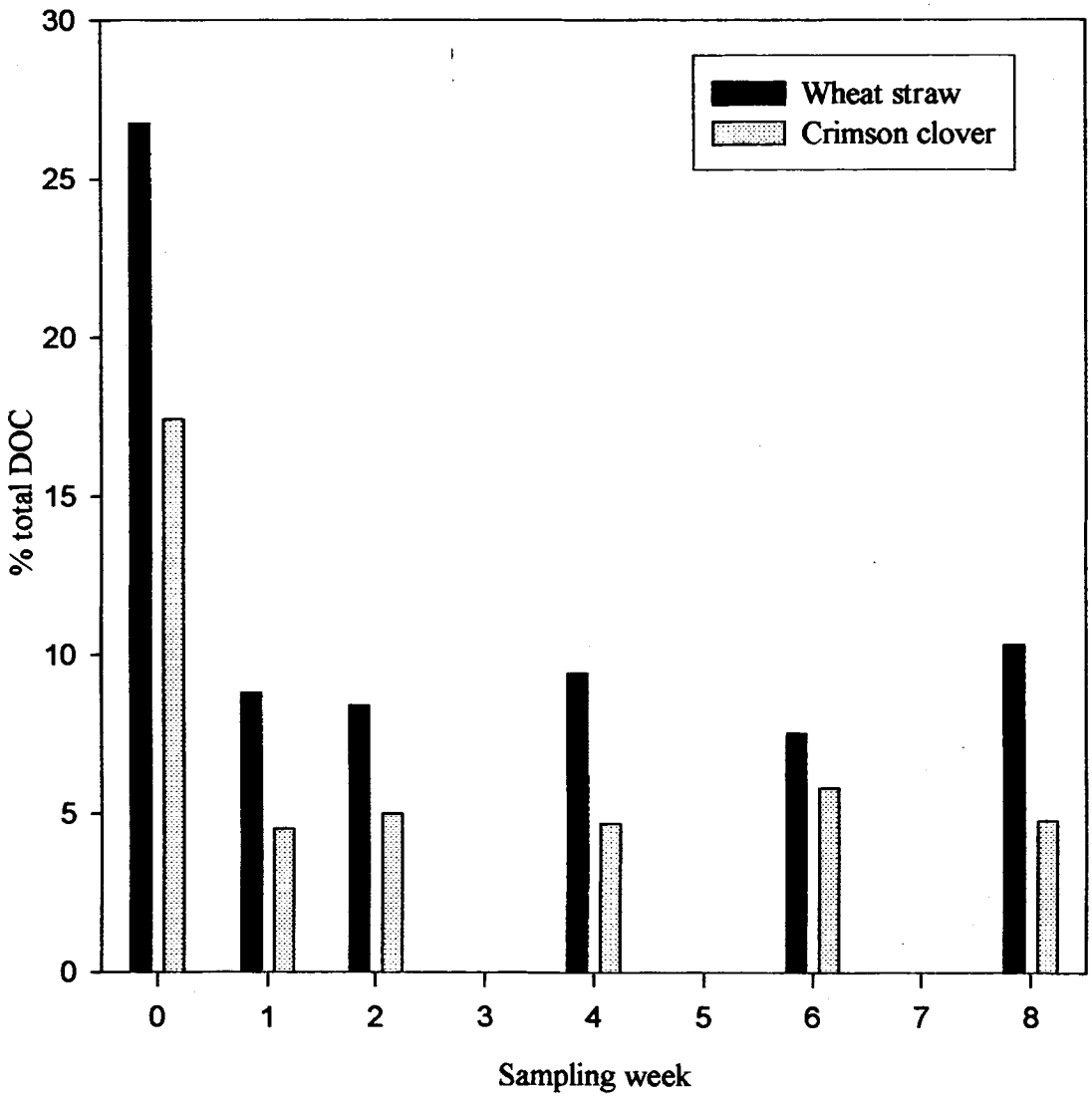

Figure 7. Anthrone-reactive soluble hexose sugars expressed as a percentage of DOC.

carbohydrates. Martens (2000) chromatographically analyzed the carbohydrate concentration of agricultural crop residues and found it to range from 4.7% (red clover) to 17.4% (canola). As the anthrone reagent is adversely affected by high nitrate concentration no time 0 ARS measure was made for PPR or PPR+SS extracts.

Gressel et al. (1995) characterized the decomposition of OM as an evolution of DOC chemistry that occurred as a function of decomposition. The evolution of both FSP and ARS were thus examined to explore the changing nature of specific DOC fractions. FSP, when examined over the length of the incubation, generally increased as a percentage of DOC for both wheat straw and crimson clover. In each case maximum values were reached at week 4 (11.5% and 13.5% of extract DOC, for wheat straw and crimson clover, respectively), falling to 10.8% and 11.2%, respectively, by the final week of the experiment. FSP concentration did not exceed 4% for either PPR or PPR+SS and was no longer detectable in PPR extracts by week 4. Both Blum et al. (1991) and Cilliers et al. (1990) have concluded that as the F-C reagent also reacts with hydroxyl substituted amino and nucleic acids, it overestimates the true concentration of phenolic acids by an approximate factor of 13. If values here are recalculated in light of this correction, initial time 0 soluble phenolic acid concentrations decline to <0.5% of extract DOC for wheat straw and crimson clover, respectively. Maximum soluble phenolic acid concentrations (i.e. calculated at week 4) are <1.0% for each amendment. Utilizing this correction, the soluble phenolic acid content of PPR and PPR+SS is indistinguishable from 0% at every sampling interval.

Over the course of the incubation, ARS remained a measurable percentage of both wheat straw and crimson clover DOC. Wheat straw ARS decreased to <9% of extract DOC after 1 week, and remained between 7-10% of extract DOC throughout the remainder of the incubation. Crimson clover ARS decreased to 5% of extract DOC after 1 week, and remained between 4-6% of extract DOC for the duration of the incubation. ARS values for PPR and PPR+SS DOC extracts were indistinguishable from 0% for weeks 1-8 of the incubation. The values for ARS are generally consistent with those calculated by Aoyama (1996) in a 28 day incubation of rapeseed meal, orchard grass shoots, and rice straw. Using size exclusion chromatography this researcher isolated a DOC fraction containing carbohydrates and peptides. This fraction initially represented between 2 and 6% of extract DOC in all incubations and was still present at day 28, representing between 1.0 and 3.5% of DOC.

Both Collins (1990) and Eijsackers et al. (1990) placed such compositional percentages into wider context by defining decomposition as progressing from water soluble components to structural carbohydrates to lignin. Eijsackers et al. (1990) calculated predicted breakdown rates for major leaf litter components, focusing on sugars, phenols, and cellulose. They concluded that these components represented 15%, 5%, and 20% of leaf litter, respectively, and that over 1 year would be 99%, 10%, and 75% degraded, respectively. Collins (1990) specifically differentiated between structural and non-structural carbohydrates (NSC), defining the majority of NSC as fructose polymers with terminal glucose groups. While the majority of NSC are water soluble, the researchers found that <35% of the NSC present in wheat residues were released during a single cold-water extraction.

Reinertsen et al. (1984) concluded that the rate of plant material degradation was dependent initially on the size of the soluble C pool plus the presence of a simultaneously degrading "intermediately-available pool." This pool was defined as containing polysaccharides and/or oligosaccharides linked in slowly-degrading polymers. The researchers believed that as the soluble C pool was exhausted, these materials gained an increasing relative significance as a source of available carbon. Other research has concluded that this microbially accessible fraction of plant residue is to some degree persistent in soils. Sikora et al. (1990) conducted a hot H_20 extraction of soil TOC and concluded that the anthrone-reactive portion (12.4 mg C kg^{-1} soil) represented 10% of soil solution carbon. Qualls et al. (1992) analyzed a commercial fulvic acid and found it to contain 16% carbohydrate. Vance and David (1991) reported that monosaccharides represented between 3.4-4.4% of forest floor DOC. Guggenberger and Zech (1994b) concluded that carbohydrates represented a significant percentage of soil solution hydrophobic (i.e. humic) acids and that the persistence of these otherwise labile materials was explained by the formation of ligno-carbohydrate polymers. The slow degradation of polysaccharide-containing complexes is a plausible explanation for the measurable ARS content of wheat straw and crimson clover DOC throughout this incubation.

Total respired C equaled 60 mg CO_2-C g^{-1} C for PPR, 70 mg CO_2-C g^{-1} C for PPR+SS, 159 mg CO_2-C g^{-1} C for wheat straw, and 214 mg CO_2-C g^{-1} C for crimson clover (Figure 8). Over a 57 day incubation, Martens (2000) calculated cumulative CO_2-C evolution of between 100-200 mg C for prairie grass and oats, respectively, with a 175 mg C evolution for clover. Collins et al. (1990) conducted a 30 day

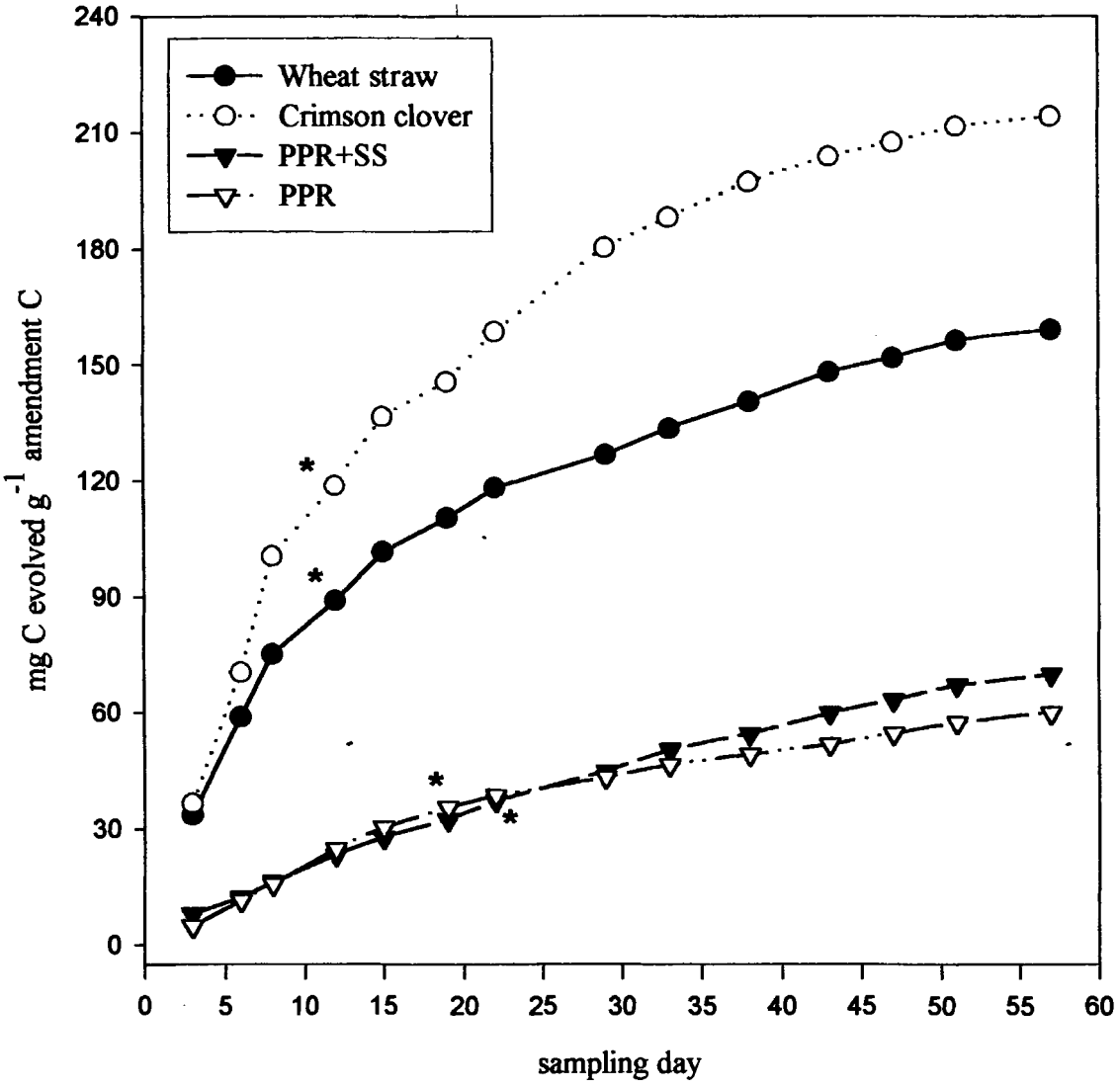

Figure 8. Cumulative CO_2 respired over an 8 week incubation. **(*)** represents the point at which 50% total C respired was reached for each amendment. PPR = Primary Papermill Residue; PPR+SS = Primary Papermill Residue + Secondary Sludge

incubation and concluded that total respired C was greater for wheat leaf blade (325 mg CO_2-C g^{-1} leaf) then for wheat chaff or wheat stem (225 mg CO_2-C g^{-1} chaff or stem). Bremer et al. (1991) studied the degradability of wheat straw, lentil straw, and lentil green manure over a 98 day incubation. CO_2 respired ranged from 250 mg CO_2-C g^{-1} residue for both lentil straw and wheat straw to 275 mg CO_2-C g^{-1} residue for lentil green manure. Sakala et al. (2000) incubated senesced pigeonpea, green pigeonpea, and N-amended maize stover for 60 days and calculated a cumulative CO_2 release of 332 mg C g^{-1} C, 376 mg C g^{-1} C and 407 mg C g^{-1} C, respectively. Factors responsible for variation between experiments include the C:N of bulk substrate, the DOC:TDN ratio of the water-soluble fractions, the size and composition of the labile and intermediately available C pools and amendment:soil ratios that may vary by several orders of magnitude.

Watkins et al. (1996) modeled decomposition as a two-phase process, observing that CO_2 respiration experiments frequently document an initially rapid rate with up to 70% of the labile DOC utilized, followed by decreasing activity during the degradation of more recalcitrant components. The researchers defined the rapid phase as involving the degradation/utilization of free amino acids, amino sugars, carbohydrates, and both cell membrane and cytoplasmic constituents. The slower phase corresponded to both the degradation of more recalcitrant primary components and decomposition of the secondary, stabilized products produced during the first phase. Ajwa and Tabatabai (1994) reported that total CO_2 evolution in a 30 day incubation ranged from 27% of corn residue C to 58% of alfalfa residue C and that greater then 50% of total CO_2 evolution occurred within the first 6 days of the

incubation. Martens (2000) observed that for lentil green manure > 50% of initial substrate C was respired within the first 14 days of a 98 day incubation. Sakala et al. (2000) concluded that CO_2 evolution was the most rapid during the first 10 days of their incubation and that 25%-30% of residue total C had been respired by the end of the experiment. These values can be compared with the present incubation in which, following a 57 day incubation, 16% of wheat straw, 22% of crimson clover, 8% of PPR+SS and 7% of PPR total C had been respired. The >50% evolution was reached in 12 days for wheat straw and crimson clover, 19 days for PPR, and 22 days for PPR+SS.

As is clear from these figures, the size of the initial C pool and the absolute concentration of both CO_2-C and sequentially extracted DOC varied considerably between amendments. It was assumed that microbes initially utilized the most labile/water soluble plant components and a measure of CO_2 respired allowed examination of the efficiency of utilization of this pool. Weekly extraction of DOC explored the progressive solubilization that occurred as this pool was consumed as a source of substrate. These two processes, when taken together, accounted for >100% of the initially available DOC pool for wheat straw, PPR and PPR+SS, and roughly 90% of the initial pool for crimson clover. It appears thus that only in the case of crimson clover was the initial DOC pool (week 0) larger than the total extracted C pool (weeks 1-8). It must be recognized, however, that without the use of radio-isotope labeling techniques it is difficult to clearly define the fractionation of DOC between respiration, incorporation into substrate, and mobilization or leaching loss. In

terms of total substrate C, total respired + solubilized C accounted for 23%, 30%, 8%, and 7%, respectively, of wheat straw, crimson clover, PPR and PPR+SS substrate.

Reinertsen et al. (1984) examined wheat straw degradation under three different N regimes and observed that though the initial C:N ratio of each mixture was adjusted to 10:1, the leachate C:N varied from 8:1 to 63:1 and increased with the increasing concentration of fertilizer added to standardize the bulk C:N ratio. Leachate N was measured as inorganic N, assuming explicitly that loss of both dissolved organic nitrogen (DON) and gaseous N species was insignificant. The authors concluded (1) that no additional source of N was required during microbial utilization of substrates with a 10:1 C:N leachate ratio and (2) that unless bulk C:N was > 40:1, the absence of an external N supply did not necessarily hinder plant residue decomposition. In the current experiment, crimson clover had an initial bulk C:N of 12:1 with an initial leachate C:N of 365:1 (Figure 9). Following this initially high value for leachate C:N, the ratio fell to 22:1 in the first week and was < 4:1 for each subsequent week of the experiment. Interestingly, though the leachate C:N at week 2 was only 4:1, the majority of N was present as NH_4^+. While this high concentration of NH_4^+ further hints at the suppression of N mineralization, neither the evolution profile of DOC during the 8 week incubation nor CO_2 respired data strongly support this suggestion. Furthermore, it must be recognized that as the leachate C:N ratio specifically excludes organic N, this measure may only poorly define the decomposition potential of N-rich amendments like crimson clover. For wheat straw, the leachate C:N ratio increased initially from its time 0 value (11:1) to a week 4 maximum (125:1), before decreasing to 58:1 by the end of the incubation. Though extract DOC concentration

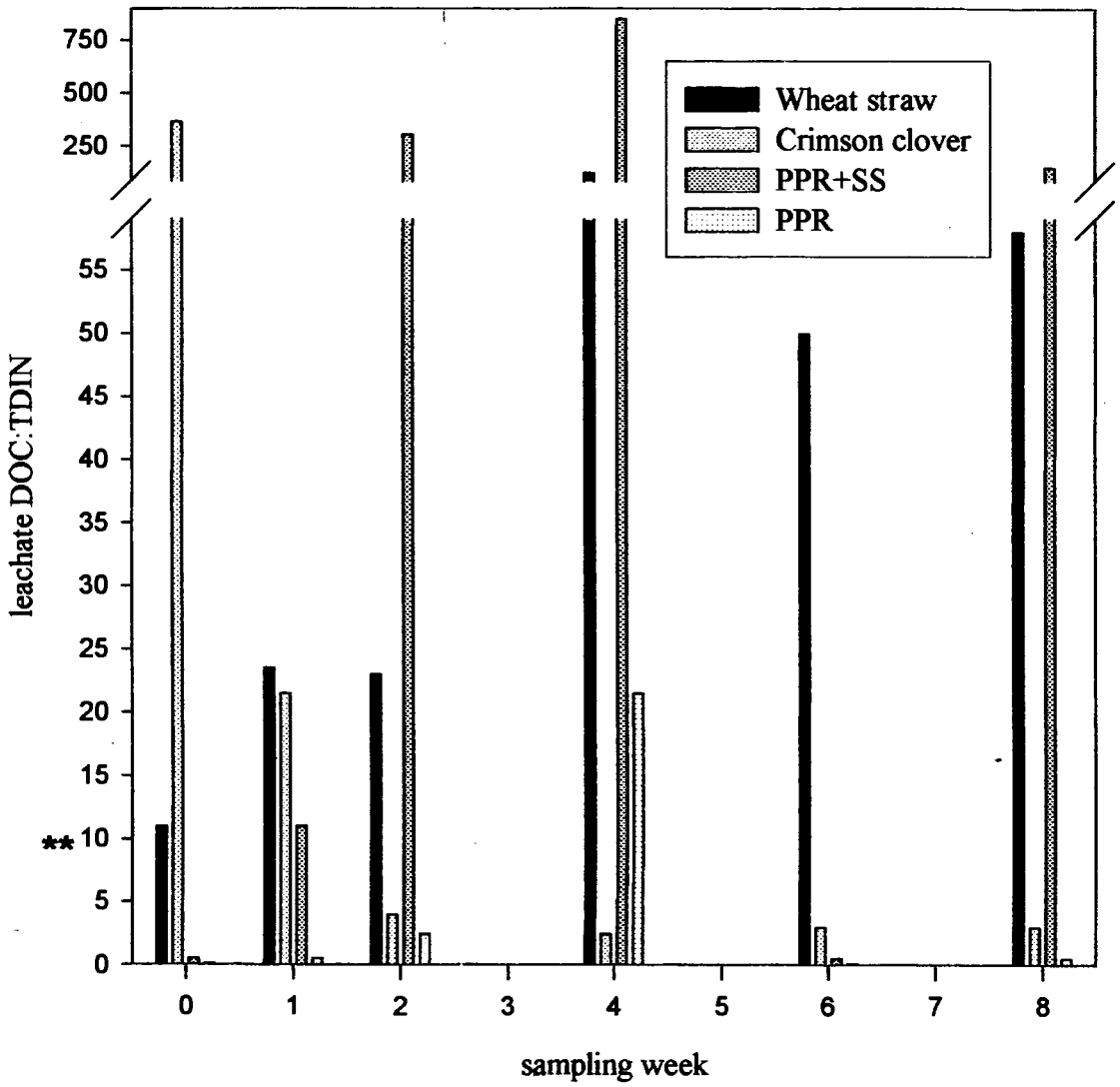

Figure 9. Extract DOC:TDIN (total dissolved inorganic N) over an 8 week incubation. (**) corresponds to the DOC:TDIN ratio above which amendment decomposition is likely hindered by N immobilization (Reinertsen et al., 1984). PPR = Primary Papermill Residue; PPR+SS = Primary Papermill Residue + Secondary Sludge

still exceeded 4 mM at week 8, these high ratios coupled with the relatively shallower CO_2 evolution profile and the lack of a significant organic N pool indicate that N limitation could have hindered wheat straw decomposition as the incubation progressed.

The papermill residues, in contrast, were pre-processed materials in which both the labile C and indigenous N pools had likely been depleted. Initial bulk C:N was standardized at 40:1 for both PPR and PPR+SS, creating an initial leachate C:N of 0.7:1 and 0.1:1, respectively. In each case the low initial ratio was due to the small quantity of readily accessible DOC relative to the high concentration of added NH_4NO_3. While the addition of fertilizer may have initially stimulated the decomposition of the PPR (as DOC concentration increased during the first week of the incubation), DOC concentration never exceeded 5 mM. For PPR+SS the highest DOC concentration, approximately 7 mM, occurred at time 0. Evolution of the leachate C:N ratio mirrored the addition schedule of fertilizer, i.e. <1:1 at time 0 and week 6, and increasing to as high as 854:1 at week 4. When compared with crimson clover and wheat straw, the CO_2 and DOC extraction profiles were markedly different for these products, a contrast that likely resulted from a combination of N immobilization and the absence of a labile C pool.

Qualls et al. (1992) observed that an increasing DOC:TDN ratio over an incubation suggested either (1) enzymatic cleavage of N-containing functional groups from the remainder of soluble molecules and/or (2) selective decomposition of entire N-rich molecules. Mary et al. (1996) concluded that the effect of N availability on the decomposition rate of residues was not described by a simple, linear function and

instead depended on the relative stage of decomposition of the residue. These researchers observed that while N addition increased the decomposition rate of the initial, soluble fraction (though the C:N ratio of that fraction might already be quite low), N addition at a later-stage likely impeded the degradation of more recalcitrant components. While ratios including N:polyphenol and N:lignin have been utilized in predictive models, it has proven difficult to model the decomposition kinetics of an amendment in terms of either its N or lignin content. In the current experiment, the lignin content of the PPR+SS was significantly higher than in the plant residues (8.4% versus 5.1% and 4.2% for wheat straw and crimson clover, respectively), though there was little difference in the lignin content of the plant residues and PPR (4.3%) (Table 1). Both PPR and PPR+SS, however, contained a significant percentage of cellulose (63.7% and 43.7%, respectively), suggesting that residue decomposition could have been hindered by the absence of a degrader community able to affect its breakdown. Furthermore, cellulose content differed widely between the plant residues (17.5% versus 33.5% for crimson clover and wheat straw, respectively), and could help explain the relatively higher CO_2 respiration rate of the crimson clover incubation.

To assess the evolution of DOC humification, various parameters were explored. Spectrophotometric absorption at 285nm (A_{285}) revealed that time 0 absorbance ranged from 24 to 134 L mol C^{-1} cm^{-1} (Figure 10). PPR displayed the highest and the PPR+SS displayed the lowest initial A_{285} with both crimson clover and wheat straw displaying similar intermediate values. While A_{285} increased for wheat straw, crimson clover, and PPR+SS over the length of the incubation, it declined for PPR. Wheat

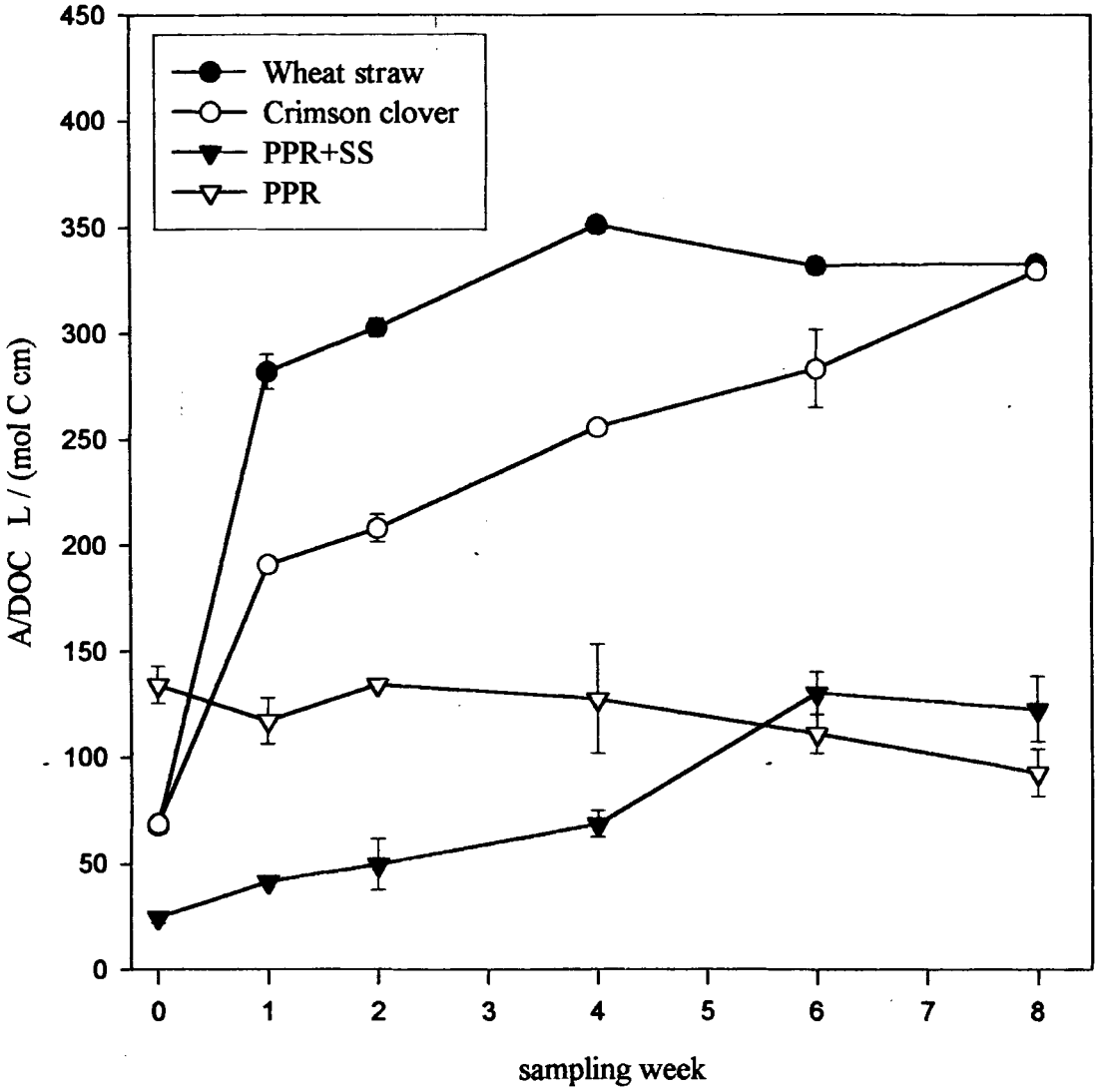

Figure 10. UV spectrophotometric absorbance (defined for a cm cell and per mole C at 285 nm) over an 8 week incubation. Values presented are mean values for two replicates. Error bars represent one standard deviation. PPR = Primary Papermill Residue; PPR+SS = Primary Papermill Residue + Secondary Sludge

straw and crimson clover displayed the greatest overall increases (from approximately 70 L mol C^{-1} cm^{-1} to 330 L mol C^{-1} cm^{-1} over 8 weeks), with the greatest incremental increase for both amendments occurring during the first week of the incubation. A_{285} may be converted to an estimate of percent aromaticity using the regression equation of Chin et al. (1994). When calculated weekly for this 8-week incubation, decomposition resulted in an aromaticity increase of from 10% to 23% for both wheat straw and crimson clover extracts, from 8% to 13% for PPR+SS extracts, and a decrease from 14% to 11% for PPR. The substantial increase in absorption seen for crimson clover and wheat straw within the first week corresponded to an increase in relative aromaticity of from 10% to 16% and 21%, respectively. These values may be compared with published values generated using the same technique. Brunner et al. (1996) calculated a 23% aromaticity (A_{285} =330 L mol C^{-1} cm^{-1}) for an extract of degraded chestnut leaf litter. Traina et al. (1990) calculated percent aromaticity for a marine and a suite of soil humic acids as 20% and 33-43%, respectively. Chin et al. (1994) calculated percent aromaticity for river water, groundwater, and Antarctic lake water fulvic acids (FA) as 23%, 13%, and 13%, respectively (A_{285} = 308 L mol C^{-1} cm^{-1}, 122 L mol C^{-1} cm^{-1}, and 150 L mol C^{-1} cm^{-1}, respectively). Rostan et al. (1995) concluded that when A_{285} exceeded 220 L mol C^{-1} cm^{-1} (18% aromaticity) it signaled the presence of fulvic-like refractory organic compounds.

Absorption results are best analyzed by considering the plant and papermill residues separately. For wheat straw, the rapid A_{285} increase within the first week likely corresponded to the initial degradation of labile, non-humic components such as soluble sugars. With these materials rapidly degrading, A_{285} increased to 282 L

mol C^{-1} cm^{-1} at week 1 followed by continued slow increase through the remainder of the experiment. Regression of increasing A_{285} against decreasing anthrone reactive C/DOC_{total} revealed a poor data distribution (Figure 11a). Regression of A_{285} against FSP-C/ DOC_{total} revealed a likewise poor distribution (Figure 12a). While poor data distribution makes it difficult to draw conclusions regarding the composition of the UV-absorbing DOC pool, it does appear that, following the degradation of the most labile components, wheat straw DOC A_{285} was equivalent to values calculated for riverine and terrestrial fulvic and humic acids. A similar trend was seen for crimson clover, with the increase over time in A_{285} occurring more gradually. This rate contrast may result from factors including a smaller initial pool of carbohydrate C or a smaller initial increase in UV-absorbing aromatic breakdown products. Poorer correlation for crimson clover A_{285} regressed against either ARS-C/DOC_{total} (r^2=0.66) (Figure 11b) or FSP-C/DOC_{total} (r^2=0.46) (Figure 12b) suggested the likely involvement of both factors. By week 2, however, crimson clover A_{285} was > 200 L mol C^{-1} cm^{-1} and was quantitatively similar to riverine and terrestrial fulvic and humic acids.

These results are conceptually consistent with the conclusions of Aoyama (1996) and Gregorich et al. (1996). Aoyama employed size exclusion chromatography to monitor the evolution of plant-derived DOC. He observed that UV-absorbing organic compounds were formed early in the decomposition process and often remained in solution throughout the incubation. In an incubation of rapeseed meal, he further observed that in as few as 3 days a chromatographic peak signaling the presence of water-soluble humic substances appeared. Though the evolution of the humic peak

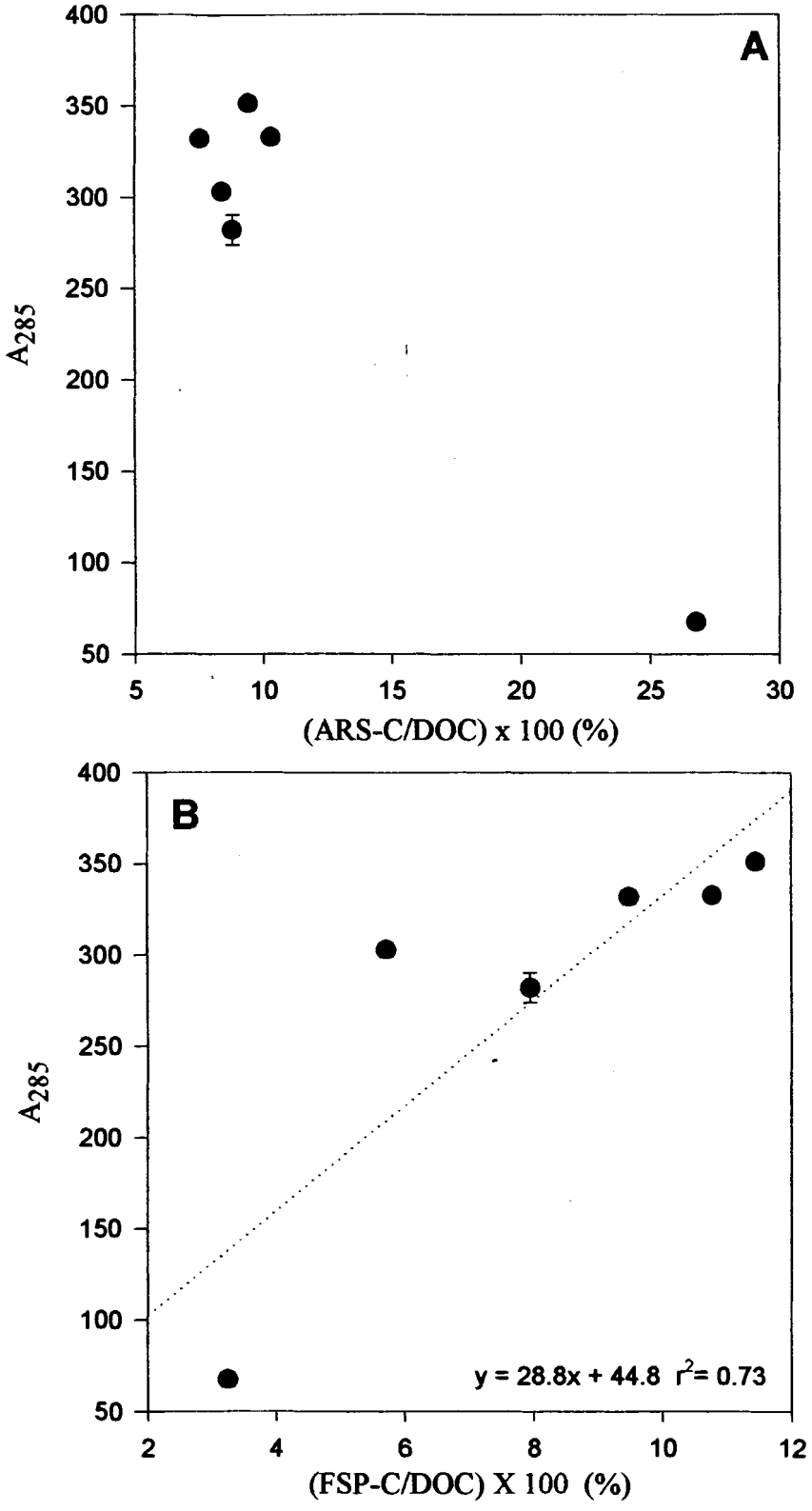

Figure 11. Anthrone reactive soluble sugars versus molar absorptivity at 285 nm. Values presented are mean values for two replicates. Error bars represent one standard deviation. A) Wheat straw B) Crimson clover

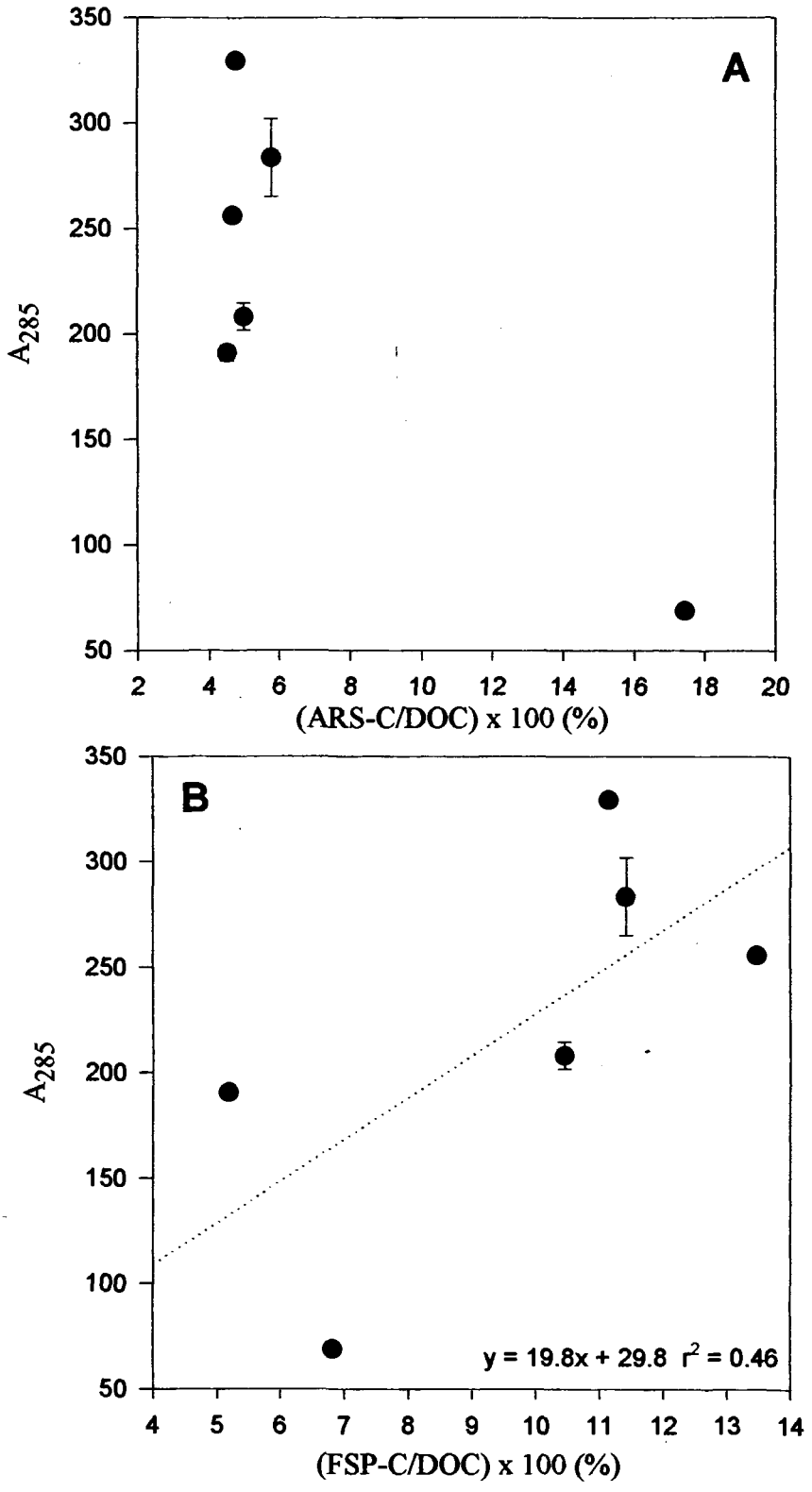

Figure 12. Folin-Ciocalteu reactive soluble phenolic acids versus molar absorptivity at 285 nm. Values presented are mean values for two replicates. Error bars represent one standard deviation. A) Wheat straw B) Crimson clover

was inconsistent between materials incubated, its signature increased throughout the experiment for all materials studied. Gregorich et al. (1996) analyzed maize leaves and the light fraction (LF) of soils previously amended with maize residues under the hypothesis that the LF represented maize residues that had begun the humification process. While Py-FIMS analyses revealed significant percentages of carbohydrates, lignin monomers and phenols in both fractions, both the C:N and amino:total N ratios decreased from fresh leaf to LF (56:1 versus 22:1 and 0.56:1 versus 0.43:1, respectively). Relative aromaticity, determined using ^{13}C NMR and considered a measure of material recalcitrance, increased from 11% in maize leaf to 17% in the LF. Correspondingly, the percentage of total scan intensity corresponded to the o-alkyl/carbohydrate region decreased from 77% in leaf residue to 60% in the LF.

The papermill residues behaved in a distinctly different fashion, with aromaticity remaining at less than 14% throughout the experiment. Aromaticity values increased toward this maximum for PPR+SS and fell from it for PPR. Both residues represent highly processed materials generating low DOC concentrations. For PPR+SS, the addition of sewage sludge likely contributed a small pool of humifying materials and was responsible for the aromaticity progression that occurred. It is also possible that the A_{285} signature was generated by the breakdown products of the limited microbial community able to utilize these materials. Interestingly, though PPR had the highest time 0 A_{285}, it had the lowest concentration of Folin-Ciocalteau reactive soluble phenolic acids (FRP) and contained no aromatic amino acid fluorescence signature. This lack of specific correlation between A_{285} and either aromatic N or FRP suggests that the π-π^* transitions measured likely resulted from the cumulative presence of

condensed aromatic structures rather than the specific existence of simple aromatic moieties.

The low molecular weight (LMW) fraction of residue DOC was examined to determine whether progressive humification resulted in increasing average molecular size. As discussed for the bulk DOC extracts, there were large differences in the absolute concentrations of DOC from which relative percentage data were generated. The DOC concentration of the ultrafiltration membrane permeate (<1000 MWCO) at time 0 accounted for 71%, 62%, 73%, and 30% of the wheat straw, crimson clover, PPR+SS, and PPR bulk extract DOC, respectively. If these data are viewed not in terms of membrane permeate, but in terms of its retentate, a retention percentage (RP) is generated that allows examination of change, over time, in average molecular size. For wheat straw, as example, if the time 0 LMW fraction accounted for 71% of bulk extract DOC, this corresponds to a 29% retention percentage (Figure 13).

For wheat straw, the RP increased significantly over the incubation and by week 8, while the bulk extract still contained 4.4 mM C, the LMW fraction contained no C (i.e. RP =100%). Crimson clover followed a different pattern, with the RP increasing from 38% to 72% by the conclusion of the incubation and demonstrating that, even after 8 weeks of decomposition, a soluble LMW fraction of DOC was still present. For both amendments, the most significant increase in the RP occurred during the first 7 days. Weekly retention percentages for both amendments were regressed against A_{285} to determine whether increasing aromaticity was correlated with increasing average molecular size. As with other regressions involving wheat straw, data distribution was poor (Figure 14a). While data distribution was broader

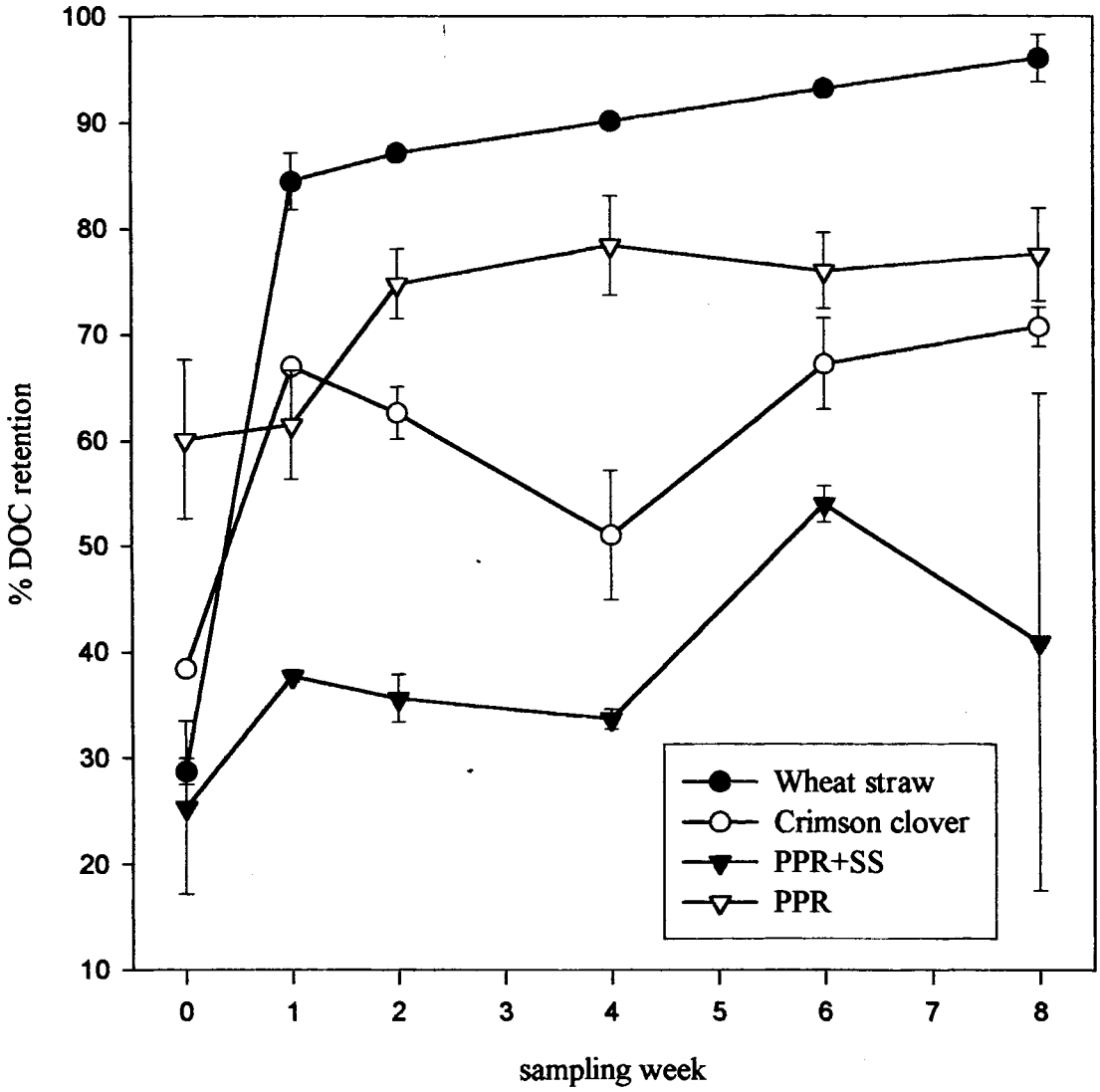

Figure 13. DOC retention percentage over an 8 week incubation. Retention defined as not passing through a 1000 MWCO ultrafiltration membrane. Values presented are mean values for two replicates. Error bars represent one standard deviation.
PPR = Primary Papermill Residue; PPR+SS = Primary Papermill Residue + Secondary Sludge

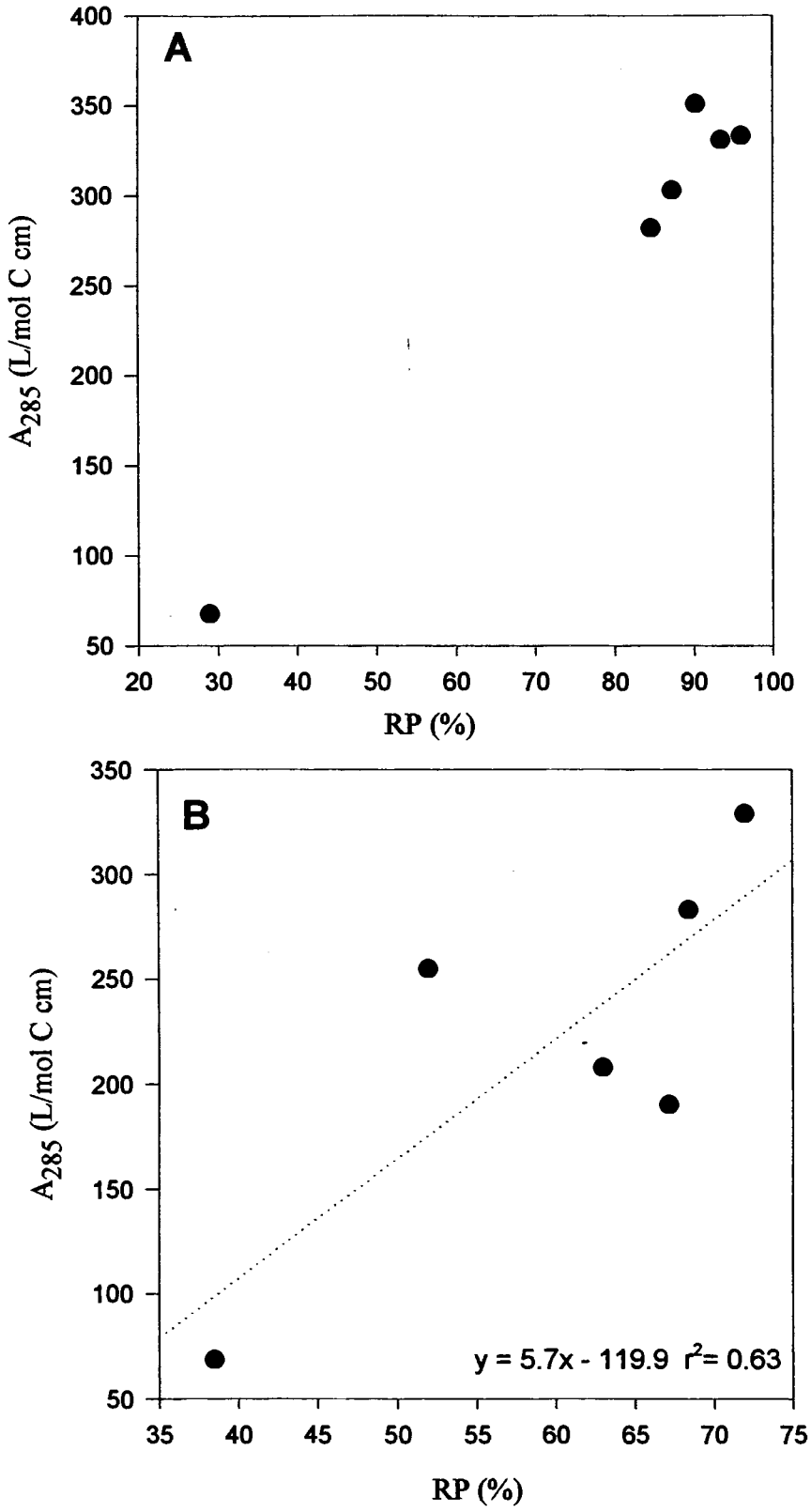

Figure 14. Molar absorptivity at 285 nm versus the retention percentage (RP) of DOC. Retention defined as not passing through a 1000 MWCO ultrafiltration membrane. A) Wheat straw; B) Crimson clover

for crimson clover, the correlation was poor (r^2=0.63) (Figure 14b). This poor correlation suggests that for crimson clover, LMW DOC contributed to aromaticity as measured throughout the incubation. For PPR, the RP increased slowly over the incubation, though the low absolute concentration of bulk and LMW DOC hindered interpretation. The RP for PPR+SS was not significantly different at week 8 than at week 0.

Folin-Ciocalteu reactive soluble phenolic acids (FSP) were likewise analyzed in terms of evolving retention percentages (RP). The initial (time 0) FSP retention percentages for both wheat straw and crimson clover were approximately 30% (Figure 15). For both amendments, RP values increased most significantly during the first week before leveling ultimately at 100% and approximately 90% for wheat straw and crimson clover, respectively. The RP for PPR+SS increased over the first week from approximately 55% to 100% where it remained for the duration of the incubation. While the application of the Blum et al. (1991) correction suggests that the actual phenolic acid concentration of wheat straw and crimson clover DOC was negligible after the first week, the Folin-Ciocalteu reagent clearly continued reacting. Though it is not possible to say with which aromatic compounds, increasing RP values suggest the presence of, and reaction with, higher molecular weight hydroxyl-substituted aromatic moieties. That such compounds are the product of linkages between semi-labile phenolic monomers was explored by Martin and Haider (1976). ^{14}C was utilized to analyze the relative decomposition potential of ferulic acid ring or side chain C versus aliphatic acid or glucose C. Results suggested both that ferulic acid and its demethoxylation products were stabilized in the soil relative to the other

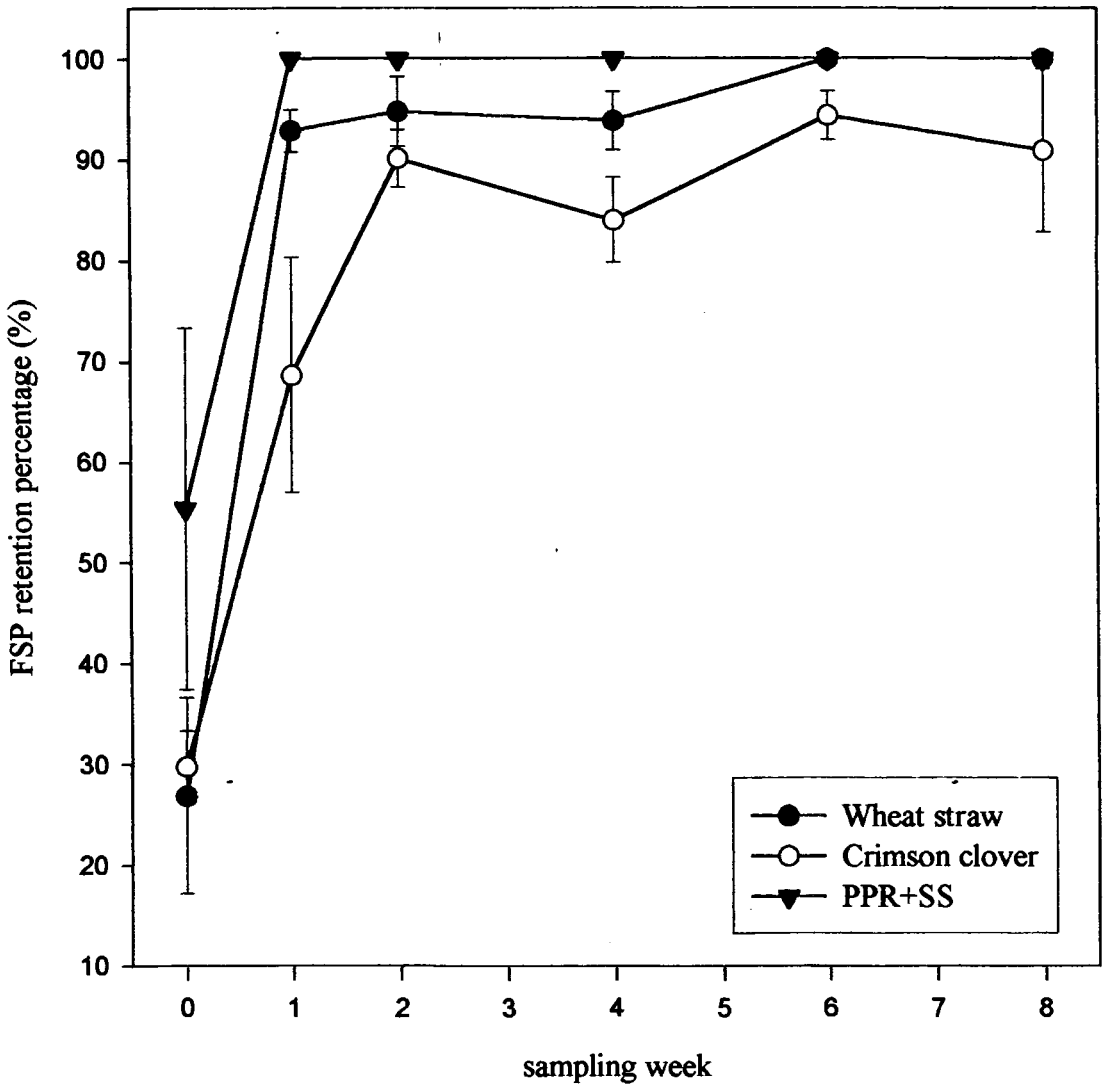

Figure 15. Folin-Ciocalteu reactive soluble phenolic acids (FSP): retention percentage over an 8 week incubation. Retention defined as not passing through a 1000 MWCO ultrafiltration membrane. Value presented are mean values for two replicates. Error bars represent one standard deviation. PPR+SS = Primary Papermill Residue + Secondary Sludge

substrates and that polymerization stabilized significantly more aromatic substrate than adsorption to either clay surfaces or existing soil humus.

Fluorescence scans were employed to further explore the aromatic nature of amendment DOC. Fluorophores were examined that corresponded to peaks defined in the published literature. Fluorophore A ($\lambda_{ex}/\lambda_{em}$ = 270-280/335-350) was present in week 0 scans of wheat straw, crimson clover and PPR+SS DOC, but was absent from PPR DOC (Table 3 and Appendix C). Peak intensity was highest for the crimson clover extract. This peak has been identified in the literature as arising from the aromatic amino acid tryptophan (Coble et al., 1990). Fluorophore B ($\lambda_{ex}/\lambda_{em}$ = 250-260/440-460) was present in time 0 scans of wheat straw, crimson clover, and PPR DOC but was absent from PPR+SS. Peak intensity was highest for PPR, and while this peak has been observed by other authors, it has not been positively identified. There is debate as to how to define the excitation and emission boundaries for fluorophore C. If the broad region $\lambda_{ex}/\lambda_{em}$ = 310-350/435-445 is considered the provenance of a single fluorophore (C) then this peak is present in the DOC extracts of all four amendments. Ohno and Cronan (1997) concluded, however, that fluorophore C was characterized by an $\lambda_{ex} \leq 320$ nm, with an $\lambda_{ex} > 320$ nm signaling the presence of a distinct fluorophore D. This designation is supported by the results of both Ohno and Crannell (1996) and Yang et al. (1994). Ohno and Crannell (1996) contrasted DOC extracted from fresh plant residues and animal manures and observed that λ_{ex} increased from 310 nm (crimson clover and hairy vetch) to 349 nm (poultry and cattle manure) with λ_{em} remaining unchanged (435-450 nm). Yang et al. (1994) analyzed DOC extracted from pine needles and a forest soil O horizon and observed

Table 3. Location and intensity of primary peaks in fluorescence spectra of DOC extracts; bulk extract[a]

a. Wheat straw

	Fluorophore								
	A			B			C-D		
Time (wks)	Excitation λ	Emission λ	I[b]	Excitation λ	Emission λ	I	Excitation λ	Emission λ	I
0	277 (nm)	333 (nm)	121	253	438	200	316	435	333
1		NP[c]		253	450	155	343	438	165
2		NP		256	453	160	340	441	193
4		NP		253	447	141	343	438	166
6		NP		253	441	150	343	435	150
8		NP		259	453	150	343	438	149

b. Crimson clover

	Fluorophore								
	A			B			C-D		
Time (wks)	Excitation λ	Emission λ	I	Excitation λ	Emission λ	I	Excitation λ	Emission λ	I
0	277	348	491	253	441	131	313	444	131
1	280	351	137	253	462	184	343	441	198
2		NP		253	450	187	340	444	168
4		NP		253	444	207	340	438	254
6		NP		256	453	203	340	444	228
8		NP		253	441	196	343	444	251

[a]All scans at 3mM C; 0.01 M IS; pH 5.5

[b]Intensity in arbitrary units

[c]No peak present

Table 3 (cont.). Location and intensity of primary peaks in fluorescence spectra of DOC extracts; bulk extract[a]

c. PPR[c]

	Fluorophore								
	A			B			C-D		
Time (wks)	Excitation λ	Emission λ	I[b]	Excitation λ	Emission λ	I	Excitation λ	Emission λ	I
0		NP[e]		255	447	320	324	443	387
1		NP		256	452	309	327	440	417
2		NP		262	446	356	339	435	556
4		NS[f]			NS			NS	
6		NP		258	450	304	330	435	465
8		NS			NS			NS	

d. PPR+SS[d]

	Fluorophore								
	A			B			C-D		
Time (wks)	Excitation λ	Emission λ	I	Excitation λ	Emission λ	I	Excitation λ	Emission λ	I
0	280	342	292		NP		319	428	114
1		NP		255	438	359	325	431	261
2		NP		252	435	313	347	429	290
4		NS			NS			NS	
6		NP		253	449	189	327	435	378
8		NP		259	449	233	333	435	344

[a]All scans at 3mM C; 0.01 M IS; pH 5.5
[b]Intensity in arbitrary units
[c]Primary Papermill Residue
[d]Primary Papermill Residue + Secondary Sludge
[e]No peak present
[f]No scan generated: <3mM C

that for the λ_{em} range 450-460 nm the λ_{ex} increased from 310 nm in the needle extract to 338 nm at the base of the O horizon. They noted as explanation that excitation peaks shift to longer wavelengths for fluorophores with extended conjugated structures and that the shift witnessed here likely resulted from increasing humification of simple aromatic compounds. If this distinction is adopted in the current experiment, fluorophore C (arising from fresh DOC) appeared in the time 0 extracts of wheat straw, crimson clover, and PPR+SS while fluorophore D (arising from complexed/humified DOC) appeared in the time 0 extract of PPR.

It is difficult to construct a clear picture of fluorophore transition over time as sometime low C concentration precluded the generation of a full suite of standardized scans. Fluorophore A disappeared from wheat straw and PPR+SS DOC after time 0, and from crimson clover DOC following week 1. Fluorophore B was present in wheat straw and crimson clover DOC throughout the incubation, though its intensity began initially higher for wheat straw and ending ultimately higher for crimson clover. Fluorophore B intensity was consistently high for PPR, though low C concentration precluded the generation of week 4 and 8 scans. While this peak was initially absent from the PPR+SS time 0 scan, it subsequently appeared and was present at all sampling intervals where there was sufficient C concentration to generate scans. Fluorophore C disappeared from wheat straw, crimson clover, and PPR+SS scans after week 0 and was replaced by fluorophore D. This peak was present in all subsequent wheat straw and crimson clover scans. Fluorophore D was likewise present in all PPR and subsequent PPR+SS extracts with sufficient C for scan

generation. Fluorophore D intensity followed a nonlinear trajectory over the length of the incubation, initially rising then falling for all four amendments.

When analyzed in the context of progressing humification, the fluorescence trends for wheat straw and crimson clover were consistent with the general trends observed via other parameters. Fluorophore C has been observed in leaf and needle extracts and correlated with the EEM peak location of pure mono- and di-hydroxybenzoic, salicylic, and hydroxycinnamic acids (Wolfbeis, 1985). Fluorophore D has been widely correlated in the literature with both terrestrial and aquatic fulvic and humic acids. If these two fluorophores represent simple and humified aromatic materials, respectively, then the shift in excitation wavelength occurring between week 0 and week 1 represents a transition toward monomer polymerization. Following the first 7 days, the most significant further transitions involved peak intensity and as this phenomenon is sensitive to multiple factors, it cannot be explicitly correlated with peak source concentration. Fluorescence within the C-D peak region underwent a less definitive evolution in papermill residue DOC. For PPR, the analyses already presented reveal an initially higher aromaticity (*vis a vis* the other amendments) and a FSP pool concentrated within the HMW fraction. This picture is corroborated by the time 0 DOC presence of fluorophore D. For PPR+SS, a weak transition from fluorophore C to D occurred during week 1 consistent with both the small recorded increase in bulk aromaticity and the disappearance of FSP from the LMW fraction.

The humification index (HIX) of Zsolnay et al.(1999) was applied to the DOC extracts utilizing the equation correction of Ohno (2002). Initial (time 0) HIX values

ranged from 0.33 for crimson clover to 0.91 for PPR (Figure 16). The low value for crimson clover was consistent with the high intensity of its aromatic amino acid fluorescence peak. Over the 8 week incubation, HIX values increased for crimson clover, wheat straw and PPR+SS, and were unchanged for PPR. Week 8 HIX values ranged from 0.84 (PPR+SS) to 0.89 (crimson clover) with the single most rapid incremental increase occurring over the first 7 days of the incubation. Results can be compared with the results of Ohno (2002) for materials representing stages in the humification continuum. Values for field corn residue, soil DOM and a purified soil fulvic acid (FA) were 0.57, 0.84, and 0.94, respectively, with DOM and FA results bracketing all week 8 values for the current experiment.

Specific interpretation of this index is rendered difficult by the lack of identity of the 435-480 nm peak. As both Coble et al. (1990) and Ohno and Cronan (1997) have concluded that the peak in question is not a reflection of the FA peak, the HIX ratio specifically excludes a humification-related fluorophore that is well defined in the literature (i.e. fluorophore D). Wolfbeis (1985) did observe a humic acid peak at $\lambda_{ex}/\lambda_{em}$ = 270/460 though noted that the scan was conducted at pH 10.0. The literature is inconclusive regarding the effect of pH >7.0 on the intensity and location of fluorophores. In this experiment, it is clear that low HIX values at time 0 resulted from the presence and concentration of aromatic amino N. Following the utilization of this material, HIX values generally increased in all extracts with sufficient C for analysis.

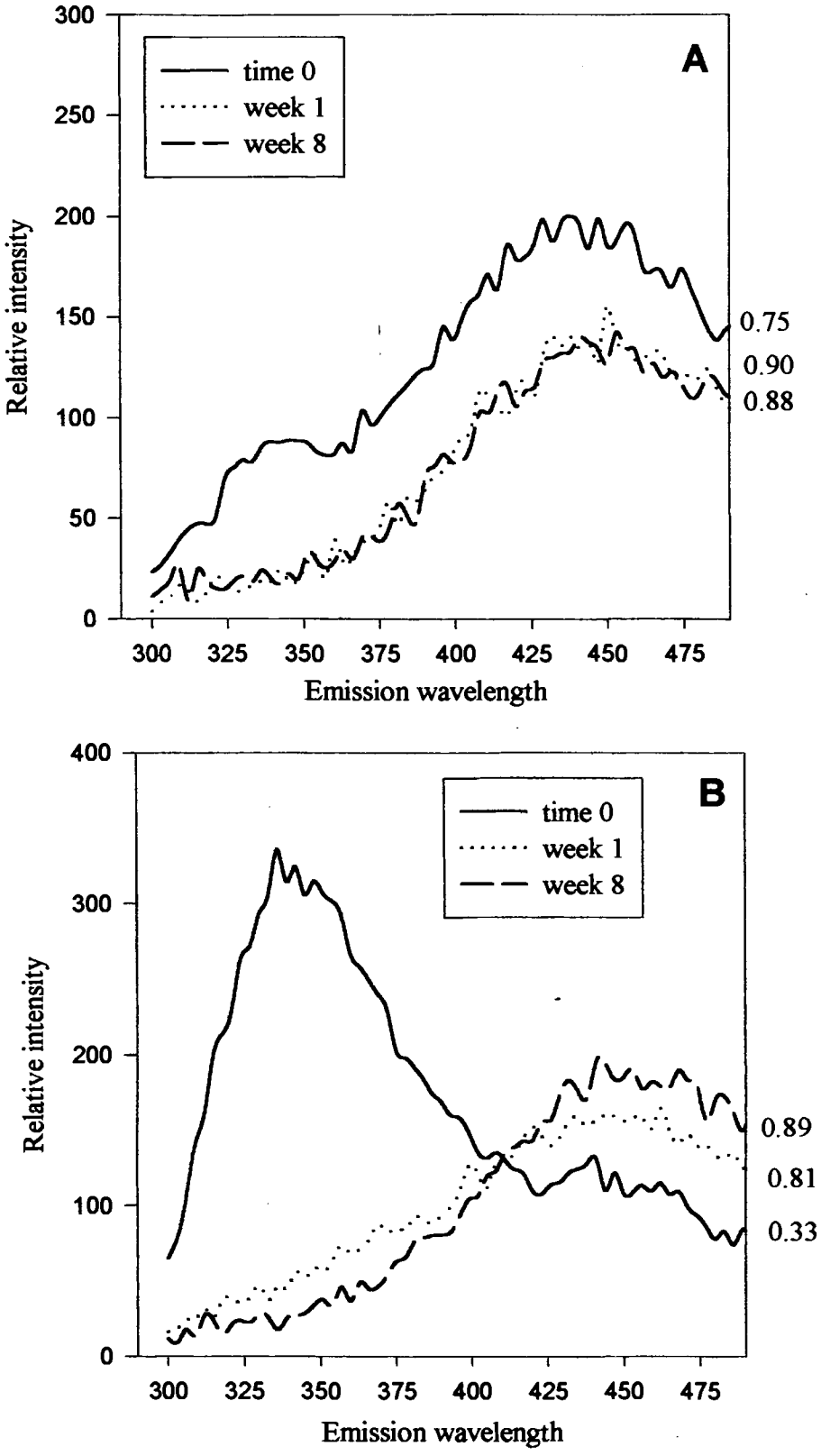

Figure 16. HIX trends for wheat straw (A) and crimson clover (B). Values on right margin correspond to relative humification on a scale of 0-1 as defined by Ohno (2002).

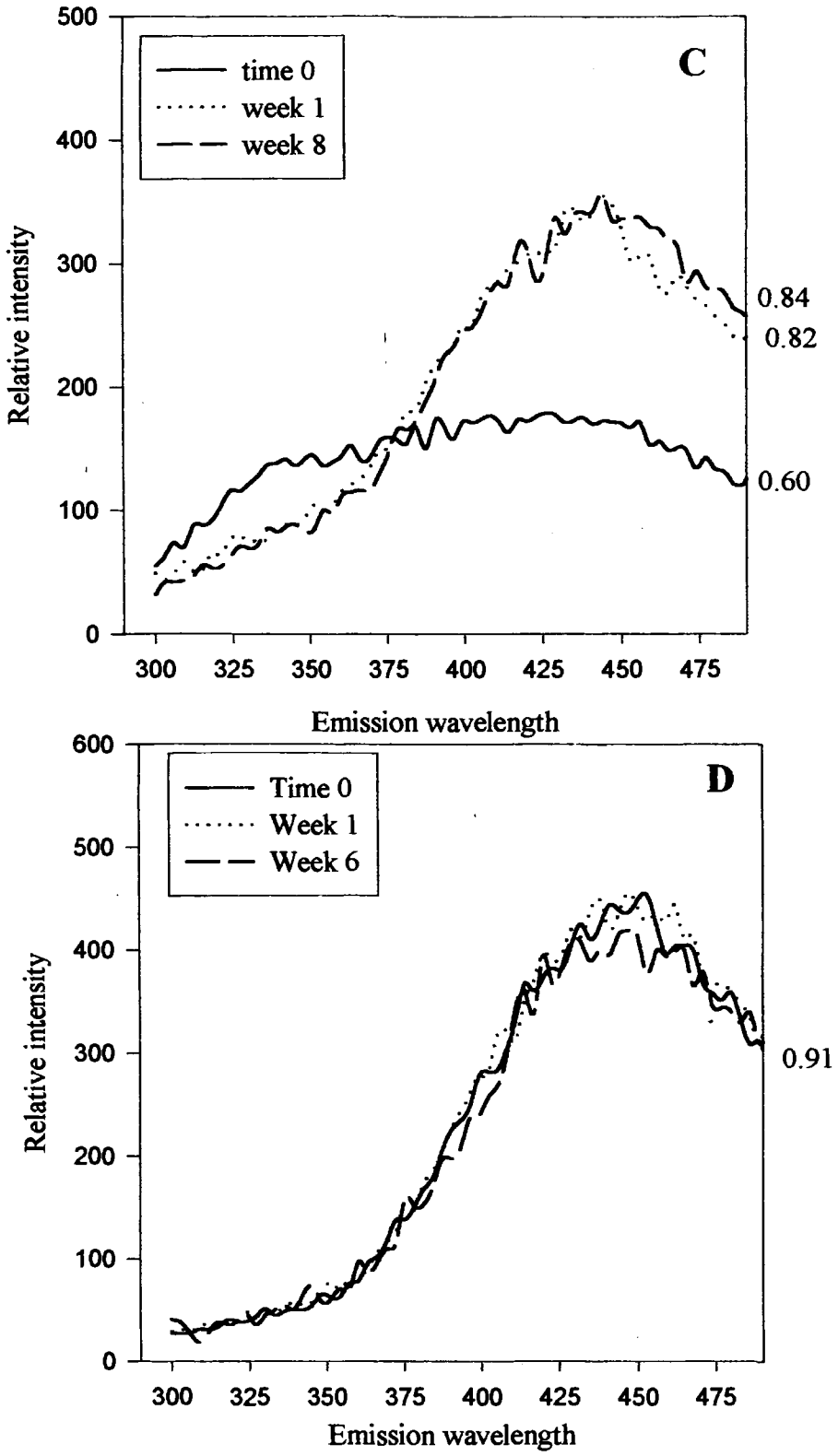

Figure 16 (cont.). HIX trends for PPR+SS (C) and PPR (D). Values on right margin correspond to relative humification on a scale of 0-1 as defined by Ohno (2002). PPR = Primary Papermill Residue; PPR+SS = Primary Papermill Residue + Secondary Sludge

Low Molecular Weight Fraction (<1000 MWCO)

The low molecular weight (LMW) fraction of the extracted DOC was examined as this fraction may significantly affect soil processes. The DOC concentration of the ultrafiltration membrane permeate (<1000 MWCO) at time 0 accounted for 71%, 62%, 73%, and 30% of the wheat straw, crimson clover, PPR+SS, and PPR bulk extract DOC, respectively. As a relative percentage of bulk extract DOC this fraction varied both over time and between amendments during this incubation (Figure 17). For wheat straw, the LMW fraction of bulk DOC decreased consistently over the incubation, falling sharply to 15% at week 1 followed by a slow subsequent decrease to 0% by week 8. For crimson clover, the LMW fraction decreased to 33% of total DOC by week 1, and 28% of total extract DOC by the conclusion of the incubation. Following the initial sampling, the LMW DOC concentration for both papermill residue extracts was too low for reliable measurement.

Folin-Ciocalteu reactive soluble phenolic acids (FSP) were measured in the LMW fraction to test for the presence of LMW hydroxyl-bearing aromatic moeities. As a percentage of total soluble LMW C, FSP accounted for 3.3% of wheat straw, 7.8% of crimson clover and 2.0% of PPR+SS DOC (Figure 18a). The C concentration of the PPR permeate was too low for component analysis. For the wheat straw LMW fraction, FSP were present through week 4 as a relatively increasing percentage of an absolutely decreasing C pool. FSP were present in the crimson clover LMW fraction throughout the incubation. Following time 0, there was insufficient C in the PPR+SS LMW fraction for further analysis. If the Blum et al. (1991) correction is applied, the

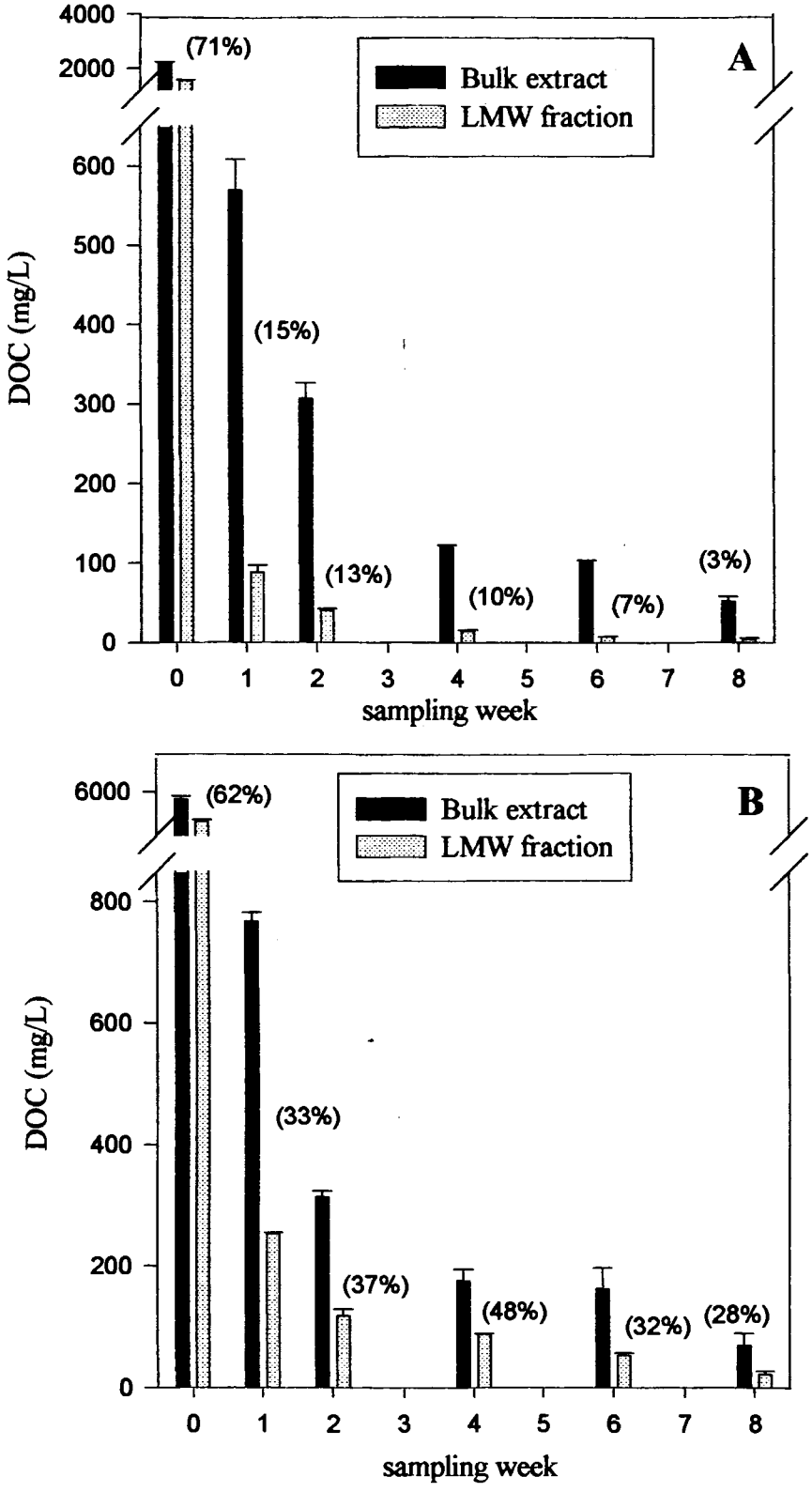

Figure 17. DOC concentration in amendment bulk extracts and LMW (<1 kDa) fraction. Values in parenthesis correspond to the relative percent LMW DOC. A) Wheat straw; B) Crimson clover

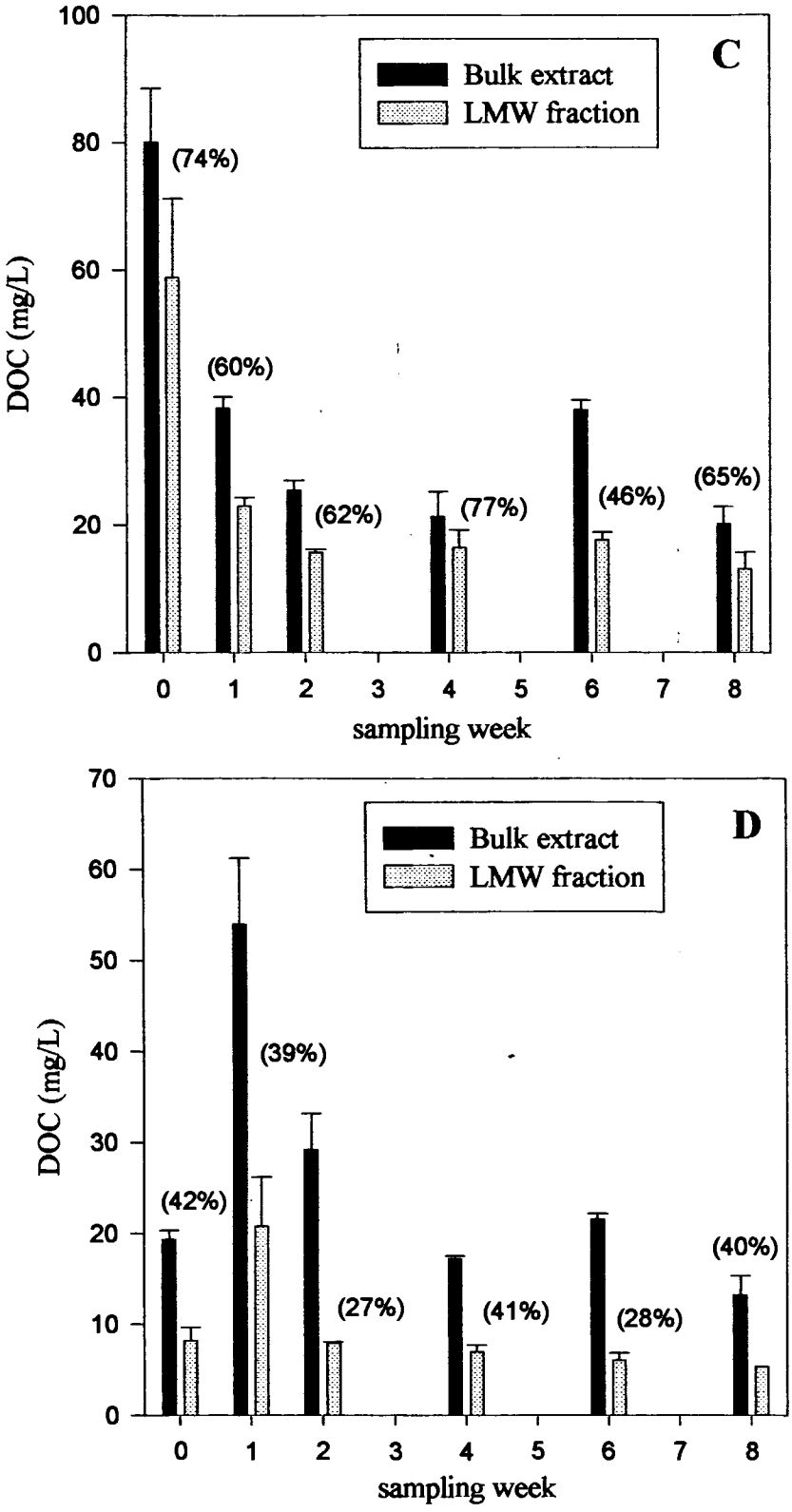

Figure 17 (cont.). DOC concentration in amendment bulk extracts and LMW (<1 kDa) fraction. Values in parenthesis correspond to the relative percent LMW DOC. C) Primary Papermill Residue + Secondary Sludge (PPR+SS); D) Primary Papermill Residue (PPR)

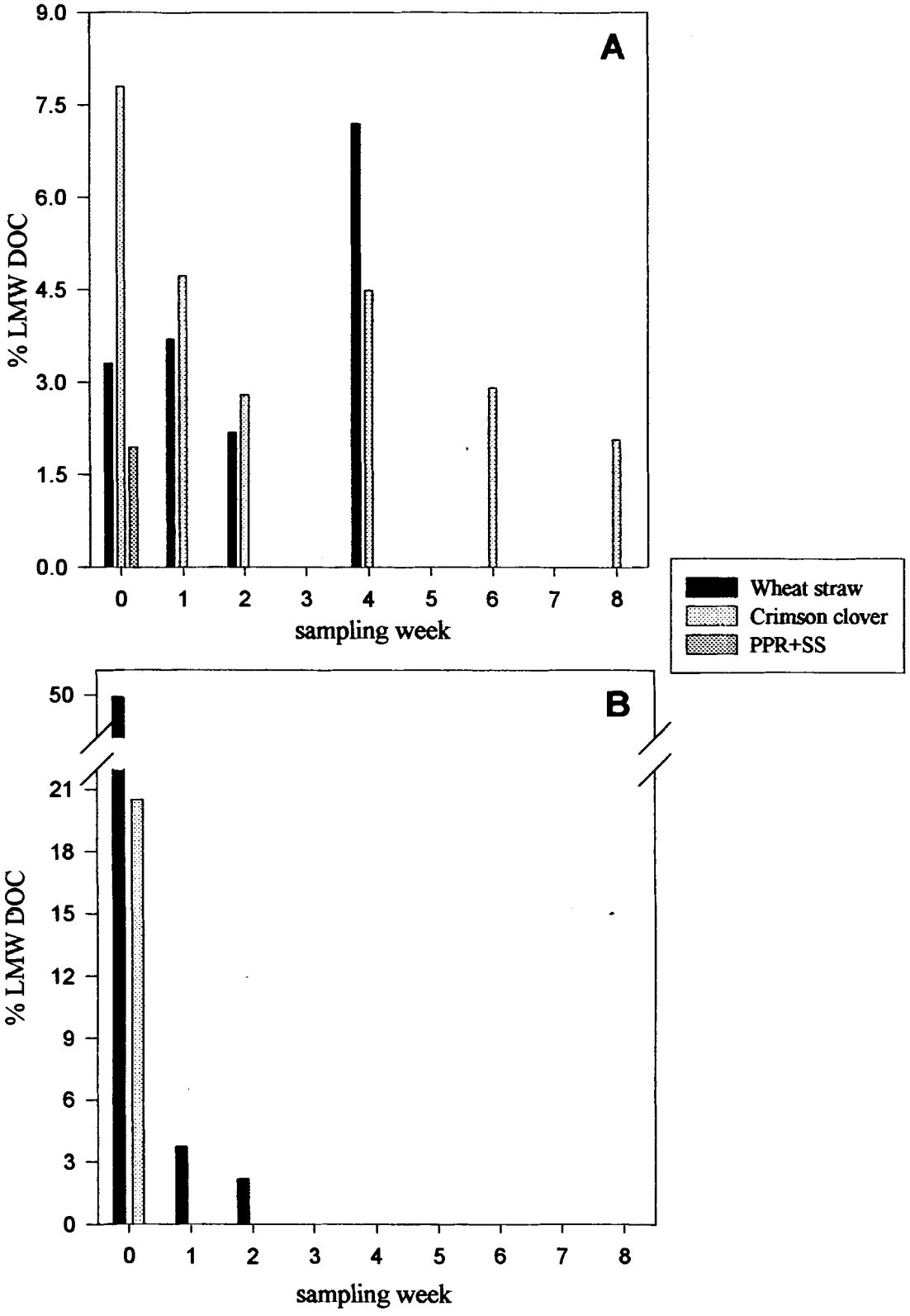

Figure 18. Low molecular weight (<1 kDa) fraction of amendment DOC over an 8 week incubation. A) Folin-Ciocalteu reactive soluble phenolic acids as a percentage of LMW DOC; B) Anthrone reactive soluble sugars as a percentage of LMW DOC.

time 0 concentration (expressed in terms of ferulic acid-C) falls to 0.2% of wheat straw, 0.6% of crimson clover, and 0% of PPR+SS LMW DOC.

Anthrone reactive sugars (ARS) were also measured in the wheat straw and crimson clover LMW fractions (Figure 18b). While high nitrate interference precluded their measure in PPR and PPR+SS time 0 extracts, low C concentrations in all subsequent extractions was taken as evidence of their absence. Calculated as a percentage of permeate DOC, ARS accounted for 49.6% and 20.5% of time 0 wheatstraw and crimson clover extracts, respectively. ARS accounted for less than 4% of week 1 wheat straw DOC, approximately 2% of week 2 DOC and following week 2 was no longer measurable. In crimson clover extracts, ARS were no longer measurable after the initial time 0 extraction. Meyer et al. (1987) observed a strong association between molecular size and biodegradability. In an incubation of tannin-rich river water, 86% of the less abundant LMW (<1000) fraction was microbially degraded while <5% of the HMW (>10000) fraction was degraded over the same time interval. Subsequent hydrolysis of the HMW fraction revealed that its small degradable fraction was comprised of complexed LMW compounds. Results from the current experiment support their conclusions, namely that the LMW fraction may be significantly enriched in more easily degradable components.

For the LMW fraction, the generation of fluorescence scans and the examination of peak transitions were complicated by low C concentrations (Table 4). Fluorophore A was present in the time 0 LMW scans of wheat straw, crimson clover, and PPR+SS with an intensity that was enhanced relative to the bulk extract scans. Fluorophore B was only clearly present in the time 0 scan for wheat straw though at a

Table 4. Location and intensity of primary peaks in fluorescence spectra of DOC extracts; LMW fraction (<1 kDa)[a]

a. Wheat straw

	Fluorophore								
	A			B			C-D		
Time (wks)	Excitation λ	Emission λ	I[b]	Excitation λ	Emission λ	I	Excitation λ	Emission λ	I
0	280 (nm)	324 (nm)	167	250	456	123	313	436	323
1		NP[c]		253	441	548	333	420	700
2		NP		250	440	631	343	428	785
4		NS[d]			NS			NS	
6		NS			NS			NS	
8		NS			NS			NS	

b. Crimson clover

	Fluorophore								
	A			B			C-D		
Time (wks)	Excitation λ	Emission λ	I	Excitation λ	Emission λ	I	Excitation λ	Emission λ	I
0	276	351	672		NP		315	441	150
1		NP		252	435	357	325	414	412
2		NP		250	438	476	349	438	475
4		NP		252	440	350	341	429	332
6		NP		252	443	455	333	428	410
8		NP		250	432	307	331	420	312

[a]All scans at 3mM C; 0.01 M IS; pH 5.5
[b]Intensity in arbitrary units
[c]No peak present
[d]No scan generated: <3mM C

Table 4 (cont.). Location and intensity of primary peaks in fluorescence spectra of DOC extracts; LMW (<1 kDa)[a]

c. PPR[c]

	Fluorophore								
	A			B			C-D		
Time (wks)	Excitation λ	Emission λ	I[b]	Excitation λ	Emission λ	I	Excitation λ	Emission λ	I
0		NS[e]			NS			NS	
1		NS			NS			NS	
2		NS			NS			NS	
4		NS			NS			NS	
6		NS			NS			NS	
8		NS			NS			NS	

d. PPR+SS[d]

	Fluorophore								
	A			B			C-D		
Time (wks)	Excitation λ	Emission λ	I	Excitation λ	Emission λ	I	Excitation λ	Emission λ	I
0	280	366	410		NP		319	437	73
1		NS			NS			NS	
2		NS			NS			NS	
4		NS			NS			NS	
6		NS			NS			NS	
8		NS			NS			NS	

[a]All scans at 3mM C; 0.01 M IS; pH 5.5
[b]Intensity in arbitrary units
[c]Primary Papermill Residue
[d]Primary Papermill Residue + Secondary Sludge
[e]No scan generated: <3mM C

lower intensity than found in the bulk extract scan. Fluorophore C was present in the time 0 scans for wheat straw, crimson clover and PPR+SS at an intensity that was, in all cases, similar to the bulk extract scans. In terms of peak development, fluorophore A disappeared after time 0 for all amendments. Fluorophore B was present in wheat straw DOC at increasing intensities until low C concentration at week 4 precluded the generation of further scans. Fluorophore B was consistently present in crimson clover at higher intensities than found in the bulk extract. As occurred in bulk extract scans for all amendments, fluorophore C disappeared after week 0 and was replaced by fluorophore D. No scans were generated for PPR or PPR+SS permeate during weeks 1-8 due to low C concentration.

For both wheat straw and crimson clover, the intensity of fluorophore D was markedly higher in the LMW than in the bulk scans. An explanation for the relatively enhanced peak intensity in the LMW fraction lies in the absence of HMW polymerized materials. Such materials would likely play a role in quenching the fluorescence of simple aromatic moeities. The week 1 appearance of the structurally condensed fluorophore D in the LMW fraction is explained by Fox (1995) who observed that small fulvic acid molecules exist within the general size range of simple organic acids. This transition (i.e. from fluorophore C to D) within the LMW fraction suggests that the UV-absorbing DOC pool (as defined by A_{285}) is dynamic, progressing through varying aromatic components. A similar conclusion was reached by Mathur et al. (1993), using A_{280} as an optical means of testing compost maturity. While the concentration of DOC extracted weekly from farmyard manures decreased over time, day-60 A_{280} values were not significantly different than day-0 values. They

concluded that while the bonds absorbing at this wavelength were still clearly present, the compounds involved had shifted from simple aromatics toward those with more complex, humified structures.

Charge Density

Both carboxyl charge (defined as titratable acidity between pH 3.0-7.0) and total charge density were calculated at time 0 and following a 7 day incubation for wheat straw and crimson clover DOC. Neither PPR nor PPR+SS were included in this analysis due to low DOC concentration. Initial carboxyl charge values calculated for wheat straw and crimson clover were consistent with published values (Table 5). Ohno and Crannell (1996) calculated values ranging from 4.6 $mmol_c\ g^{-1}C$ for wheat straw to 8.0 $mmol_c\ g^{-1}$ C for crimson clover, including 6.2 $mmol_c\ g^{-1}$ C for field corn residue and 7.5 $mmol_c\ g^{-1}$ C for hairy vetch. Ionization products (pKa) calculated for both amendments were likewise consistent with published values. Cronan et al. (1992) calculated pKa values ranging from 3.9-4.2 for soil solution DOC extracts with carboxyl charge densities ranging from 4.8-6.0 $mmol_c\ g^{-1}$ C. Vance and David (1991) calculated pKa values ranging between 3.8-4.8 for hydrophobic and hydrophilic acids extracted from forest soil DOC.

Charge density values determined for the bulk extracts were compared both temporally (i.e. time 0 versus week 1) and relative to the LMW fraction (Figure 19). For crimson clover (time 0), both the carboxyl and the total charge density of the LMW fraction were significantly greater than for the bulk extract. Carboxyl charge defined as a percentage of total charge density (C/T), however, was equivalent (i.e. 60%) between size fractions. For wheat straw (time 0), while carboxyl charge density

Table 5. The effect of incubation on charge density

Plant material	Fraction	Week	Carboxyl charge[a]	pKa	Total charge density	C/T[b]
			$mmol_c\ g^{-1}C$		$mmol_c\ g^{-1}C$	%
Wheat straw	LMW fraction[c]	0	5.27[d] (0.23)	3.8	5.64 (0.00)	93.5 (4.1)
		1	9.95 (0.47)	3.8	33.5 (0.81)	29.7 (0.8)
	Bulk extract	0	4.72 (0.00)	3.8	5.60 (0.00)	84.2 (0.0)
		1	5.10 (0.23)	3.8	12.2 (0.23)	42.0 (1.1)
Crimson clover	LMW fraction[d]	0	8.62 (0.23)	4.0	14.3 (0.02)	60.2 (1.5)
		1	11.4 (0.24)	4.2	25.0 (1.74)	45.8 (2.9)
	Bulk extract	0	6.99 (0.47)	4.0	11.6 (0.47)	60.1 (1.6)
		1	6.45 (0.00)	4.1	12.7 (0.00)	50.8 (0.0)

[a]defined as titratable acidity between pH 3.0-7.0

[b](carboxylate charge density/total charge density) x 100

[c]defined as passing through a 1000 MWCO ultrafiltration membrane

[d]values presented are mean values for three replicates + (one standard deviation)

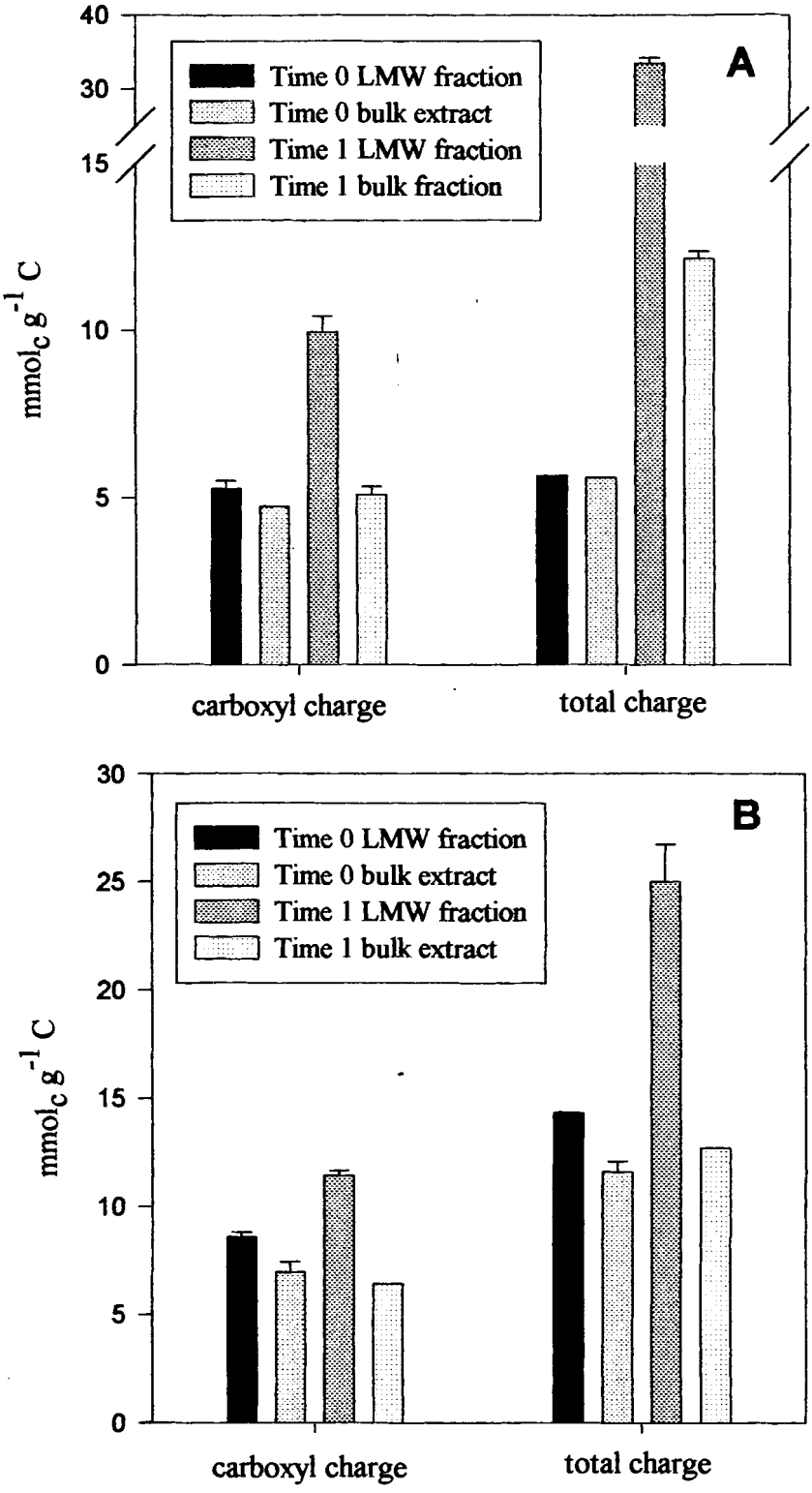

Figure 19. Charge density differences over time (time 0 versus week 1) and between the bulk extract versus the LMW (<1000 MWCO) fraction. Error bars represent one standard deviation. A) Wheat straw; B) Crimson clover

was significantly higher in the LMW fraction than the bulk extract, total charge density was equivalent. The C/T ratio was, correspondingly, higher for the LMW fraction than for the bulk extract (94% versus 84%, respectively). In contrasting the amendment bulk extracts, while both the initial carboxyl and total charge density were, in absolute terms, significantly greater for crimson clover DOC, the C/T ratio was higher for wheat straw (84% versus 60%, respectively).

For the crimson clover bulk extract, there was no significant temporal (i.e. time 0 versus week 1) change in carboxyl charge density. Total charge density increased, however, leading to a decrease of from 60% to 51% in the C/T ratio. For the LMW fraction, while both carboxyl and total charge density increased significantly over time, the C/T ratio decreased from 60% to 46%. When the week 1 bulk extract was compared with the LMW fraction, both the carboxyl and the total charge density had increased significantly, with the C/T ratio decreasing from 51% to 46%. For the wheat straw bulk extract, while there was no change over time in carboxyl charge density, total charge density increased significantly, leading to a decrease of from 84% to 42% in the C/T ratio. For the LMW fraction, both carboxyl charge and total charge density increased significantly over the time interval, resulting in a decrease of from 94% to 30% in the C/T ratio. When the bulk extract (week 1) was compared with the LMW fraction, both the carboxyl and total charge density had increased significantly, with the C/T ratio decreasing from 42% to 30%.

In comparing bulk extracts at week 1, while both carboxyl and total charge density were greater for crimson clover than wheat straw the difference relative to its extent at time 0 had decreased dramatically. Total week 1 carboxyl charge density for

both amendments was between 5.1-6.5 $mmol_c\ g^{-1}$ C. These values are consistent with the calculations of Cronan and Aiken (1985) for the total carboxyl charge density of extracted and purified O/A horizon forest floor leachates (i.e. between 5.6-8.3 $mmol_c\ g^{-1}$ C, with an average over all sites of 6.5 $mmol_c\ g^{-1}$ C). Total week 1 charge density for both amendments was between 12.2-12.7 $mmol_c\ g^{-1}$ C. These values are consistent with the calculations of Vance and David (1991) for forest soil solution hydrophobic and hydrophilic acids (10.5 $mmol_c\ g^{-1}$ C and12.3 $mmol_c\ g^{-1}$ C, respectively) and Romkens and Dolfing (1998) for agricultural soil DOC (8.5-11.2 $mmol_c\ g^{-1}$ C).

Over the incubation it thus appears that (1) there was a significant increase in non-carboxyl acidity that was more pronounced in the LMW fraction than in the bulk extracts, (2) a significant increase in carboxyl acidity that was only visible in the LMW fraction, and (3) all acidity increases were more pronounced in the wheat straw than in the crimson clover extracts. These conclusions can be viewed in the context of results already presented. The more significant increase in total titratable acidity seen for wheat straw is consistent with the more rapid increases seen regarding the humification indices presented here. For both amendments, it was clear that an increase in phenolic acidity occurred during the 1 week incubation. This is consistent with the increases measured in Folin-Ciolcateu reactive phenolic acids (FSP) and is likely consistent with the increases measured via relative aromaticity indices (i.e. A_{285}, HIX, and the peak excitation wavelength shift from fluorophore C to D).

Following incubation, the non-carboxyl acidity of the LMW fraction increased to 13.6 $mmol_c\ g^{-1}$ C and 23.6 $mmol_c\ g^{-1}$ C for crimson clover and wheat straw DOC, respectively, with total titratable acidity increasing to 25.0 $mmol_c\ g^{-1}$ C (crimson

clover) and 33.5 $mmol_c\ g^{-1}$ C (wheat straw). These values can be compared with the results of Evangelou and Marsi (2001) and Bergelin et al. (2000). After decomposing corn stalks and leaves for 8 months, Evangelou and Marsi (2001) extracted and size fractionated soluble humic materials. Both phenolic and total acidity showed relative enrichment per gram C as size fraction decreased, with values in the lowest molecular weight fraction reaching 19.7 $mmol_c\ g^{-1}$ C (phenolic) and 57.5 $mmol_c\ g^{-1}$ C (total titratable acidity). Bergelin et al. (2000) analyzed the buffering capacity of LMW organic acids in podzolic soils and calculated a specific buffering capacity of 40 $mmol_{H+}\ g^{-1}$ DOC.

The apparent concentration of both carboxyl and non-carboxyl acidity in the LMW fraction may be interpreted in several ways. While humification clearly involves a transition toward higher molecular weight components, this process also involves breakdown steps in which simple molecules are enzymatically cleaved from larger compounds. These molecules would exist ephemerally as intermediary products prior to the reactive formation of humic structures. While the significant increase in non-carboxyl charge density may correspond to the liberation of monomeric phenols, this interpretation contradict both the steep increase in the FSP retention percentage (RP) and the shift in the fluorescence excitation wavelength seen over this interval for both amendments. A second possibility involves Fox's (1995) identification of low molecular weight fulvic acids. As humification progresses through even low molecular weight condensation reactions involving acid functional group-bearing aromatic monomers, total charge density could increase with the increasing structural complexity that accompanies polymerization. This view is

consistent with the conclusion of Krosshavn et al. (1992) regarding the relationship between degree of humification and OM functional group distribution within forest soils. Utilizing ^{13}C NMR, these researchers concluded that the process of plant material humification involved relative increases in the overall proportions of alkyl and carboxyl C. That such an increase in carboxyl charge was more pronounced for the LMW fraction than for the bulk extract during this incubation may suggest that the early stages of humification are more initially visible in lower molecular weight components.

It is difficult to define what percentage of the increase in titratable acidity resulted from the generation of acidic breakdown products/LMW fulvic acids and what resulted simply from the degradation of non-titratable components (i.e. the simple sugars). As all titrations were conducted at a standard C concentration, the degradation of labile materials would leave a C pool enriched by default in acid functional group-bearing components. Vance and David (1991) concluded, for example, that roughly 90% of forest soil DOC was comprised of hydrophobic and hydrophilic organic acids. In the context of this experiment, not only did the wheat straw extract contain a higher percentage of anthrone-reactive sugars, but the wheat straw LMW fraction was preferentially enriched relative to the bulk extract in this labile substrate. The removal of such materials would clearly play a role in the acidity increase measured, though to what extent it is the explanatory mechanism and through what means it would favor the dramatic increase in phenolic acidity is uncertain. In the context of defining early-stage humification, however, the categorical distinction between LMW polymerization and simple component

degradation may prove arbitrary. These two processes, operating in tandem, may describe complementary facets of the same DOC transition occurring during the early stages of humification.

Copper Binding Capacity

Evangelou and Marsi (2001) observed that the high O-bearing functional group content of humic materials likely explains the affinity between humics and both solution phase and adsorbed metals. Young et al. (1982) observed that at low solution metal concentrations N-bearing compounds displayed strong Cu binding potential. Luster et al. (1994 and 1996) studied the Cu binding capacity of both juniper and chestnut leaf litter DOC and concluded that strong Cu binding was definable in terms of inner-sphere quasiparticle complexes involving either 4 O or 2 O + 2 N ligand sites. Sposito (1986) defined quasiparticles as hypothetical, non-interacting macromolecules bearing single functional group classes. Both Luster et al. (1994 and 1996) and Sposito (1986) defined weak Cu binding in terms of outer-sphere complexes involving 2 O + 2 H_2O molecules.

Results in the current experiment suggest that multiple factors influence both the strength of Cu-ligand stability constants (log ^cK) and the maximum binding capacity (B_{max}) of the ligand. At time 0, stability constants for both quasiparticle complexes (L1 and L2) were greater for wheat straw bulk extract than for crimson clover bulk extract (Table 6). Following incubation, stability constants decreased for wheat straw and increased for crimson clover, such that ultimate log $^cK_{L1}$ (i.e. strong binding) values were similar between amendments while log $^cK_{L2}$ (i.e. weak binding) values were higher for crimson clover than for wheat straw.

Table 6. The effect of incubation on Cu binding parameters

Plant material	Fraction	Week	log $^{c}K_1$	B_{max1} [a]	log $^{c}K_2$	B_{max2} [a]
Wheat straw	LMW fraction[b]	0	6.61	0.11	5.35	0.53
		1	6.96	0.35	3.05	32.1
	Bulk extract	0	7.21	0.16	5.79	0.22
		1	6.96	0.29	4.55	1.48
Crimson clover	LMW fraction[b]	0	7.00	0.24	4.37	6.28
		1	6.78	0.55	5.17	1.26
	Bulk extract	0	6.59	0.41	2.98	46.4
		1	6.78	0.34	5.17	2.27

[a] mmol Cu g^{-1} C

[b] defined as passing through a 1000 MWCO ultrafiltration membrane

Analyzed in terms of maximum binding capacity (B_{max}), it appeared that (1) both strong and weak maximum binding capacities were initially greater for crimson clover, (2) the binding capacity increased over the incubation interval for wheat straw and decreased over the same interval for crimson clover, but (3) after 1 week the maximum Cu binding capacity of crimson clover DOC was still greater. Maximum binding capacities for the bulk extracts ranged from 0.16 mmol Cu g^{-1} DOC for wheat straw [time 0-L1] to 46.4 mmol Cu g^{-1} DOC for crimson clover [time 0-L2]. Strong binding site values are reasonably consistent with published values. Luster et al. (1994) calculated B_{max1} = 0.13 mmol Cu g^{-1} DOC for chestnut leaf litter DOC. Kuiters and Mulder (1992) calculated B_{max} of between 0.75-0.91 mmol Cu g^{-1} DOC for leaf and needle litter DOC and between 2.58-5.96 mmol Cu g^{-1} DOC for a forest soil O horizon. No published values were found for agricultural materials though Romkens et al. (1998) calculated B_{max} for the LMW fraction of an agricultural soil DOC as 0.45 mmol Cu g^{-1} DOC. While functional group chemistry is generally consistent within the plant kingdom, there are clearly differences in absolute and relative abundance of functional groups between distinct types of plant residues (Stroebel, 2001). Such differences would explain the variation in B_{max1} observed between agricultural crops and forest leaves and needles. In terms of binding site L2, as already discussed, the degree of data variance at the highest applied Cu concentration (1 mM Cu) suggests that interpretation of both weak binding site affinity and saturation binding capacity may be subject to greater error than interpretation of strong binding site data. As example, the maximum weak binding capacity value calculated by the model (46.4 mmol Cu g^{-1} DOC) appears unrealistic

in light of the significantly lower total charge density calculated for this amendment (i.e., 11.6 $mmol_c\ g^{-1}$ C).

Conditional stability constants for wheat straw and crimson clover DOC were compared with published values to explore the functional group composition of each class of quasiparticle binding complexes. In analyzing strong, inner-sphere binding, Luster et al. (1996) recorded a log $^cK = 7.5$ for Cu complexation with either catechol or histidene. They further noted a log $^cK = 6.9$ for strong Cu binding in a juniper leaf litter extract. This value was correlated with electron spin resonance (ESR) study of the leaf litter extract with results suggesting that such binding involved 1,2-dihydroxyphenols and amino acids. Martell and Smith (1977) calculated log $^cK = 6.9$-8.3 for Cu binding with aromatic amino acids. Taga et al. (1991) analyzed the stability of complexes formed between Cu and a peat derived humic acid (HA). They calculated a log $^cK = 6.2$-7.0 for Cu:HA and when correlated with infrared (IR) spectroscopic analysis concluded that strong binding was predominantly a function of carboxyl group content. Brown et al. (1999) recognized that aromaticity was not a necessary precondition for strong Cu binding by calculating a log ^cK for Cu-citric acid of 6.55. The researchers noted as explanation that this acid is tri-carboxylic and thus has a strong potential to form Cu-ligand chelates. Evangelou et al. (2001) size fractionated maize tissue DOC and concluded that as size fraction decreased Cu-binding stability constants increased from 6.9 to 7.3. Infrared spectroscopic analysis revealed that carboxyl groups dominated binding, but that amine and hydroxyl groups were also involved. Values presented for strong binding stability constants were all calculated at pH 6.0 and 0.01-0.1 M ionic strength.

Strong binding at low solution Cu concentrations thus appears to be mediated by multi-carboxylic acids, aromatic hydroxyl-substituted monomers, and N-containing functional groups. For crimson clover, the greater time 0 B_{max1} (relative to wheat straw) was likely a function of a higher concentration of aromatic amino N, greater total and carboxyl charge density and/or higher initial percentage of Folin-Ciocalteu reactive soluble phenolic acids (FSP). The relative decrease in crimson clover B_{max1} over the 7 day incubation was likely affected by the diminishing concentration of aromatic N. As this was coupled with increases in both acidity and humification, the decrease in B_{max1} plausibly corresponds to a shift from 2 O + 2 N to a 4 O binding geometry. While this suggests that the maximum binding capacity of N-bearing sites is higher, binding affinity appears to increase with the domination of O-controlled geometry. For wheat straw, while aromatic N was present in the time 0 extract it was found at a relatively low concentration and suggests that a larger percentage of time 0 strong Cu binding involved 4 O binding geometry. This dominance of O-bearing sites may explain the greater Cu binding affinity of wheat straw DOC.

Luster et al. (1996) defined weak binding (i.e. with high [Cu] relative to DOC concentration) as involving ketonic, phenolic and, likely, carboxylic functional group linkages and as corresponding to log ^{c}K values within the range 3.0-6.0. Luster et al. (1994) found Cu:oxalate and Cu:salicylate stability constants of 5.8-5.9, suggesting that both aromatic and aliphatic carboxyl group-bearing acids play some role in weak metal binding. These researchers further utilized fluorescence scans to explore Cu binding capacity in the context of aromatic peak location. They calculated a log ^{c}K =

4.8 for Cu binding with a fluorophore centered at $\lambda_{ex}/\lambda_{em}$ = 323/448. This peak was identified as the fulvic acid peak (fluorophore D) and the authors suggested that weak binding was correlated with the higher molecule weight, more conjugated nature of this fluorophore. The association between weak binding and fulvic acids was supported by Brown et al. (1999) who calculated log ^{c}K = 5.2-5.9 for Cu binding with a Suwannee River fulvic acid. Taga et al. (1991) defined weak binding as hydroxyl binding and found log ^{c}K values between 4.9 and 5.6. Martell and Smith (1977) calculated stability constants for Cu binding with a suite of dicarboxylic acids and found log ^{c}K values ranging between 2.6-5.1. Buffle et al. (1980) studied oak, beech, larch and chestnut leaves and concluded that the Cu:DOC stability constants ranged between 4.1-4.9. All values were calculated at pH 5.5-6.0 with 0.1-0.01 M ionic strength.

In terms of weak binding for wheat straw and crimson clover bulk extracts, a clear pattern could not be discerned from the parameters studied here. In the current experiment, factors leading to a change in log $^{c}K_{L2}$ between time 0 and week 1 would include a shift toward fluorophore D and increasing retention percentages for both DOC and soluble phenolic acids (i.e., increasing polymerization/humification). If the anomalously high value for crimson clover time 0 is excluded, bulk extract stability constants are between 4.6 and 5.8. Such values for weak ligand binding are supported by the literature, and suggest that at high soil Cu burdens, outer sphere chelation with organic ligands may lead to the solubilization of low mM concentrations of Cu. While both carboxyl and phenol O likely play a role in outer sphere binding, it is the relative

arrangement of these functional groups on increasingly polymerized macromolecules that determines affinity for soil metals (Kubicki et al, 1997).

Discussion exists as to whether metals preferentially bind to the LMW fraction. Vulkan et al. (2002) analyzed the solution-phase speciation of metals extracted from soils amended with sewage sludge and concluded both that the majority (91%) of solution-phase Cu was complexed, and that the predominant complexing agent was LMW (<1 kDa) DOC. While comparisons in the current experiment are not strictly between distinct molecular weights, the LMW fraction can be analyzed versus the bulk extract to compare relative strong binding capacities per standardized C concentration. For crimson clover at time 0, the LMW fraction strongly bound 28 μmol Cu $mmol^{-1}$ carboxyl charge (both defined per gram C) versus 58 μmol Cu $mmol^{-1}$ carboxyl charge (c_c) for the bulk extract. Strong binding is defined here as inner-sphere binding. Following the 7 day incubation, binding capacity per LMW charge site increased significantly to 48 μmol Cu $mmol^{-1}$ c_c while binding capacity per bulk extract charge site decreased slightly to 53 μmol Cu $mmol^{-1}$ c_c. This increase represents an increase in the relative contribution of LMW binding sites to the total Cu chelating capacity of crimson clover DOC. For wheat straw at time 0, the LMW fraction bound 22 μmol Cu $mmol^{-1}$ c_c versus 34 μmol Cu $mmol^{-1}$ c_c for the bulk extract. Following the 7 day incubation, binding capacity per LMW and bulk extract charge sites both increased (to 36 μmol Cu $mmol^{-1}$ c_c and 56 μmol Cu $mmol^{-1}c_c$, respectively). While binding capacity per charge site increased absolutely for the LMW wheat straw extract following incubation, the percentage contribution to bulk extract charge did not change. For both amendments, however, following the 7

day incubation, the LMW fraction was responsible for at least 64% of total strong binding capacity as defined per standard concentration of carboxyl charge (i.e. per mmol c_c). As the contribution of LMW DOC to total DOC decreased significantly over this time interval (from 71% to 15% for wheat straw and 62% to 33% for crimson clover) this suggests that the LMW fraction is a disproportionately effective strong metal chelator.

One likely explanation for the observation that LMW fulvic acids contribute significantly to chelation capacity is that polymers of smaller size have enhanced surface area to volume ratios and thus carry a greater percentage of their acidic functional groups exposed on the surface of the polymer. While higher molecular weight polymers may have a greater *overall* charge density, many potential binding sites will be folded within the interior of humic polymers and geometrically restricted in their interactions. Kubicki et al. (1997) modeled the effect that adjacent functional groups have on metal binding. The researchers concluded that, at an environmentally relevant pH (6.0), salicylic acid-Al^{3+} interactions result from either monodentate complexes involving one carboxyl oxygen supported by one hydroxyl group H-bond, or covalently bonded bidentate complexes involving adjacent carboxyl and hydroxyl substituents. In both instances it is the proximity of a second acidic functional group that stabilizes the chelate. In the current experiment, the increase in LMW binding capacity per unit charge plus the significant increase in non-carboxyl charge density following incubation suggest that the model results of Kubicki et al. (1997) may explain the □ignificant chelating capacity of humified, LMW wheat straw and crimson clover DOC.

Conclusions

Solution-phase condensation reactions have been correlated with the stabilization of DOC and likely play a significant role in long-term carbon sequestration in soils. While such concepts have traditionally influenced research into the C dynamics of undisturbed ecosystems, there have been fewer studies of C stabilization in agricultural systems following organic or conservation-oriented production methods. While humification is clearly a bulk process, with all stages of material breakdown and re-polymerization occurring simultaneously in the soil, this experiment has demonstrated that its early stages can be effectively tracked for organic carbon leached from fresh plant materials. Monitoring the progression of humification in papermill residues, however, proved more difficult. These potential amendments were pre-processed materials with both the labile C and indigenous N pools already depleted. As these materials were relatively inert, little evidence of structural or chemical transition was visible in the soluble C pool.

For wheat straw and crimson clover, however, early-stage humification progressed through increasing molar absorptivity, averaged molecular size, and both phenolic and total charge density, and through the polymerization of originally monomeric plant breakdown products as determined through transitions in fluorescence properties. While no individual technique employed in this study demonstrated this process conclusively, the cumulative picture generated was of an increase over time in the structural complexity of the DOC pool. Interestingly, when the low molecular weight (LMW) fraction of the DOC pool was examined, both the condensation of aromatic monomers and the corresponding increase in charge density

were accentuated. Evidence of humification in even LMW materials suggests both the scale invariance of the polymerization process and provides an explanation for the enhanced Cu binding capacity of LMW DOC following incubation.

Examining the metal complexation capacity of amendment DOC provided a relevant means of assessing the environmental effects of early-stage humification. For wheat straw and crimson clover, DOC extracted both initially and following a 7 day incubation successfully desorbed and complexed resin-bound Cu. Interpretation of weak, outer-sphere binding was complicated by poor replicability at high solution Cu concentrations. Strong, inner-sphere binding, however, was responsible for 0.11-0.55 mmol Cu bound per g C with the higher values corresponding to the LMW fraction of amendment extracts. For the bulk extracts, while there was no clear and consistent pattern of increasing binding capacity following incubation (i.e., with increasing degree of early-stage humification), factors affecting strong binding capacity likely included the availability of aromatic amino N, differences in both total and carboxyl charge density, and the relative percent concentration of Folin-Ciocalteu reactive soluble phenolic acids (FSP). For the LMW fraction, maximum strong binding capacities increased with increasing degree of humification. One plausible explanation for this increase (relative to inconclusive results for bulk extracts) is that LMW humic materials likely carry a greater relative percentage of surface-exposed acidic functional groups and experience less geometric or steric hinderance to metal binding. One potential concern regarding such LMW soluble Cu complexes is that their size likely renders them mobile in soil solution. This mobility may have

implications for soil organism toxicity, the inhibition of plant root development, and the leaching or runoff potential of Cu complexes into adjacent water bodies.

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APPENDIX A: QC Report for Selected Chemical Characteristics of Amendment DOC

Table A1. QC report for selected chemical characteristics of amendment DOC

Week		Ca	Mg	K	Al	Fe	P	NO_3^- - N	NH_4^+ - N
		mg/L							
0	**known**	**10.0**	**1.0**	**50.0**	**0.5**	**0.1**	**1.0**	**4.5**	**5.0**
	recovered	9.7	1.0	45.1	0.5	0.1	1.0	4.5	5.0
	% recovered	96.6	96.5	90.2	104.8	109.0	95.3	99.3	99.6
1	**known**	**10.0**	**1.0**	**50.0**	**0.5**	**0.1**	**1.0**	**4.5**	**5.0**
	recovered	10.1	1.1	47.2	0.6	0.1	1.1	4.5	5.0
	% recovered	101.3	107.0	94.4	121.6	105.0	109.0	99.6	99.6
2	known	**100.0**	**10.0**	**500.0**	**0.5**	**1.0**	**10.0**	**4.5**	**5.0**
	recovered	96.1	9.3	468.4	0.5	0.9	10.1	4.5	5.0
	% recovered	96.1	92.8	93.7	97.4	94.9	101.0	99.8	99.4
4	known	**100.0**	**10.0**	**500.0**	**0.5**	**1.0**	**10.0**	**4.5**	**5.0**
	recovered	94.6	9.1	460.9	0.4	0.9	9.8	4.5	4.9
	% recovered	94.6	90.9	92.2	78.2	92.8	97.9	100.0	98.4
6	known	**100.0**	**10.0**	**500.0**	**0.5**	**1.0**	**10.0**	**4.5**	**5.0**
	recovered	97.0	ND[a]	ND	0.5	1.0	10.1	4.5	4.9
	% recovered	97.0	ND	ND	100.2	99.8	10.0	99.6	98.4
8	known	**100.0**	**10.0**	**500.0**	**0.5**	**1.0**	**10.0**	**4.5**	**5.0**
	recovered	92.5	ND	ND	0.5	0.9	9.6	4.5	4.9
	% recovered	92.5	ND	ND	96.2	92.0	95.5	99.6	98.4

[a]missing data

APPENDIX B: Scatchard Distribution Plots of Cu-Binding Capacity

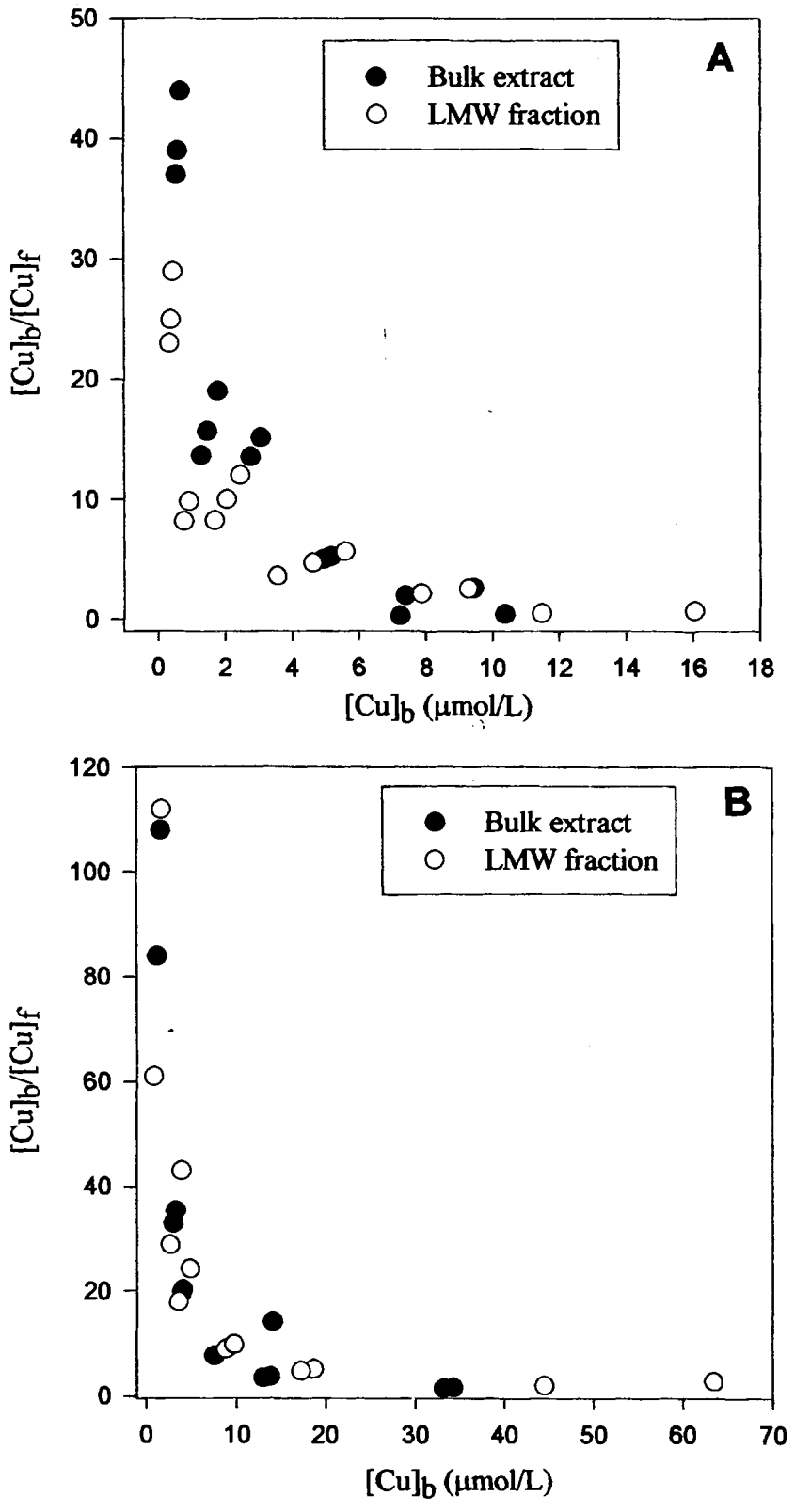

Figure B.1. Scatchard distribution plots for Cu binding capacity of time 0 bulk extract and LMW (<1 kDa) fraction. A) Wheat straw B) Crimson clover

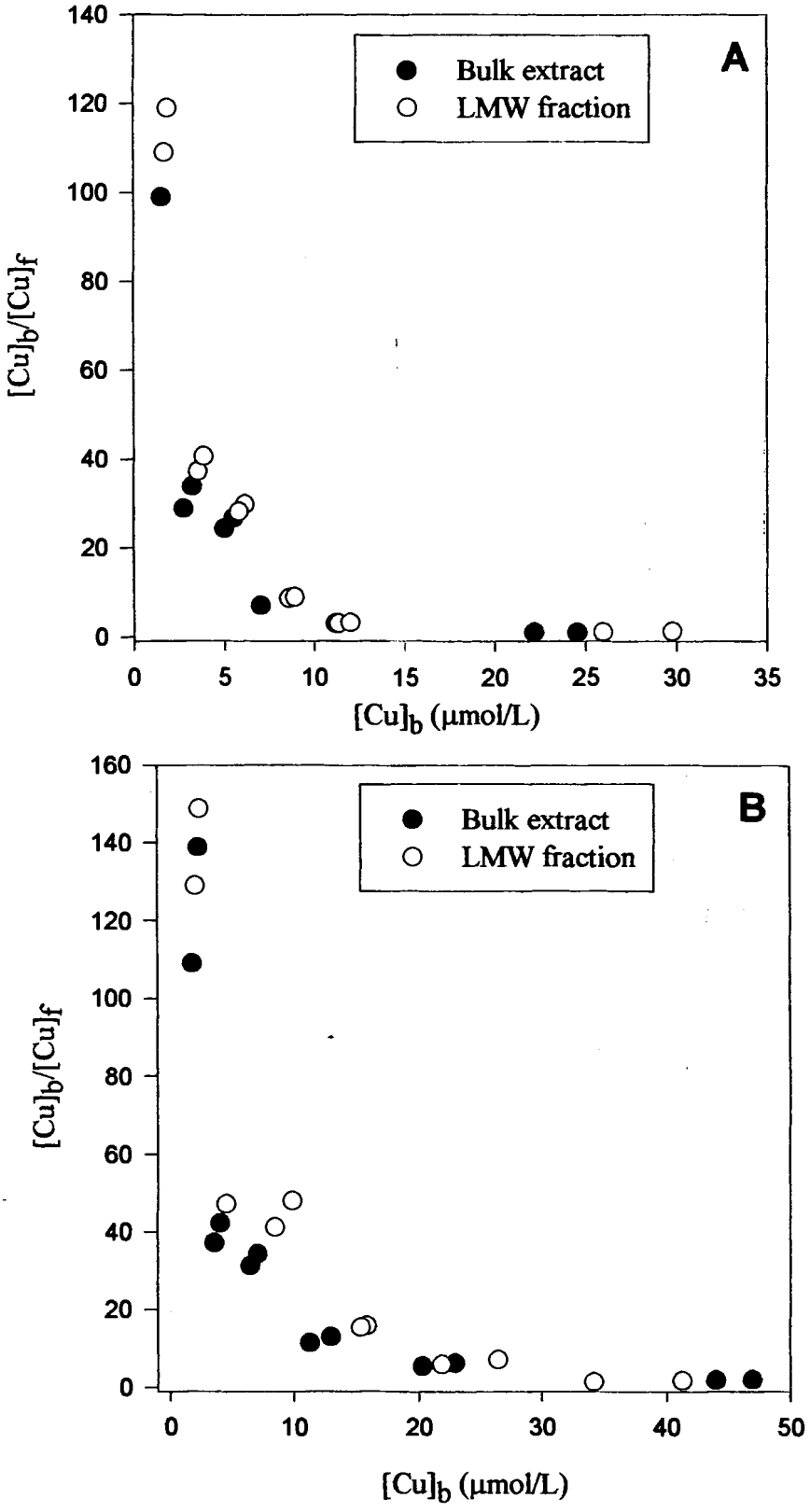

Figure B.2. Scatchard distribution plots for Cu binding capacity of week 1 bulk extract and LMW (<1 kDa) fraction. A) Wheat straw B) Crimson clover

APPENDIX C: Fluorescence Scans

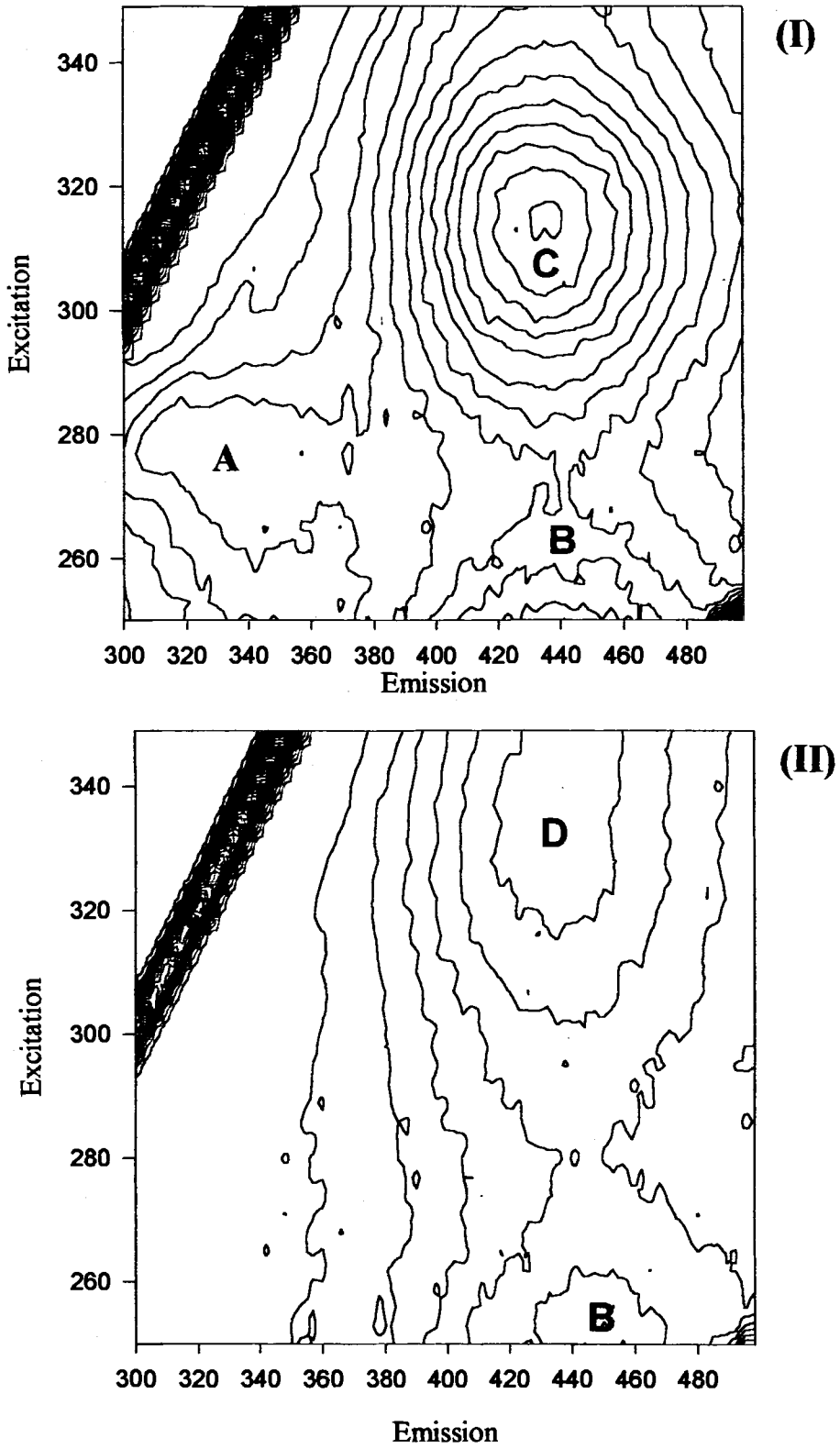

Figure C.1. Wheat straw bulk extract. Transition of key fluorophores between time 0 (I) and week 1 (II). Contour interval = 25 RIU (relative intensity units).

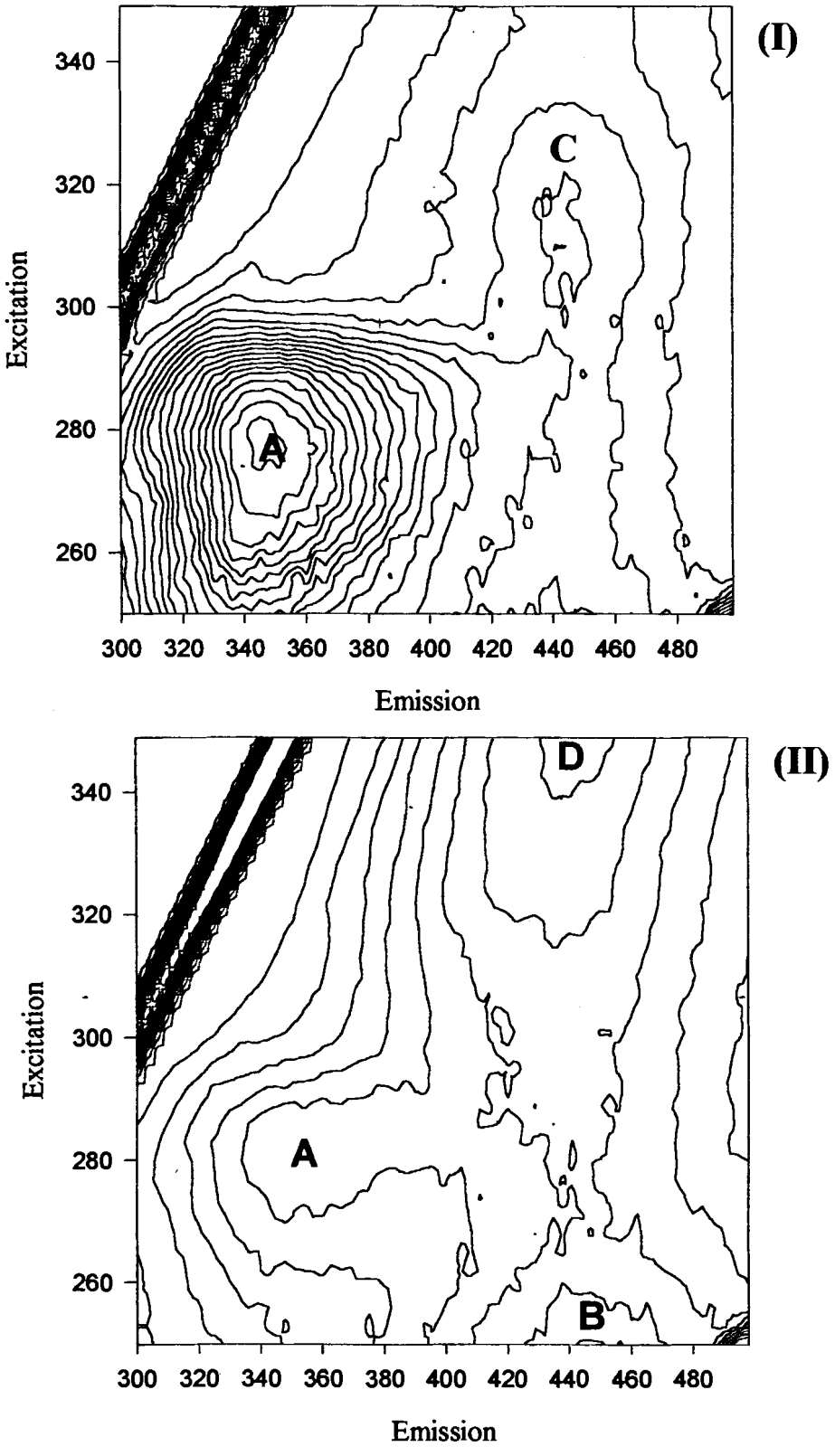

Figure C.2. Crimson clover bulk extract. Transition of key fluorophores between time 0 (I) and week 1 (II). Contour interval = 25 RIU (relative intensity units).

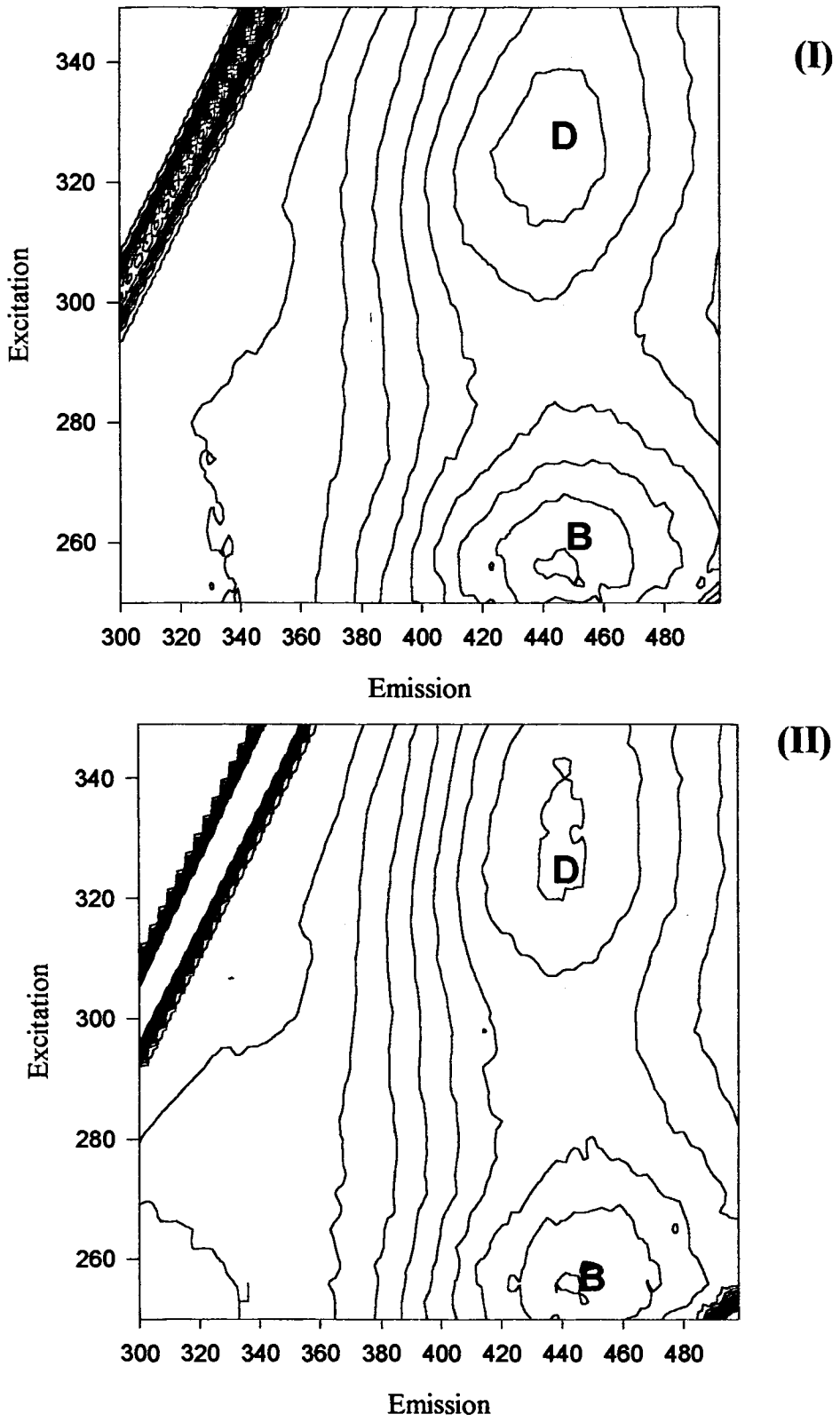

Figure C.3. PPR bulk extract. Transition of key fluorophores between time 0 (I) and week 1 (II). Contour interval = 50 RIU (relative intensity units). PPR = Primary Papermill Residue

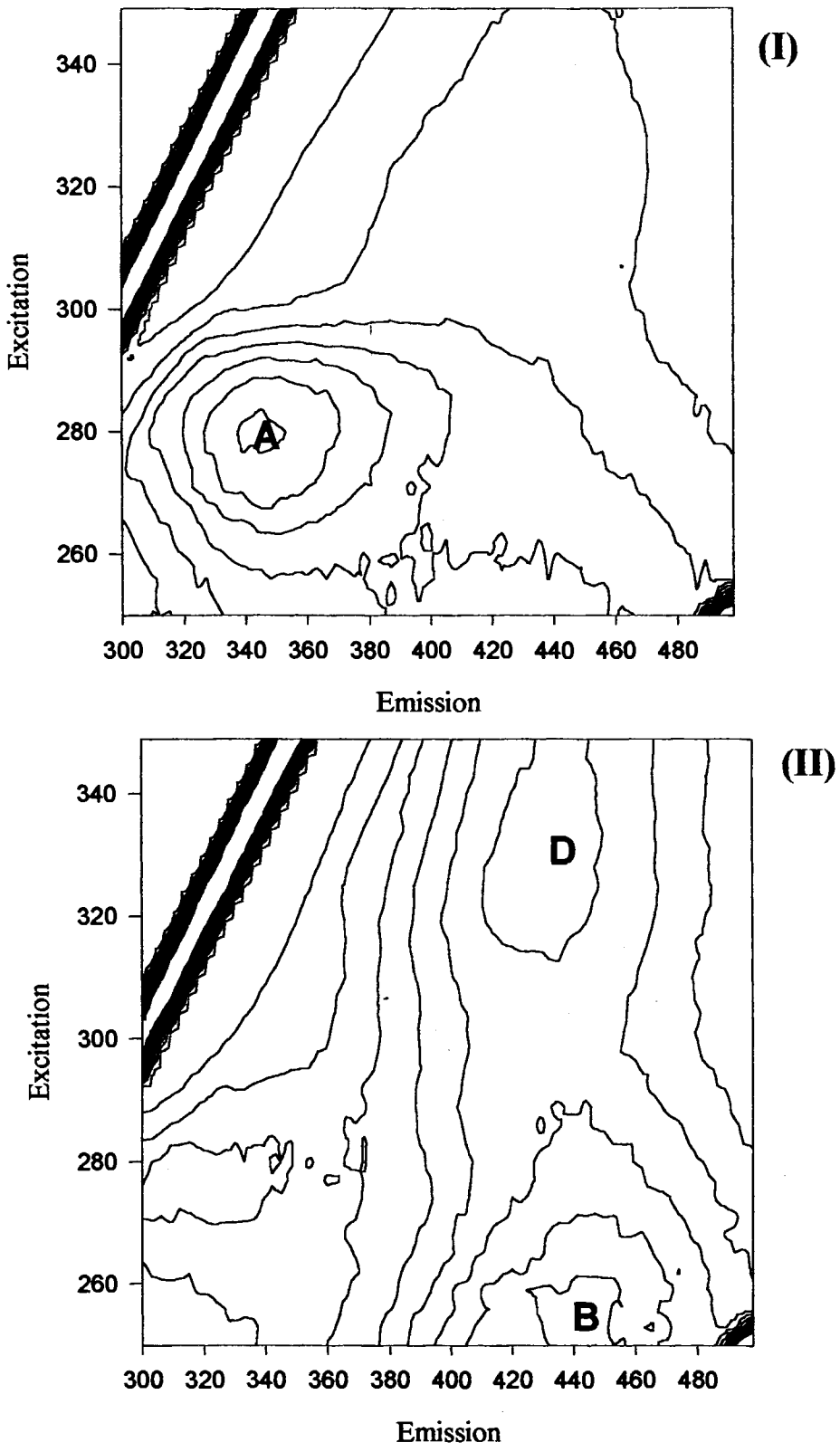

Figure C.4. PPR+SS bulk extract. Transition of key fluorophores between time 0 (I) and week 1 (II). Contour interval = 50 RIU (relative intensity units). PPR+SS = Primary Papermill Residue + Secondary Sludge

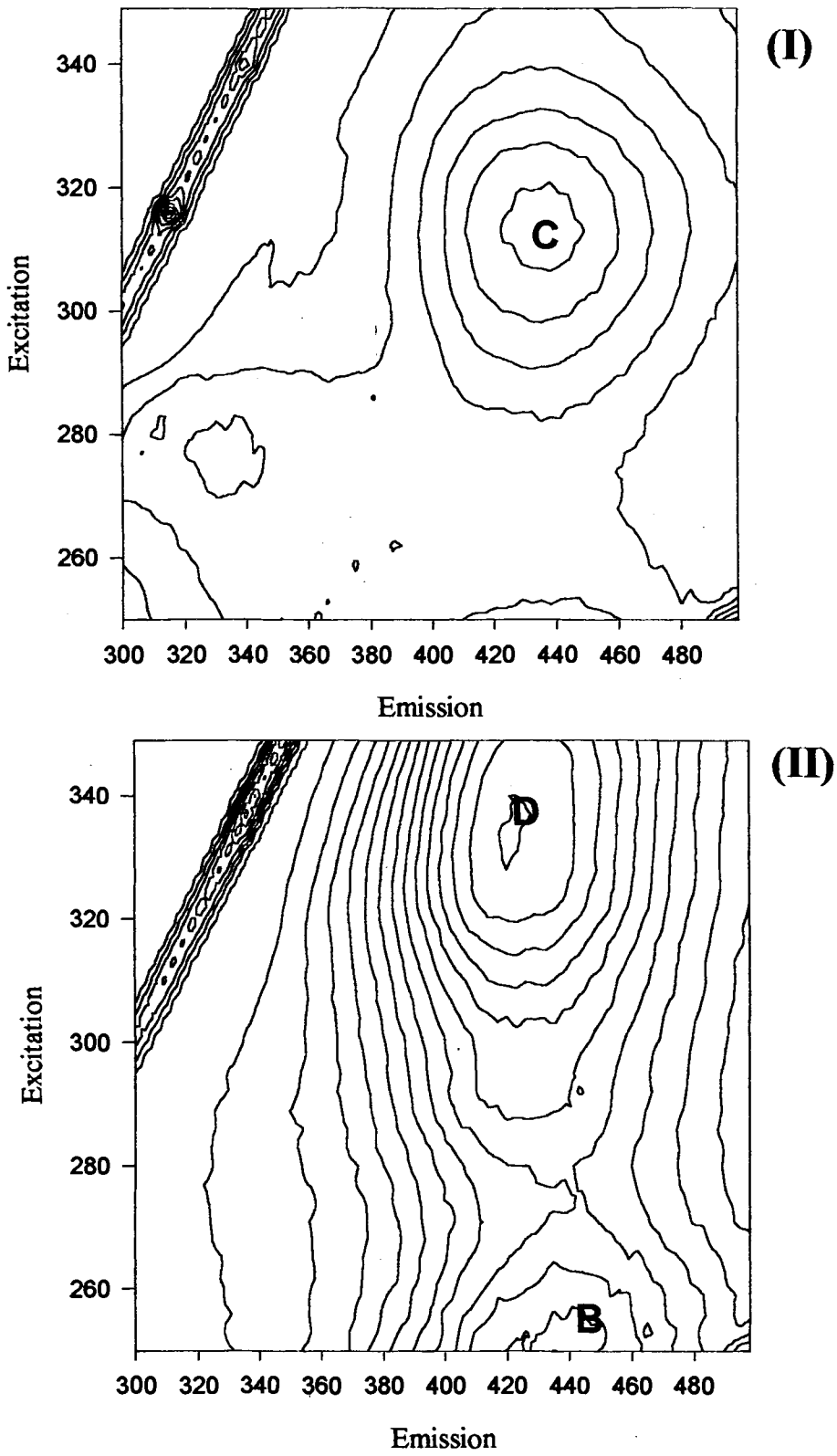

Figure C.5. Wheat straw LMW (<1 kDa) fraction. Transition of key fluorophores between time 0 (I) and week 1 (II). Contour interval = 50 RIU (relative intensity units).

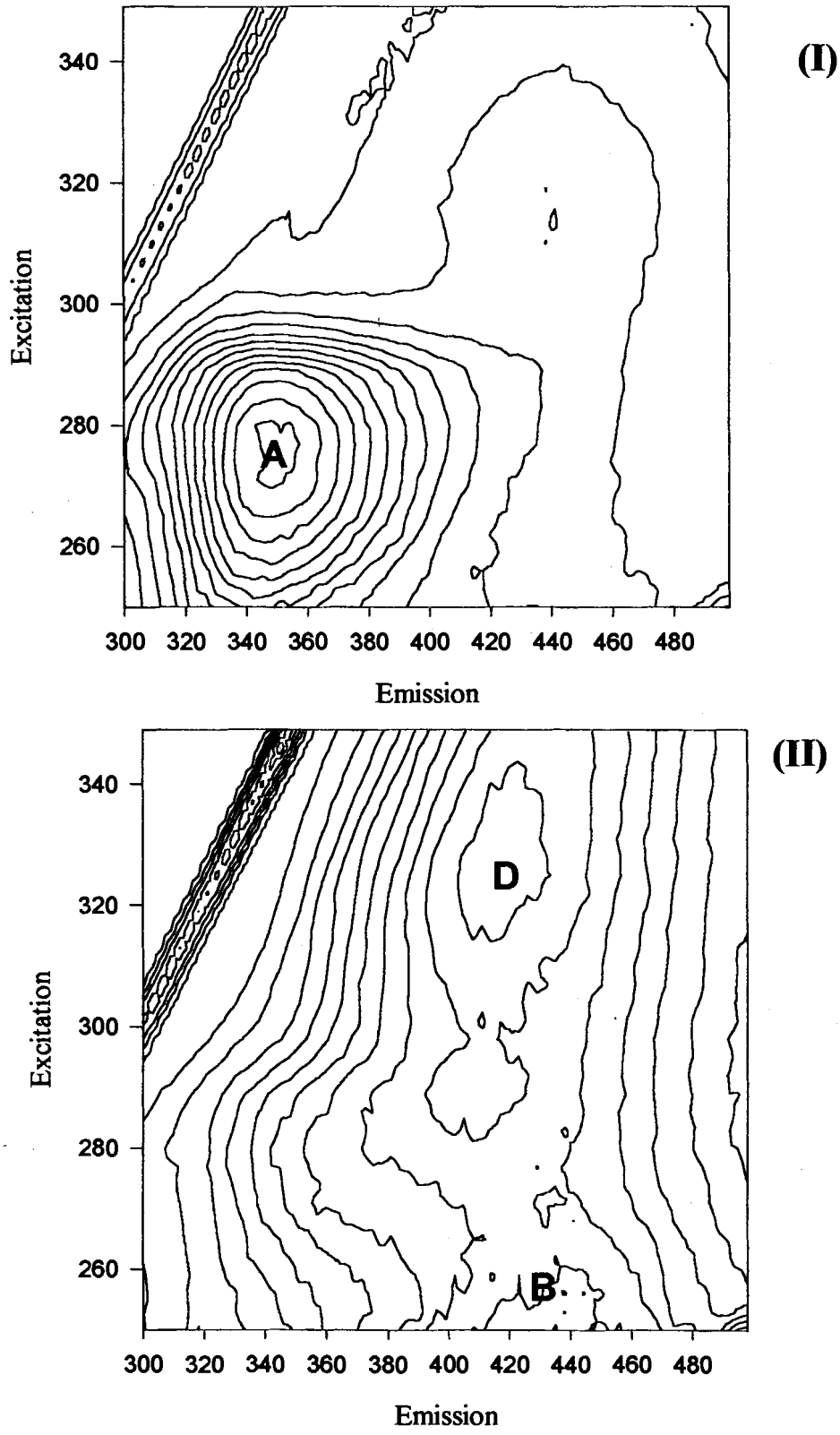

Figure C.6. Crimson clover LMW (<1 kDa) fraction. Transition of key fluorophores between time 0 (I) and week 1 (II). Contour interval = 50 RIU (relative intensity units).

BIOGRAPHY OF THE AUTHOR

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