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Contribution of amino compounds to dissolved organic nitrogen in forest soils

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Abstract. Dissolved organic nitrogen (DON) may play an important role in plant nutrition and nitrogen fluxes in forest ecosystems. In spite of the apparent importance of DON, there is a paucity of information concerning its chemical composition. However, it is exactly this chemical characterization that is required to understand the importance of DON in ecosystem processes. The primary objective of this study was to characterize the distribution of free amino acids and hydrolyzable peptides/proteins in the DON fraction of Oa horizon leachates along an extreme edaphic gradient in northern California. *In situ* soil solutions were extracted by centrifugation from Oa horizons collected beneath *Pinus muricata* (Bishop pine) and *Cupressus pygmaea* (pygmy cypress) on slightly acidic/fertile and highly acidic/infertile sites. DON accounted for 77 to 99% of the total dissolved nitrogen in Oa horizon leachates. Nitrogen in free amino acids and alkyl amines ranged from 0.04–0.07 mg N/L on the low fertility site to 0.45–0.49 mg N/L on the high fertility site, and accounted for 1.5 to 10.6% of the DON fraction. Serine, glutamic acid, leucine, ornithine, alanine, aspartic acid and methylamine were generally the most abundant free amino compounds. Combined amino acids released by acid hydrolysis accounted for 48 to 74% of the DON, suggesting that proteins and peptides were the main contributor to DON in Oa horizon leachates. Together, nitrogen from free and combined amino compounds accounted for 59 to 78% of the DON. Most of the DON was found in the hydrophobic fraction, which suggests the presence of protein/peptide-polyphenol complexes or amino compounds associated with humic substances. Because free and combined amino acids can be an important nitrogen source for some plants, soil DON may play an important role in plant nutrition and ecosystem function.

Introduction

The importance of dissolved organic nitrogen (DON) in ecosystem nitrogen fluxes and plant nutrition is only beginning to be appreciated. Dissolved organic nitrogen is often the dominant nitrogen form leached from the forest floor of both deciduous and coniferous forests (Van Cleve and White 1980; Sollins and McCorison 1981; Fahey and Yavitt 1988; Dahlgren and Ugolini 1989; Qualls and Haines 1991; Northup et al. 1995; Michalzik and Matzner 1999; Michalzik et al. 2001). Similarly, nitrogen export in stream waters from many forested watersheds, especially those not affected by elevated atmospheric deposition of nitrogen, is often domi-

nated by DON (Sollins and McCorison 1981; Qualls 1989; Hedin et al. 1995; Arheimer et al. 1996; Chapman et al. 1998; Campbell et al. 2000; Hagedorn et al. 2000; McHale et al. 2000). Leaching of DON may have several ecological consequences, such as constraining N accumulation in terrestrial ecosystems (leading to N limitations) and enhancing N bioavailability to aquatic ecosystems.

The export of DON from ecosystems having a wide range of nitrogen availability has led to the suggestion that DON losses are not exclusively subject to the traditional mechanisms of direct biotic control, such as mineralization and mineral N uptake (Hedin et al. 1995). This implies that DON is a rather recalcitrant material that is not readily available to the biotic community. However, certain plants have been shown to utilize some forms of dissolved and detrital organic nitrogen, suggesting that DON may be important in plant nutrition. For example, several studies have shown that plants can utilize amino acids irrespective of their different types of root-mycorrhizae associations (Melin and Nilsson 1953; Chapin et al. 1993; Raab et al. 1996, 1999; Näsholm et al. 1998; Lipson et al. 1999). Some plants also utilize proteins and protein-tannin complexes as a nitrogen source (Bajwa and Read 1985; Abuzinadah and Read 1986a, 1986b, 1989; Leake and Read 1989; Finlay et al. 1992; Griffiths and Caldwell 1992; Bending and Read 1996).

Plants with ericoid mycorrhizae are purported to be especially efficient at using organic nitrogen forms (Read 1991). Ericoid mycorrhizae produce extracellular enzymes, such as polyphenol oxidases, peroxidases or tannin carboxyl esterases, which degrade the polyphenols resulting in bound protein becoming available to proteolytic enzymes. (Leake and Read 1989; Bending and Read 1996). Direct utilization of organic nitrogen may result in a 'short-circuiting' of the nitrogen cycle, bypassing the mineralization pathway which is often the major bottleneck restricting the supply of nitrogen to plants (Chapin 1995; Northup et al. 1995). The ability of some species (e.g., *Ericaceous*) to utilize DON in nitrogen deficient ecosystems may provide a competitive advantage that allows these species to alter or inhibit forest succession (Prescott and Weetman 1994; Schimel et al. 1996, 1998; Titus et al. 1995).

The mobility of DON appears to be regulated by sorption to mineral soil components and, to a lesser degree, by biodegradation and uptake by biota (Qualls and Haines 1992). Thus, the mobility of DON will be strongly a function of its chemical composition. In general, hydrophobic compounds (e.g., protein-tannin complexes, amino acids complexed with humic substances) are selectively sorbed in mineral soil horizons, which causes a relative enrichment of the more mobile hydrophilic substances (e.g., amino acids, free peptides and free proteins) in solutions with increasing soil depth (Jardine et al. 1989; Kaiser and Zech 1998). As a result, losses of DON are likely to be strongly linked to hydrologic parameters, such as variations in water flow paths through soils and dissolution kinetics of humic soil components (Hedin et al. 1995). This is especially important in forest soils where subsurface lateral flow through the porous upper horizons (O and A horizons) effectively bypasses the soil zone having the greatest sorption capacity (B horizons). Once in the aquatic ecosystem (streams and lakes), the chemical forms of DON will affect N bioavailability and possibly aquatic primary productivity.

There are many important ecological consequences of DON in terrestrial and aquatic ecosystems. However, the importance of DON in the N cycle is difficult to assess because of uncertainties regarding its composition, sinks, sources, and bioavailability. While several studies have measured bulk DON concentrations, or utilized specific amino acids in plant uptake studies, there is a paucity of information concerning the chemical composition of DON in forest ecosystems. Knowing the chemical forms of nitrogen in DON is essential for elucidating the role of DON in ecosystem processes. Thus, the primary objective of this study was to characterize the quantities and distribution of free and combined (hydrolyzable) amino compounds in the DON fraction of *in situ* soil solutions collected from an extreme edaphic gradient. Dissolved organic matter was separated into hydrophobic and hydrophilic fractions to further characterize DON and to examine its relationship to dissolved organic carbon.

Materials and methods

Research was conducted at the Ecological Staircase located in the Jug Handle Reserve on the northern California coast about 200 km north of San Francisco. The Ecological Staircase consists of a series of five marine terraces ranging in age from about 100,000 to 500,000 years (Merritts et al. 1991). Because all terraces occur within 5 km of each other, they all experience the same Mediterranean-type climate with frequent fog during spring and summer. The mean annual temperature is 12.5 °C and the mean annual precipitation is 983 mm (data for the period of record at nearby [5 km] Fort Bragg, CA; National Oceanic and Atmospheric Administration 1998–99). About 80% of the precipitation occurs between November and March. Soil and plant communities vary dramatically among terraces. Soils on the youngest terrace (Inceptisols) are slightly acidic and fertile supporting highly productive mixed-conifer forests. In contrast, soils on older terraces (Spodosols and Ultisols) are highly acidic and infertile supporting pygmy forests of dwarf (< 3 m) conifers and *Ericaceae* species. The pygmy forest has been described as being as close to a final successionary ecosystem as can be found in nature and the soils supporting it are among the most infertile ever reported (Sholars 1982). This extreme edaphic/biotic gradient provides an ideal opportunity to examine changes in nutrient cycling as soils become progressively older and less fertile.

To examine the effects of soil fertility and acidity on nitrogen cycling, we collected the Oa horizon beneath *Pinus muricata* and *Cupressus pygmaea* on terrace 1 (T1) and terrace 4 (T4). The Oa horizon was chosen because it is the dominant rooting zone for fine roots in the pygmy forest. The Oa horizon consisted of highly decomposed organic materials having no recognizable plant remains. The two sites were selected because they represent end-members of the soil fertility/acidity gradient: T1 = fertile/slightly acid (tall forest site) and T4 = infertile/acidic (pygmy forest site). Samples were collected in mid-April of 1998 and 1999. Replicate Oa horizon samples (5 replicates in 1998 and 3 replicates in 1999) were collected from

beneath the canopy of each species on each terrace. Samples were immediately placed on ice and stored at 3 °C before extraction of soil solutions within 48 hours. To characterize the effect of soil fertility/acidity on plant nutrient status, foliage samples were collected from the same trees where Oa horizon samples were collected. Total carbon and nitrogen in the foliage and Oa horizons were determined by combustion using a Carlo Erba C/N analyzer. Mineral nitrogen (NH_4^+ and NO_3^-) was extracted with 2 M KCl using a 1:10, soil:solution ratio and shaking time of one hour.

Soil solution was extracted from the Oa horizons by centrifugation using double-bottom nylon centrifuge tubes (Dahlgren 1993). The sample was centrifuged (3 °C) for 30 min at an average RCF of 15,530 g calculated at the midpoint of the soil-holding cup. The 1998 Oa horizon samples were split into two subsamples. One subsample was extracted fresh (within 48 hr of collection) while the other subsample was incubated for 7 days at 15 °C (field temperature) prior to soil solution extraction. The rationale for examining the incubated samples was to determine whether amino acid concentrations increased in the absence of plant uptake. Extracted soil solutions were filtered through a pre-rinsed 0.2 μm membrane filter (Nuclepore Track-Etch). Mineral nitrogen (NH_4^+ and NO_3^-) in the extracts was measured using a conductimetric nitrogen analyzer (Carlson 1978, 1986). Total dissolved nitrogen (DON + mineral N) was determined conductimetrically following persulfate oxidation (Yu et al. 1993). DON was calculated by subtracting mineral nitrogen from the total dissolved nitrogen. Dissolved organic carbon (DOC) was measured using persulfate oxidation/IR detection of CO_2 on a Tekmar–Dohrmann (Phoenix 8000) carbon analyzer. Total phenol content of the *in situ* soil solutions was measured by the Folin-Ciocalteau method (Scalbert et al. 1989). Because this technique is based on the reduction of a phosphotungstic-phosphomolybdic reagent, it will also yield a positive result for humic substances that have reducing capacity.

In 1998, 2 or 3 of the five replicates from each sampling site were randomly selected and analyzed for amino compounds. In 1999 all three replicates were analyzed for amino compounds. A total of 21 dissolved primary free amino acids and alkyl amines were quantified by high performance liquid chromatography (HPLC) using a pre-column derivatization method adapted from Jones et al. (1981). We use the term "free amino acids" to refer to amino acids that do *not* exist in peptide linkages. Sigma AA-S-18 amino acid standard solution (2.5 μmol/mL) and individual amino compounds (> 98 % purity, Sigma or Aldrich) were used to make aqueous standard solutions, which were stored at −20 °C. O-phthaldialdehyde/mercaptoethanol (OPA) derivatization reagent was made from 50 mg o-phthaldialdehyde, 50 μL mercaptoethanol, 1.25 mL methanol and 11.2 mL 0.4 M sodium borate buffer (pH 9.5±0.1). All solutions were prepared in purified water ("Milli-Q water"; $\geqslant$18.2 MΩ-cm) from a Millipore Milli-Q Plus system.

For analysis, typically a 200 μL aliquot of standard or sample was derivatized with 25 μL OPA, shaken for 1.0 min, neutralized with 8 μL of 10% acetic acid and injected into the HPLC analytical system. The HPLC system consisted of a C-18 analytical column (Keystone BetaBasic-18, 5 μm bead, 3.0×250 mm) with accompanying guard column, two Shimadzu pumps (LC-10AT and LC-10ATVP), and a

Shimadzu RF-551 fluorescence detector (detection and emission wavelengths of 330 and 420 nm, respectively). Derivatized amino acids and alkyl amines were eluted at a flow rate of 0.44 mL/min using a gradient program with two eluents: Eluent A = methanol and 0.05 M sodium acetate buffer (pH 6.1±0.1) (20:80 v:v); Eluent B = methanol, 0.04 M sodium acetate buffer (pH 6.1±0.1) and tetrahydrofuran (80:19:1 by volume).

Method detection limits for individual amino compounds ranged from 3 to 22 nM, with a mean value of 12 nM. Because glycine and threonine co-elute using this method, their concentrations are reported as the total of the two compounds. Concentrations of individual amino compounds in procedural blanks were always less than detection limits. The average relative percentage difference in the concentrations of individual amino compounds in replicate injections was 9.4% (range = 5 – 17%).

Combined amino compounds (e.g., proteins and peptides) were determined by first hydrolyzing the sample using the method of Tsugita et al. (1987), as modified by Keil and Kirchman (1991) and Confer et al. (1995). The hydrolyzed samples were analyzed for free amino compounds by HPLC as described above. We tested the efficiency (recovery) of our methods by hydrolyzing and analyzing the free amino acid concentrations of Cytochrome *c* (Cyt *c*). For Cyt *c*, there was excellent recovery of amino N from the protein (92% of theoretical) and very good agreement between the known protein sequence and the amounts of recovered free amino acids, with an average relative percent difference between known and recovered amounts for individual compounds of 9.0%.

Dissolved organic matter was fractionated into operationally defined hydrophobic and hydrophilic components using Amberlite XAD-8 resin following a procedure adapted from Leenheer and Huffman (1979). Due to the small soil solution volume (5 – 10 mL) extracted by centrifugation, a batch reaction was employed rather than the traditional flow-through column method. At pH 2, the carboxylic acid groups of the hydrophobic fraction are protonated and the molecules are uncharged, resulting in their retention on the hydrophobic XAD-8 resin. Based on this methodology, hydrophobic substances include humic substances, humic and fulvic acids, tannins and polyphenols (Qualls and Haines 1991). Amino acids, peptides and proteins associated with these hydrophobic substances will also be retained on the resin and therefore included as part of the hydrophobic fraction. The hydrophilic materials that are not retained by the resin include carbohydrates, small carboxylic acids, humic substances with a high COOH/C ratio, and nitrogen-containing compounds such as aromatic amines and amino-sugar polymers. In addition, because they are hydrophilic and positively charged at pH 2, most amino acids, free peptides, and free proteins are expected to be in the hydrophilic fraction (Kroeff and Pietrzyk 1978; Qualls and Haines 1991).

The XAD-8 macroporous resin (40–60 mesh) was cleaned prior to fractionation following the procedure of Thurman and Malcolm (1981). Following cleaning and draining (by gravity), 2 g of XAD-8 resin was added to 2 mL of acidified sample (pH adjusted to 2 with ~ 10 μL of 2 M HCl) and shaken for 5 min. The hydrophobic fraction of the dissolved organic matter was retained on the XAD-8 resin.

The solution containing the hydrophilic fraction was filtered through a 0.2 μm membrane filter (Nuclepore Track-Etch) and analyzed for total nitrogen, DOC, and mineral nitrogen as described above. Concentrations of hydrophobic DON and DOC were calculated by subtracting the values of hydrophilic DON and DOC from the total DON and DOC, respectively.

Results and discussion

The Ecological Staircase edaphic gradient

There are large differences in soil acidity and fertility between the tall forest (T1) and pygmy forest (T4) sites (Table 1). The pH(H_2O) of Oa horizons at T1 (6.0) was about two units higher than T4 (4.1) reflecting the strong acidification that has occurred with increasing soil age. Foliar and Oa horizon nitrogen concentrations in samples from T1 were nearly a factor of two greater than those from T4. At a given site, foliar N concentrations were 33 to 46% greater in *P. muricata* than in *C. pygmaea*. Oa horizons beneath *P. muricata* showed slightly higher N concentrations than Oa horizons sampled beneath *C. pygmaea*. Mineral nitrogen concentrations (NH_4^+ and NO_3^- extractable with 2 M KCl) in Oa horizons were 4 to 7 times greater on T1 compared to T4. In contrast to nitrogen, foliar C concentrations were similar for both *P. muricata* and *C. pygmaea* on T1 and T4. Organic C concentrations in Oa horizons were similar between species; however, the T1 Oa horizon had appreciably higher C concentrations (540 – 550 g/kg) than the T4 Oa horizon (400 – 410 g/kg).

As with foliage and Oa horizon samples, N concentrations in Oa horizon leachates were higher in T1 compared to T4. For example, concentrations of dissolved organic nitrogen (DON) were 1.8 to 3.3 times higher on T1 (*P. muricata* = 4.91 mg/L and *C. pygmaea* = 4.21 mg/L) than leachates from T4 (*P. muricata* = 1.51 mg/L and *C. pygmaea* = 2.40 mg/L). The C/N ratio of the dissolved organic matter (DOC/DON) was about twice as high on T4 (103–125) as compared to T1 (50–55). Soluble mineral nitrogen (NH_4^+ and NO_3^-) concentrations were much lower but exhibited the same trend as DON. Concentrations ranged from 0.58 to 0.94 mg/L on T1 and from 0.03 to 0.07 mg/L on T4. Thus, DON was the dominant nitrogen form in Oa horizon leachates, accounting for 77 to 99% of the total dissolved nitrogen. The dominance of DON relative to mineral nitrogen, especially in the pygmy forest samples (T4), suggests that DON may be an important nitrogen source for biota in these ecosystems.

Contribution of free amino acids to DON

We began the characterization of DON by examining the prevalence of free amino acids and alkyl amines. A typical chromatogram of free amino compounds in an Oa horizon extract is shown in Figure 1A, along with a sample chromatogram of the

Table 1. Concentrations (mean±standard deviation) of nitrogen and carbon in foliage, Oa horizon and Oa horizon leachates collected from *Pinus muricata* and *Cupressus pygmaea* on the Ecological Staircase (T1 = terrace 1, T4 = terrace 4).

Species	Sample type		Nitrogen		Carbon		C/N[a]	
			T1	T4	T1	T4	T1	T4
Pinus muricata	foliage	Total (g/kg)	15.2±1.5	8.8±0.7	496±7	499±4	33	57
	Oa horizon	Total (g/kg)	12.1±0.2	6.3±0.5	551±7	414±4	46	66
		Mineral[b] (mg/kg)	29.4±2.2	4.38±0.66	N.M.[c]	N.M.		
	leachate[d]	Total (mg/L)	5.49±0.23	1.58±0.17	N.M.	N.M.		
		DON/DOC (mg/L)	4.91±0.19	1.51±0.13	244±8	156±26	50	103
		Mineral (mg/L)	0.58±0.14	0.07±0.03	N.M.	N.M.		
Cupressus pygmaea	foliage	Total (g/kg)	11.4±1.2	6.0±1.8	491±14	490±9	43	82
	Oa horizon	Total (g/kg)	10.4±0.3	5.7±0.2	540±15	403±4	52	71
		Mineral (mg/kg)	24.4±3.12	5.53±0.74	N.M.	N.M.		
	leachate	Total (mg/L)	5.48±0.45	2.43±0.23	N.M.	N.M.		
		DON/DOC (mg/L)	4.21±0.21	2.40±0.14	231±1	302±34	55	125
		Mineral (mg/L)	0.94±0.26	0.03±0.01	N.M.	N.M.		

[a]Calculated on a mass basis
[b]NH_4^+ and NO_3^- extracted with 2 M KCl
[c]Leachate was collected by centrifugation
[d]N.M. = not meaningful

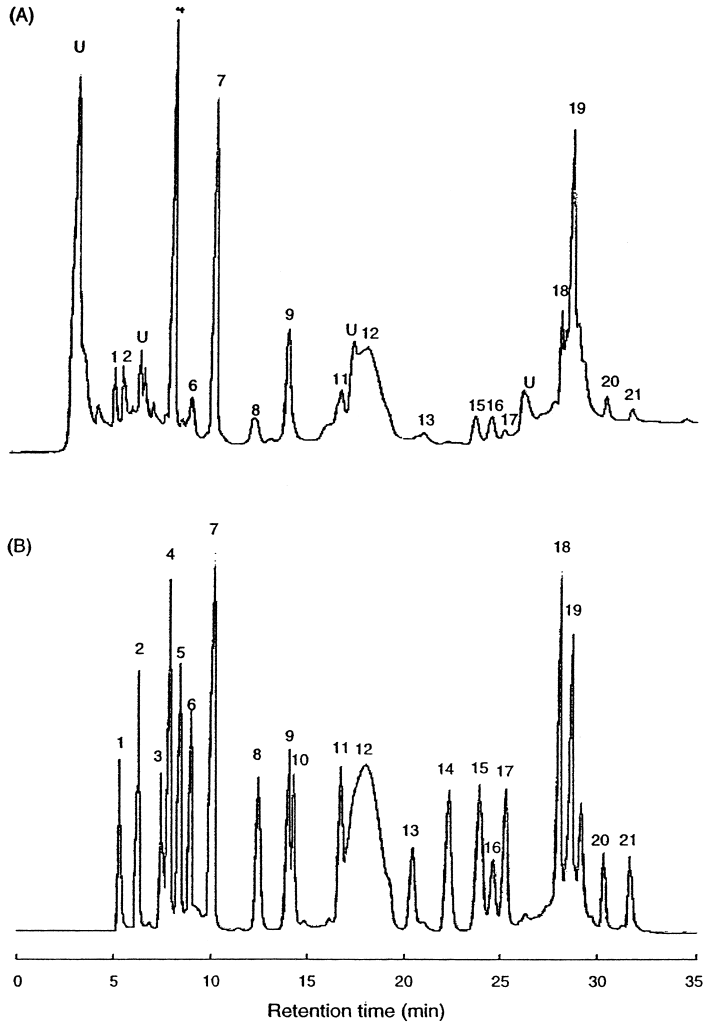

Figure 1. (A) Chromatogram of an Oa horizon extract diluted with Milli-Q water. The identities of numbered peaks are the same as listed in part (B) below. The identities of peaks marked with "U" are unknown. (B) Chromatogram of standard amino compounds: (1) aspartic acid, (2) glutamic acid, (3) histidine, (4) serine, (5) methionine sulfoxide, (6) arginine, (7) glycine and threonine, (8) tyrosine, (9) alanine, (10) 4-aminobutyric acid, (11) ethanolamine, (12) ammonium, (13) tryptophan, (14) methionine, (15) valine, (16) methylamine, (17) phenylalanine, (18) iso-leucine, (19) leucine, (20) ornithine and (21) lysine.

standard amino compound mixture (Figure 1B). Many of the amino compounds in our standard mix were found in most samples and their concentrations ranged widely. In addition, there were a significant number of unidentified peaks in the Oa horizon extracts (Figure1A).

Concentrations of individual free amino compounds in Oa horizon extracts ranged from less than the detection limit to up to 12 μM, while total concentrations (sum of all free amino compounds) ranged from 1.6 to 29.9 μM in fresh samples (Figures 2, 3, 4 and 5)(Appendix). These free amino compound concentrations are similar to the range (1 – 50 μM) of total free amino acids in soil extracts reported by Monreal and McGill (1985). Amino acids identified in this study spanned the range of acidic (e.g., aspartic acid, glutamic acid), neutral (e.g., alanine, glycine, leucine, serine) and basic (e.g., arginine, lysine, histidine) compounds. Generally serine, glutamic acid, aspartic acid, leucine, methylamine and alanine were present in the highest concentrations. On a given terrace, leachates from both *P. muricata* and *C. pygmaea* contained similar amino acid concentrations. However, while ornithine and lysine dominated in *C. pygmaea* leachates, they were generally below detection limits in *P. muricata* leachates. Free amino acid concentrations were generally greater on T1 compared to T4 and concentrations were generally higher in 1999 than 1998 (Figures 2, 3, 4 and 5).

In 1998 samples, leachates were extracted from both fresh and incubated Oa horizons (Figures 2 and 3). After 7 days of aerobic incubation, samples had increased levels of most amino compounds, especially samples associated with *P. muricata*. Because not all replicate samples were analyzed in 1998, we were not able to perform a statistical analysis to document differences between fresh and incubated Oa horizons. Ornithine, lysine and tryptophan were absent in the fresh *P. muricata* leachates, but they were major constituents in the incubated samples of both *P. muricata* and *C. pygmaea*. This suggests that in the field these amino acids might be preferentially taken up from solution by *P. muricata*.

The origin and fate of biologically active compounds, such as free amino acids, in soils are very complex. Free amino acids may originate in soils from: i) leaching of biological tissues (plant, animal and microbial remains), ii) release during the conversion of protein N to NH_3 (proteins $\rightarrow$ peptides $\rightarrow$ amino acids $\rightarrow$ NH_3) by heterotrophic organisms, and iii) plant root/microbial excretion. Once amino acids are released in soils, many factors affect their abundance, including synthesis and destruction by biota, adsorption by clay minerals and reactions with quinones and reducing sugars (Stevenson 1994).

The dominant amino acids identified in this study are consistent with the occurrence of amino acids in acid hydrolysates of soils as reviewed by Stevenson (1994). A large number of unidentified ninhydrin-reacting substances in soil hydrolysates have also been observed in previous studies. For example, Stevenson (1954) isolated 33 amino compounds of which 29 were identified, while Young and Mortenson (1958) reported 57 ninhydrin-reacting substances of which only 24 were identified. Some studies conclude that the amino acid composition from a wide range of soils is remarkably similar (Sowden et al. 1977), while others point out extreme variability between soils (reviewed by Stevenson (1994)). Similarly, cultivation and cropping often show mixed effects on amino acid distribution and abundance (Stevenson 1956; Keeney and Bremner 1964; Khan 1971; Gupta and Reuszer 1967; Senwo and Tabatabai 1998).

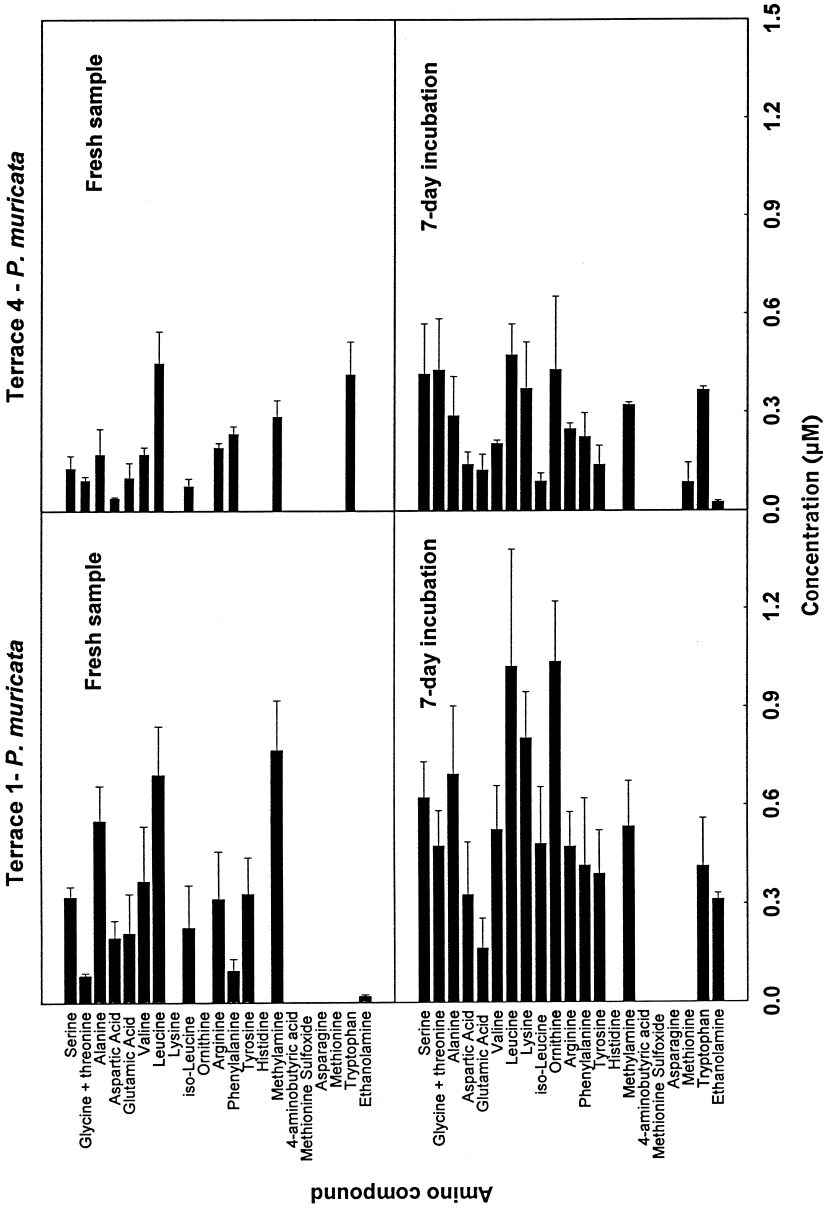

Figure 2. Amino compounds extracted from fresh and incubated *P. muricata* Oa horizon along the Ecological Staircase in April 1998.

The predominant amino acids in the solid-phase soil are often those contained in the cell walls of microorganisms, such as alanine, aspartic acid and glutamic acid. These amino acids are also among the dominant amino acids found in the DON

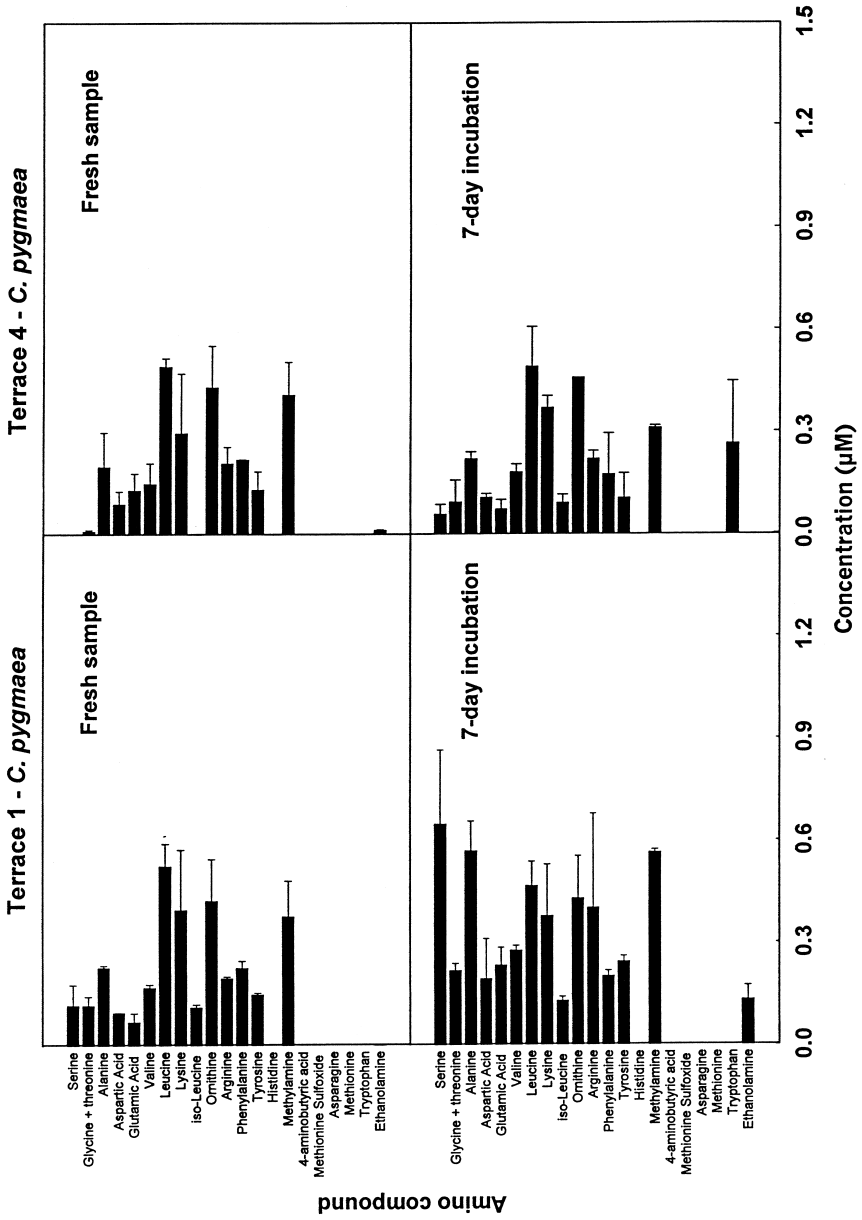

Figure 3. Amino compounds extracted from fresh and incubated *C. pygmaea* Oa horizon along the Ecological Staircase in April 1998.

fraction in this study, suggesting a possible microbial origin of amino acids. Given the large differences in soil fertility between our tall forest (T1) and pygmy forest (T4) sites, we anticipated seeing differences in composition of amino compounds

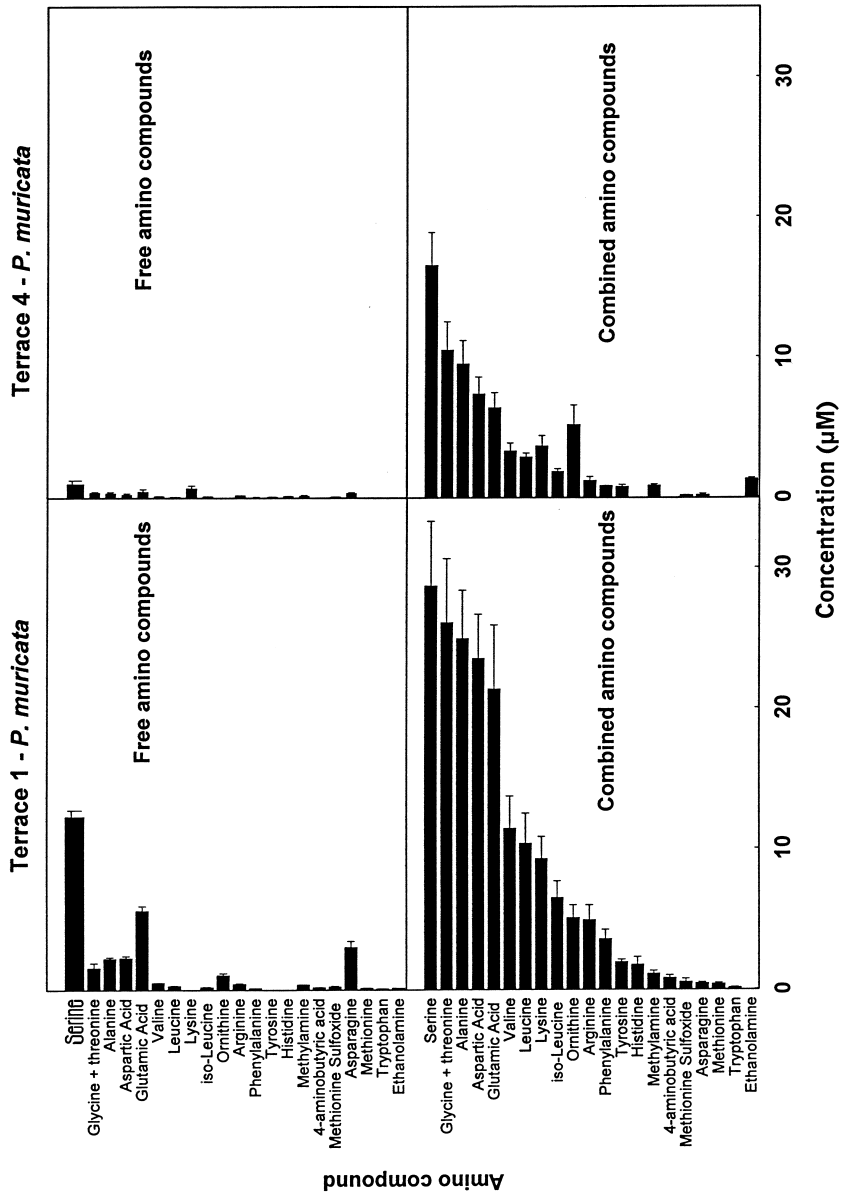

Figure 4. Free and combined amino compounds extracted from fresh *P. muricata* Oa horizon along the Ecological Staircase in April 1999.

between sites. While the composition of amino acids in Oa horizon leachates was similar between terraces in 1998 and 1999, the concentrations of individual amino acids in 1999 were higher on T1 than T4. In 1998, amino acid concentrations generally differed by less than a factor of two between the sites.

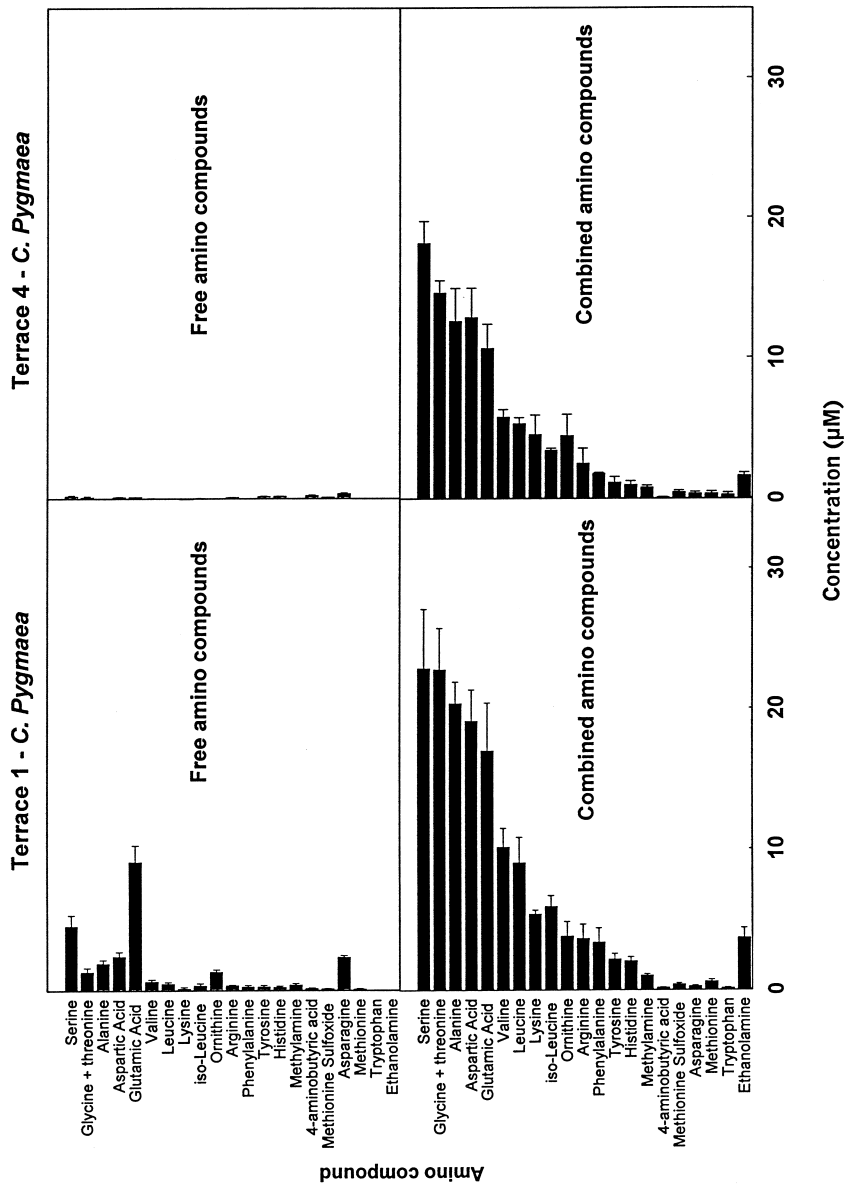

Figure 5. Free and combined amino compounds extracted from fresh *C. pygmaea* Oa horizon along the Ecological Staircase in April 1999.

Nitrogen contained in amino compounds was calculated and compared to other forms of dissolved nitrogen (Figure 6). In 1999 samples, nitrogen in free amino compounds ranged from 0.45 – 0.49 mg/L in T1 to 0.04 to 0.07 mg/L in T4, which accounted for 10 to 10.6% of the DON in T1 and 1.5 to 4.5% of the DON in T4.

For T1 and T4 unhydrolyzed soil leachates, the total height of unknown peaks represented about 10 and 40%, respectively, of the total height of the known peaks. Although we have found that the HPLC method responds to at least one non-amino compound (sulfite), overall the specificity of the derivatization and analysis suggests that these unknown peaks are probably also primary amines. In addition, while response factors (i.e., peak height per mol of N) for all of the known compounds varied over a wide range, response factors for most of the compounds (15 of the 21 studied) were within a factor of 2.5 of each other. Thus, assuming that the unknowns have a similar response factor to the known peaks, our reported values for N in free amino compounds are likely underreported by approximately 10% for T1 and 40% for T4.

Contribution of combined amino acids to DON

In addition to the free amino compounds described above, we quantified the contribution of combined amino compounds (e.g., proteins and peptides) to the DON by hydrolyzing the samples. The distribution of amino compounds following hydrolysis showed the same general trend for both species on both sites (T1 and T4) (Figures 4 and 5). Both *P. muricata* and *C. pygmaea* leachates were dominated by five amino compounds: serine, glycine+threonine, alanine, aspartic acid and glutamic acid. The same amino compounds were often dominant in both the free and combined pools. It is interesting to note that these same amino acids were found to be the five most abundant amino acids in the Williamson River of Oregon (Lytle and Perdue 1981). Concentrations of these individual compounds in combined forms ranged from about 10 to 30 μM (Appendix). The sum of all amino compounds following hydrolysis ranged from 71 to 182 μM. These values were about 6 times greater than the sum of free amino compound concentrations in T1 leachates while they were 21 to 62 times greater than the sum of free amino compound concentrations in T4 leachates.

Combined amino compounds accounted for 48 to 58% of DON in T1 leachates, and 64 to 74% of DON in T4 leachates (Figure 6). In contrast, the free amino compound contribution to DON was only 10.0 to 10.6% in T1 leachates and 1.5 to 4.5% of DON in T4 leachates. The source of combined (hydrolyzable) amino compounds is believed to be primarily proteins and peptides, as well as amino acids associated with humic substances (Stevenson 1994). Nitrogen from free *and* combined amino compounds accounted for 59 to 62% of DON on T1 and 70 to 78% of DON on T4. These results are similar to Hagedorn et al. (2000) who found hydrolyzable amino acids accounted for about 60% of the DON in the mor layer of a pine forest.

Approximately 40% of the DON in T1 leachates and about 25% of the DON in T4 leachates is not accounted for by the identified amino compounds. As seen in the fresh samples, unknown peaks were present in the hydrolyzed samples but they were quantitatively less important. For example, in the 1999 hydrolyzed samples, the total height of unknown peaks represented only 3 to 6% of the total height of the known peaks. We estimate that the unknown amino compounds from HPLC

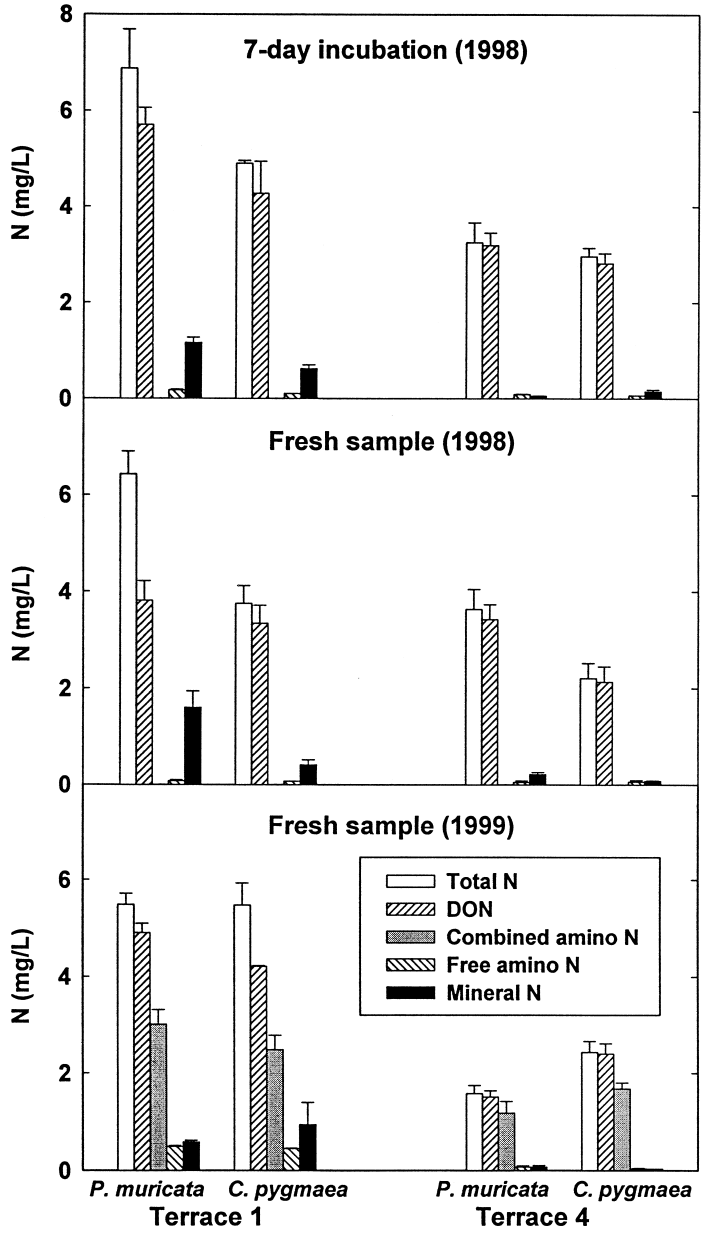

Figure 6. Nitrogen concentrations (mean±standard deviation) in Oa horizon leachates under *P. muricata* and *C. pygmaea* along the Ecological Staircase. (Combined amino compounds were not measured in the 1998 samples).

analysis (free and combined amino compounds) could account for approximately an additional 10% of the DON fraction.

Our results for the DON fraction are similar to results reported previously for acid hydrolysates of soil samples. Sowden et al. (1977) found that the proportions of unidentified hydrolyzable N (16 to 18%) and nonhydrolyzable N (11 to 16%) from the soil nitrogen pool were remarkably consistent between all soils examined. Much of the nonhydrolyzable N may be attributed to heterocyclic nitrogen compounds (Schnitzer and Spiteller 1986; Schulten and Schnitzer 1998). While combined and free amino compounds account for the bulk of DON in Oa horizon leachates from the Ecological Staircase, additional research is required to account for the unidentified organic nitrogen compounds.

Distribution of DON between hydrophobic and hydrophilic fractions

We examined DON partitioning between hydrophobic and hydrophilic fractions in 1999 Oa horizon leachates based on its reaction with XAD-8 resin. In all samples, most of the DON was found in the hydrophobic dissolved organic matter (DOM) fraction (Figure 7). In T1 leachates, 85 to 90% of the DON was hydrophobic while 60 to 75% of the DON was hydrophobic in T4 samples. The primary N-containing compounds in the hydrophobic DOM fraction are believed to be protein-polyphenol complexes and/or amino compounds complexed with humic substances (Qualls and Haines 1991). Hydrogen bonding between proteins and polyphenols tends to make these compounds more hydrophobic as the degree of complexation increases. Heterocyclic nitrogen and amino acids, peptides and proteins bound to soluble humic substances (humic/fulvic acids) are also expected to contribute to the hydrophobic DOM fraction (Schulten and Schnitzer 1998). Current methodologies do not allow for an unambiguous separation between protein-tannin complexes and amino compounds associated with humic substances. Nitrogen in the hydrophilic fraction generally consists of non-bound proteins and peptides, free amino acids, nucleic acid bases and amino sugars, along with any of these compounds associated with hydrophilic DOC compounds (Kroeff and Pietrzyk 1978; Qualls and Haines 1991; Schulten and Schnitzer 1998).

DOC in Oa horizon leachates was dominated by hydrophobic components with greater than 80% of the DOC occurring in this fraction (Figure 7). The C/N ratio of the hydrophobic DOM was generally higher than the hydrophilic fraction with the exception of *C. pygmaea* leachates from T1 where the ratios were similar. All leachates had a high total phenol content (> 20 mg/kg tannic acid equivalents) and there was a strong correlation between total phenol content and the concentration of combined amino compounds (Figure 8). This relationship is consistent with the presence of protein/peptide-polyphenol complexes that render the compounds hydrophobic. However, the inability to distinguish the contribution of phenols from plant polyphenols and humic substances prevents us from unambiguously categorizing the dominant form of hydrophobic DON. We hypothesize that the lower C/N ratios in the hydrophilic fraction result from the presence of non-complexed amino acids and proteins/peptides.

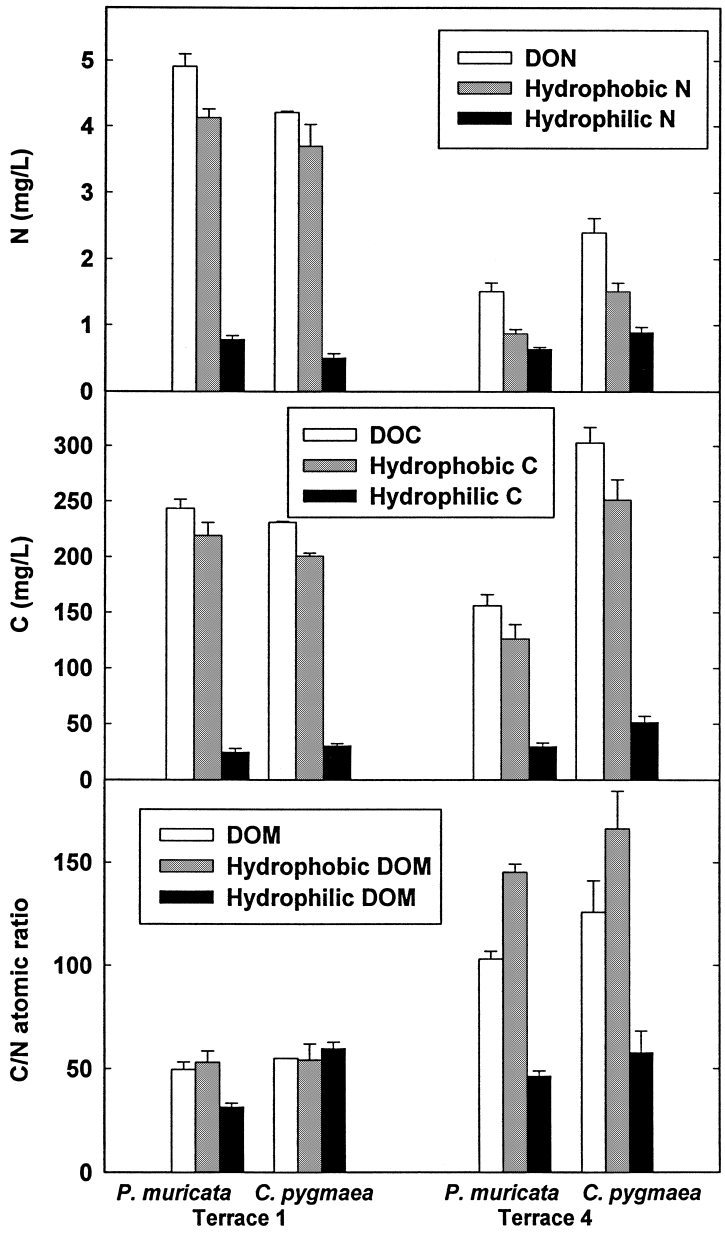

Figure 7. Hydrophobic and hydrophilic fractions (mean±standard deviation) of DON and DOC in Oa horizon leachates under *P. muricata* and *C. pygmaea* along the Ecological Staircase in April 1999.

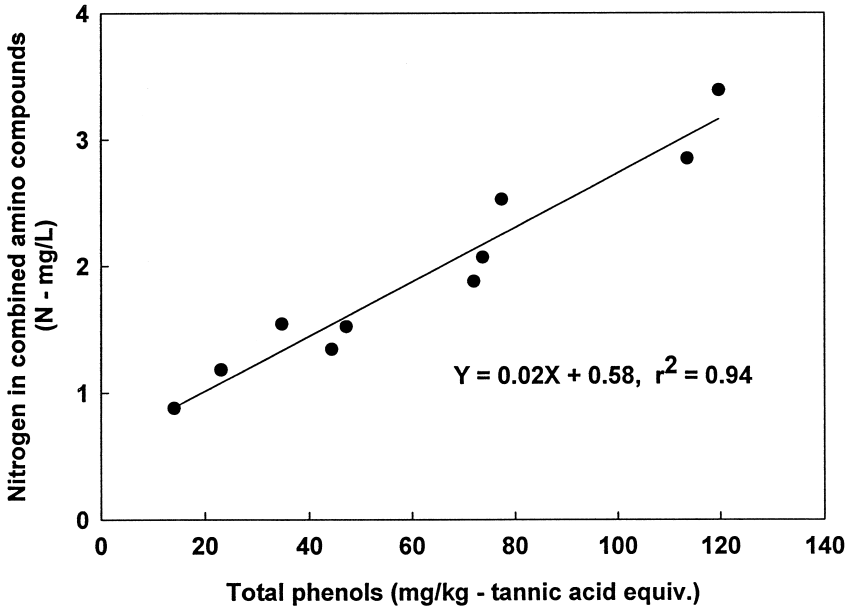

Figure 8. Correlation between the concentrations of total phenols and nitrogen contained in combined amino compounds of Oa horizon leachates under *P. muricata* and *C. pygmaea* along the Ecological Staircase in April 1999.

Implications for nitrogen cycling

In a review of DON concentrations in Oa horizon leachates from a wide range of temperate forests, Michalzik et al. (2001) reported that values ranged between 0.8 and 2.5 mg N/L. At our study sites DON concentrations ranged from 1.5 to 4.9 mg N/L. The more fertile, less acidic T1 site had DON levels about 3-fold greater than the less fertile, acidic T4 site. Higher DON concentrations on T1 may result from greater N availability or from the higher pH (T1 pH $\approx$ 6 versus T4 pH $\approx$ 4). In forest floor leachate, DOC levels were positively correlated with forest floor pH across a wide range of temperate forests (Michalzik et al. 2001). These authors found that DON concentrations were not correlated with total N pools or mineral N additions from atmospheric deposition.

Temperate forest ecosystems having a wide range of nitrogen availability all export DON. This has led to the suggestion that DON is not biologically available and not subject to direct biotic control (Hedin et al. 1995). The chemical composition of DON determined in this study can provide some evidence as to the potential bioavailability of DON. Previous studies showed that a wide range of plants have the ability to utilize free amino acids as a nitrogen source (Melin and Nilsson 1953; Chapin et al. 1993; Näsholm et al. 1998; Raab et al. 1996, 1999; Padgett and Leonard 1996; Lipson et al. 1999). Näsholm et al. (1998) showed that uptake rates for glycine by *Pinus sylvestris, Picea abies, Vaccinium myrtillus*, and the grass *De-*

schampsia flexuosa were similar to that of $^{15}NH_4^+$. Nitrogen contained in free amino compounds in Oa horizon leachates from T1 ranged from 0.45 to 0.49 mg N/L, which equals 50 to 80% of the mineral N concentrations (Table 1). In contrast, Oa horizon leachates from T4 contained 0.04 to 0.07 mg N/L in free amino compounds, which is similar to mineral N concentrations at this site. The samples that were incubated for 7 days in the absence of plant uptake showed increased total free amino compound concentrations of 16 to 60% and 87 to 108% in Oa horizon leachates from beneath *C. pygmaea* and *P. muricata*, respectively (Appendix). This increase may reflect the amount of free amino compounds that would be taken up by vegetation. Collectively, these data suggest that free amino compounds could be a source of available nitrogen in our ecosystem.

Several studies have also shown that plants can utilize proteins (Abuzinadah and Read 1986b; Finlay et al. 1992) and protein-tannin complexes (Leake and Read 1989; Bending and Read 1996) as a nitrogen source. The N contained in the hydrophilic fraction of the DOM is generally believed to exist as free amino acids, free peptides, free proteins, aromatic amines and amino-sugar polymers (Kroeff and Pietrzyk 1978; Qualls and Haines 1991). In T1 leachates, N contained in the hydrophilic fraction nearly equaled N in the free amino compound fraction. In T4 leachates, N in the hydrophilic fraction (0.7 to 0.9 mg N/L) exceeded N in the free amino compound fraction by 10-fold (0.04–0.07 mg N/L, Figures 6 and 7). This difference may reflect higher concentrations of free peptides and free proteins contained in T4 Oa horizons.

The largest quantity of N contained in DON was found in the hydrophobic DOM fraction. Protein-tannin complexes and/or amino compounds complexed with humic substances are believed to be the primary N-containing compounds in the hydrophobic DOM fraction (Qualls and Haines 1991). Current methodologies do not allow for an unambiguous separation between protein-tannin complexes and amino compounds associated with humic substances. While Ericoid mycorrhizae have been shown to utilize proteins from protein-tannin complexes (Leake and Read 1989; Bending and Read 1996), little is known about the ability of plant-mycorrhizae associations to utilize amino acids and proteins complexed with humic substances. Further research directed at the bioavailability of protein-tannin complexes and amino compounds complexed with humic substances appears warranted given the prevalence of DON contained in the hydrophobic DOM fraction of many forest soils.

The mobility of DON appears to be regulated by sorption to mineral soil components (Qualls and Haines 1992). Sorption is related to the chemical composition of DON and interactions with other DOM components (e.g., humic substances and tannins). In general, hydrophobic compounds (e.g., protein-tannin complexes, amino acids complexed with humic substances) are selectively sorbed in mineral soil horizons, which causes a relative enrichment of the more mobile hydrophilic substances (e.g., amino acids, free peptides and free proteins) in solutions with increasing soil depth (Jardine et al. 1989; Kaiser and Zech 1998). Therefore, the dominance of DON (60 to 90%) in the hydrophobic fraction may limit the loss of DON from T1 and T4 ecosystems. The hydrophilic DON fraction is the most mo-

bile and therefore the most likely to enter surface waters. Although the impacts of DON on aquatic ecosystems are not as significant as those of inorganic N forms, DON is potentially bioavailable and may affect the primary productivity of aquatic ecosystems.

The ratio of DOC to DON has been proposed as a measure of ecosystem N saturation, or the capacity of ecosystems to immobilize N into the soil N pool (Harriman et al. 1998; Campbell et al. 2000). Harriman et al. (1998) found that DOC:DON decreased with increasing N saturation and increased export of mineral N from the watershed. A DOC:DON threshold of 25–30 in catchment runoff was proposed as a value for separating N-saturated from N-limited sites. At our sites, the mean DOC:DON ratio was 52 for T1 and 114 for T4 Oa horizon leachates. Mineral nitrogen concentrations for Oa horizon leachates from T1 ranged from 0.6 to 0.9 mg N/L while those from T4 ranged from 0.03 to 0.07 mg N/L. Our data also follows a trend of decreasing DOC:DON ratios with increasing N availability, consistent with the findings of Harriman et al. (1998).

A prevalence of DON in nitrogen deficient ecosystems has been associated with a nitrogen conservation mechanism and with a mechanism that allows certain plants to compete for a limited nitrogen supply (Chapin 1995; Northup et al. 1995). Direct utilization of organic nitrogen 'short-circuits' the nitrogen cycle by eliminating the need for complete mineralization. Mineralization is often the major constraint on nitrogen supplies to plants. In nitrogen deficient ecosystems, certain plant-mycorrhizal associations capable of utilizing DON may be able to utilize the ecosystem nitrogen supply more effectively, thereby providing the symbionts with a competitive advantage. Nitrogen in a form more recalcitrant than mineral N appears to alter N availability and to attenuate N losses from leaching and denitrification.

Conclusions

The importance of DON in the N cycle is difficult to assess because of uncertainties regarding its composition, sinks, sources, and bioavailability. This study contributes important new information regarding the chemical forms of DON, which is essential for elucidating the role of DON in ecosystem processes. Dissolved nitrogen in Oa horizon leachates along the soil fertility/acidity gradient of the Ecological Staircase was dominated by DON (77 to 99%) in both the tall forest (T1) and pygmy forest (T4) sites. In the 1999 leachates, nitrogen in free amino acids and alkyl amines ranged from 0.45 – 0.49 mg N/L on T1 to 0.04 – 0.07 mg N/L on T4 and accounted for 1.5 to 10.6% of the DON fraction. Combined amino compounds accounted for 48 to 74% of the DON fraction, suggesting that protein and peptides were the main contributor to DON in Oa horizon leachates. Together, nitrogen from identified free *and* combined amino compounds accounted for 59 to 78% of the DON. We estimate that unknown free and combined amino compounds present in our HPLC analysis could account for an additional 10% of the DON fraction. Thus, approximately 10 to 30% of the nitrogen contained in the DON fraction of the Oa

horizon leachates was unaccounted for. Based on literature accounts, much of the nonhydrolyzable nitrogen may be attributed to heterocyclic nitrogen compounds. Fractionation of DOM into hydrophobic and hydrophilic fractions showed that most of the DON (60 to 90%) was hydrophobic, suggesting the presence of protein/peptide-polyphenol complexes or amino compounds associated with humic substances. Because free and combined amino acids can be an important nitrogen source for some plants, soil DON may play an important role in plant nutrition and ecosystem function.

Appendix

Table A1. Concentration (nM; mean±standard deviation) of individual amino compounds in litter leachates extracted from *Pinus muricata* (P) and *Cupressus pygmaea* (C) litter along the Ecological Staircase (T1 = terrace 1, T4 = terrace 4).

Compound	Chemical Formula	1998 samples							
		Fresh samples				7-day incubation			
		P – T1	C – T1	P – T4	C – T4	P – T1	C – T1	P – T4	C – T4
Ethanolamine	C_2H_7NO	18±6	1±0	N/D[a]	9±2	313±19	133±42	24±6	N/D
Tryptophan	$C_{11}H_{12}N_2O_2$	N/D	N/D	413±101	N/D	413±164	N/D	365±11	263±183
Methionine	$C_5H_{11}NO_2$	N/D	N/D	N/D	N/D	N/D	N/D	85±60	N/D
Asparagine	$C_4H_8N_2O_3$	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Methionine Sulfoxide	$C_5H_{11}NO_3S$	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
4-aminobutyric acid	$C_4H_9NO_2$	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Methylamine	CH_3NH_2	766±154	377±104	285±50	406±96	534±139	564±8	320±9	309±7
Histidine	$C_6H_9N_3O_2$	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Tyrosine	$C_9H_{11}NO_3$	330±112	149±5	N/D	128±54	391±132	244±17	139±58	103±73
Phenylalanine	$C_9H_{11}NO_2$	97±37	227±21	232±24	216±1	416±204	202±16	223±74	172±121
Arginine	$C_6H_{14}N_4O_2$	315±145	197±5	190±15	205±49	474±105	402±276	247±18	218±24
Ornithine	$C_5H_{12}N_2O_2$	N/D	423±122	N/D	428±122	1037±184	429±124	428±224	456±125
iso-Leucine	$C_6H_{13}NO_2$	228±130	112±8	74±23	N/D	482±173	129±12	89±25	89±24
Lysine	$C_6H_{14}N_2O_2$	N/D	396±177	N/D	294±175	804±142	377±151	371±142	367±35
Leucine	$C_6H_{13}NO_3$	691±150	524±67	448±98	489±25	1023±358	465±72	473±95	488±117
Valine	$C_5H_{11}NO_2$	369±167	170±9	170±22	145±61	525±134	277±14	203±11	179±23
Glutamic Acid	$C_5H_9NO_4$	212±121	69±26	99±46	126±50	166±91	233±52	123±48	70±28
Aspartic Acid	$C_4H_7NO_4$	198±54	96±1	37±5	86±37	329±159	194±117	140±38	105±11
Alanine	$C_3H_7NO_2$	551±108	228±7	170±79	195±102	694±209	568±88	288±120	218±21
Glycine + threonine	$C_2H_5NO_2$ / $C_4H_9NO_3$	83±9	118±26	92±12	6±4	476±107	218±21	427±158	91±65
Serine	$C_3H_7NO_3$	322±33	118±60	128±39	N/D	622±110	647±217	415±154	55±29
Total[b] (nM)		4179	3205	2337	2732	8698	5081	4360	3182

[a]N/D = Not detectable

[b]Sum of amino compound concentrations

Table A2. Concentration (nM; mean±standard deviation) of individual amino compounds in litter leachates extracted from *Pinus muricata* (P) and *Cupressus pygmaea* (C) litter along the Ecological Staircase (T1 = terrace 1, T4 = terrace 4).

Compound	Chemical Formula	1999 samples							
		Fresh samples				After hydrolysis			
		P - T1	C - T1	P - T4	C - T4	P - T1	C - T1	P - T4	C - T4
Ethanolamine	C_2H_7NO	79±9	20±5	4±2	12±7	N/D[a]	3654±702	1294±79	1565±224
Tryptophan	$C_{11}H_{12}N_2O_2$	30±16	27±13	N/D	N/D	101±21	90±36	N/D	202±175
Methionine	$C_5H_{11}NO_2$	81±20	95±55	N/D	N/D	349±79	542±166	N/D	287±164
Asparagine	$C_4H_8N_2O_3$	3008±431	2361±147	270±60	353±74	409±71	220±57	144±96	283±131
Methionine Sulfoxide	$C_5H_{11}NO_3S$	185±37	113±38	30±15	105±3	507±216	344±104	115±24	397±118
4-aminobutyric acid	$C_4H_9NO_2$	127±21	140±61	N/D	213±50	787±189	115±31	N/D	30±20
Methylamine	CH_3NH_2	337±20	390±124	97±58	N/D	1074±234	990±105	801±105	711±158
Histidine	$C_6H_9N_3O_2$	N/D	229±92	65±31	172±27	1733±554	2012±307	N/D	907±277
Tyrosine	$C_9H_{11}NO_3$	N/D	266±107	29±10	154±28	1914±181	2156±393	716±146	1074±420
Phenylalanine	$C_9H_{11}NO_2$	71±19	265±110	8±5	N/D	3570±659	3344±1014	788±14	1713±69
Arginine	$C_6H_{14}N_4O_2$	375±54	363±52	117±29	96±6	4903±1081	3613±1026	1158±278	2449±1068
Ornithine	$C_5H_{12}N_2O_2$	998±152	1333±168	N/D	N/D	5060±916	3783±1032	5149±1387	4405±1529
iso-Leucine	$C_6H_{13}NO_2$	159±26	351±168	54±21	24±9	6500±1187	5879±780	1807±199	3365±1617
Lysine	$C_6H_{14}N_2O_2$	N/D	137±106	623±193	N/D	9251±1579	5346±278	3629±746	4485±1411
Leucine	$C_6H_{13}NO_3$	225±40	490±123	16±8	17±9	10343±2148	9000±1811	2845±271	5278±431
Valine	$C_5H_{11}NO_2$	461±21	654±137	67±26	29±10	11406±2294	10119±1341	3276±567	5746±535
Glutamic Acid	$C_5H_9NO_4$	5592±346	9135±1175	389±184	111±8	21333±4559	16962±3425	6354±1087	10634±1744
Aspartic Acid	$C_4H_7NO_4$	2236±151	2430±335	162±99	102±29	23502±3168	19082±2235	7345±1206	12837±2096
Alanine	$C_3H_7NO_2$	2186±110	1925±269	269±97	N/D	24926±3464	20327±1545	9494±1662	12579±2341
Glycine + threonine	$C_2H_5NO_2/C_4H_9NO_3$	1538±344	1326±305	327±65	87±48	26065±4579	22742±2970	10477±2018	14589±880
Serine	$C_3H_7NO_3$	12253±469	4576±792	940±261	173±65	28670±46102	22838±4248	16504±2371	18133±1595
Total[b] (nM)		29942	26626	3469	1648	182404	153158	71897	101660

[a] N/D = Not detectable

[b] Sum of amino compound concentrations

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