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Long-term effects of elevated nitrogen on forest soil organic matter stability

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Abstract. Nitrogen addition may alter the decomposition rate for different organic-matter pools in contrasting ways. Using a paired-plot design, we sought to determine the effects of long-term elevated N on the stability of five organic-matter pools: organic horizons (Oe + a), whole mineral soil (WS), mineral soil fractions including the light fraction (LF), heavy fraction (HF), and a physically recombined fraction (RF). These substrates were incubated for 300 days, and respiration, mineralized N, and active microbial biomass were measured. Samples with elevated N gave 15% lower cumulative respiration for all five substrates. Over the 300-day incubation, the Oe + a gave twice the cumulative respiration ($g\,C\,kg^{-1}$ initial C) as the LF, which gave slightly higher respiration than the HF. Respiration was 35% higher for the WS than for the RF. Mineralized N was similar between N treatments and between the LF and HF. Net N mineralized by the LF over the course of the 300-day incubation decreased with higher C:N ratio, due presumably to N immobilization to meet metabolic demands. The pattern was opposite for HF, however, which could be explained by a release of N in excess of metabolic demands due to recalcitrance of the HF organic matter. Mineralized N increased with respiration for the HF but showed no pattern, or perhaps even decreased, for the LF. WS and RF showed decreasing active microbial biomass near the end of the incubation, which corresponded with decreasing respiration and increasing nitrate. Our results show that long-term elevated N stabilized organic matter in whole soil and soil fractions.

Introduction

Many short-term studies have shown increased degradation of organic matter in response to elevated N, whereas the few longer-term studies reveal the opposite response (Fog 1988). Elevated N levels can be accompanied by decreased degradation of litter (Berg et al. 2001) and soil organic matter (Preston and Newman 1995; Scott et al. 1998), as well as reduced microbial biomass and respiration (Söderström et al. 1983; Nohrstedt et al. 1989; Smolander et al. 1994). If such decreases in litter and soil organic matter (SOM) degradation yield substantial increases in soil C stores, there could be profound effects on the global C budget (Johnson and Curtis 2001).

The effect of elevated N on organic-matter degradation, however, is not consistent across all substrates nor is it consistent over time (Fog 1988; Torn et al. 1997). Harmon et al. (1990) demonstrated that litter with higher initial N

concentration decomposed faster initially, but ultimately lost less mass than litter with lower initial N concentration. Berg (1988, 2000a) attributed this effect in litter to the differential influence of N on the degradation of high- versus low-quality litter compounds; that is, N increases degradation of soluble C and non-protected cellulose and hemicelluloses, but impedes degradation of lignin and lignin-like compounds. Adding N to litter can have the same initial effect as high N concentration in the litter (Homann and Cole 1990). As with initial N concentration, the response to N addition may change through time. Microbial respiration or litter mass loss can increase at first (Fog 1988), but the long-term response can be higher litter N concentration accompanied by decreased respiration (Scott et al. 1998).

If Fog (1988) is correct that degradation rates increase with increasing N concentrations for easily degradable substrates but decrease for other substrates, it follows that increased N concentrations may disproportionately increase stability of soil fractions that include highly complex or otherwise recalcitrant organic matter. Density fractionation permits exploration of this concept by separating soil into portions of differing C quality. Density fractionation involves disrupting soil aggregates in a dense liquid, allowing a 'light fraction' to float to the surface where it can be collected separately from the sedimented 'heavy fraction' (Sollins et al. 1999). The light fraction (LF) is chemically and visually similar to litter (Spycher et al. 1983; Skjemstad et al. 1986; Golchin et al. 1994a). Organic matter in the heavy fraction (HF) is composed of organo-mineral complexes and highly humified organic matter (Young and Spycher 1979; Golchin et al. 1994b, 1995b). The LF typically has a much higher C:N ratio (Christensen 1992) and contains younger C than the HF (Gaudinski et al. 2001), likely indicating that this fraction contains C that has a higher rate of turnover in whole soil (Trumbore and Zheng 1996). Finally, the two density fractions may support microbial communities that are spatially isolated in the soil (Chotte et al. 1998) and that thus may utilize different classes of organics (Fog 1988; Ladd et al. 1993).

Our goal was to determine the effects of long-term elevated N on organic-matter stability in litter, soils, and soil density fractions. By stability we mean the tendency of the organic matter to resist further transformation or degradation (Sollins et al. 1996)–see Discussion. We incubated substrates in the laboratory to isolate them from ecosystem fluxes of water, carbon, and nutrients, and thereby better test the inherent stability of the SOM. Soils with elevated N came from a 1969-1986 experiment in second-growth Douglas-fir forests in which each of a series of paired plots had been treated periodically with urea. We collected the soil and litter a decade after final fertilization, separated whole soil into LF and HF, then incubated these fractions, Oe + a horizon, and whole soil for 300 days.

Methods

Site selection and sample collection

Soils were collected from seven forested sites in western Washington and Oregon (Table 1), all part of a network established in 1969 by the Stand Management

Table 1. Site characteristics of seven stand management cooperative installations from which litter and soils were collected.

Site[a]	Location	Great group	Parent material	Textural class	Treatment	Oe + a horizon ($g\,kg^{-1}$)			Soil (0–5 cm) ($g\,kg^{-1}$)		
						C	N	C:N	C	N	C:N
Cedar Falls (1)	47.410N	Durochrept	Glacial till	Gravelly loam	Control	190.3	8.2	23	106.0	7.2	15
	121.815W				Elevated-N	238.9	11.9	20	117.0	8.3	14
Deep Creek (20)	45.962N	Haplumbrept	Glacial	Loam	Control	288.0	12.5	22	51.0	4.5	11
	123.307W		sediments		Elevated-N	200.1	10.2	20	57.0	5.7	10
Skykomish (43)	47.735N	Haplorthod	Granite	Fine sand	Control	371.6	12.0	30	39.1	2.4	16
	121.249W				Elevated-N	356.6	16.2	22	37.0	2.7	13
Headquarter	46.219N	Haplohumult	Igneous	Loam	Control	250.5	11.7	21	103.0	7.2	14
Camp (57)	122.732W				Elevated-N	230.8	10.0	23	93.0	7.3	13
Fourth Creek (65)	43.804N	Not classified	Pumice	Loam	Control	288.8	10.5	27	81.2	4.7	17
	122.381W				Elevated-N	312.8	12.1	26	94.4	6.5	14
Elk Creek (89)	43.365N	Haplumbrept	Sandstone	Loam	Control	192.8	8.3	23	105.9	6.5	16
	123.868W				Elevated-N	186.4	9.8	19	77.1	6.5	12
Cristy Falls (103)	43.904N	Not classified	Pumice	Loam	Control	315.1	13.6	23	108.1	6.0	18
	122.301W		and ash		Elevated-N	221.0	11.0	20	112.0	7.5	15
Mean (SE)					Control	271.0 (17.5)	11.0 (0.6)	25 (1.0)	84.9 (7.5)	5.5 (0.4)	15 (0.6)
					Elevated-N	249.5 (17.1)	11.6 (0.6)	21 (0.6)	83.9 (7.5)	6.4 (0.5)	13 (0.4)

[a]Numbers in parentheses are official site numbers used by the Stand Management Cooperative, University of Washington, Seattle.

Cooperative Nutrition Project (Hazard and Peterson 1984; Edmonds and Hsiang 1987) to evaluate the response of these forests to fertilization. Overstory at these sites was 46–72 year-old second-growth Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco.) at the time of sampling.

Each site contained an N-fertilized and non-fertilized (control) plot. Fertilized plots were treated with urea periodically over a 16-year period as follows: 448 kg N ha^{-1} at plot establishment in 1969, then 224 kg N ha^{-1} in years 8, 12, and 16, for a total of 1120 kg N ha^{-1}. Sites 1 and 43 did not receive the N addition in year 16, so totals were only 896 kg N ha^{-1}. The total application of 900–1100 kg N ha^{-1} was greater than the more common addition of 150–300 kg N ha^{-1} to Douglas-fir forests in the Pacific Northwest. The lesser urea application is typically more economical from a management perspective, but the greater application was potentially more useful in addressing the goal of this study.

Samples from 14 SMC sites were collected prior to this study and analyzed for C and N (Homann et al. 2001). We examined this preliminary data (0–5 cm mineral soil) before choosing the subset of seven sites for more intensive analysis. We imposed two selection criteria to allow us to focus on the influence of N on C dynamics. First, we wanted the C concentrations of the two plots within each pair to be as similar as possible in order to reduce the likelihood that differences in C levels could confound effects of elevated N (e.g., Sollins et al. 1996). Second, we wanted the C:N ratios to differ between the two plots within each pair. We reasoned that it was necessary to use sites in which %N increased relative to %C if we were to test the effects of elevated N on soil C.

Detailed information on sample collection and storage is provided by Homann et al. (2001) and Swanston et al. (2002). Samples from up to 16 points per plot were collected and composited within each plot. Samples from the Oe + a and 0–5 cm mineral soil were used in our study, although Oi samples were collected for other purposes (Homann et al. 2001). Samples were collected in summer of 1995, sieved (2 mm), and the <2 mm fraction stored frozen at −20 °C until analysis.

Sample preparation and analysis

Density fractions and incubation substrates were prepared as described by Swanston et al. (2002). Briefly, 400–700 g of whole soil (0–5 cm) were dispersed and gravity-separated in sodium polytungstate (NaPT, Sometu-US, Van Nuys, CA; adjusted to 1.65 g mL^{-1}). Floating material (light fraction) was aspirated and rinsed with distilled water, then sedimented material (heavy fraction) was decanted and rinsed with distilled water. Percent recovery (mass) and C and N concentrations are reported in Table 2.

Five substrates were incubated: Oe + a horizon (Oe + a), whole soil (WS), LF, HF, and the physically recombined fraction (RF). For the LF and Oe + a, 2.00 g (dry weight equivalent) of substrate was mixed with 18.00 g (dry weight) of acid-washed quartz sand and placed in a glass scintillation vial. Similarly, for WS and HF, 10.00 g of substrate was mixed with 10.00 g sand. The RF was created by

Table 2. Mass recovery and C and N concentrations in density fractions.

Site	Mass recovery (%)	Light fraction (g kg^{-1} [b])				Heavy fraction (g kg^{-1} [b])				Recombined fraction (g kg^{-1} [b])		
		Mass (%)[a]	C	N	C:N	Mass (%)	C	N	C:N	C	N	C:N
1												
Control	100.7	18.3	286.2	13.0	22	81.7	56.3	4.1	14	108.0	6.3	17
Elevated-N	98.7	21.4	282.2	14.3	20	78.6	55.9	4.7	12	126.0	7.8	16
20												
Control	100.1	8.7	258.3	11.1	23	91.3	32.8	3.8	9	47.0	4.0	12
Elevated-N	100.4	8.0	243.7	13.0	19	92.0	35.9	3.7	10	54.0	4.3	13
43												
Control	101.6	16.9	162.0	6.8	24	83.1	10.9	1.1	10	37.0	2.0	19
Elevated-N	100.4	17.0	162.4	8.4	19	83.0	8.9	1.0	9	67.0	2.4	15
57												
Control	98.7	18.0	280.0	15.0	19	82.0	52.3	5.1	10	97.2	7.0	14
Elevated-N	97.5	16.0	214.0	12.5	17	84.0	44.2	4.7	9	84.9	7.0	12
65												
Control	99.0	21.0	234.9	10.5	22	79.0	31.0	2.9	11	93.2	5.0	19
Elevated-N	100.7	18.8	280.0	15.5	18	81.2	28.0	2.8	10	88.0	5.8	15
89												
Control	99.0	17.1	264.1	12.4	21	82.9	52.9	4.6	12	103.6	6.1	17
Elevated-N	99.8	15.7	224.9	13.0	17	84.3	39.0	3.9	10	81.3	6.2	13
103												
Control	105.0	32.6	244.7	10.0	24	67.4	41.0	4.1	10	113.1	5.8	19
Elevated-N	103.4	24.4	305.2	17.0	18	75.6	43.0	4.2	10	99.1	6.4	15
Mean (SE)												
Control	100.6 (0.8)	19.1 (2.3)	247.1 (11.9)	11.3 (0.7)	22 (0.6)	80.9 (2.3)	39.6 (4.2)	3.7 (0.3)	11 (0.4)	85.6 (7.9)	5.2 (0.4)	17 (0.7)
Elevated-N	100.1 (0.7)	17.3 (1.9)	244.6 (13.7)	13.5 (0.7)	18 (0.4)	82.7 (1.9)	36.4 (3.9)	3.6 (0.3)	10 (0.3)	81.4 (7.5)	5.7 (0.5)	14 (0.4)

[a] As percent of total recovered soil mass.
[b] g kg^{-1} of fraction.

recombining LF and HF in the proportions in which they were recovered from the soil (Table 2), then mixing 10.00 g of mixture with 10.00 g of sand. Masses were tracked to the nearest 1 mg. We added sand to the substrates to increase sample size, permitting more flexibility in the type and number of physical analyses that could be performed. In the case of the HF, sand also created some degree of physical structure, lessening the likelihood of anoxic conditions during the incubation. In using the sand, we assumed it was inert and underwent no appreciable weight change during the course of the incubation. Carbon and N were measured on substrates on day 1 of the incubation (see below) and values were corrected for content of the added sand. These analyses were carried out on a Leco CNS Analyzer (Leco, St. Joseph, MI) by the Central Analytical Lab at Oregon State University. Analyses for pH were conducted on air-dried samples of mineral soil and Oe + a prior to the incubation using a 10:1 water:soil ratio for the Oe + a and a 2:1 water:soil ratio for the mineral soil.

Substrates were inoculated and then incubated for 300 days at 20 °C (±1 °C) in glass scintillation vials placed in sealed 1-L mason jars (Stotzky 1965; Hart et al. 1994; Swanston et al. 2002). All vials within a jar contained the same substrate from the same site. Samples (500 μL) were taken from the headspace of the jars with an airtight syringe and injected into a GC-8A Series Shimadzu gas chromatograph with a 2 m × 2 mm (ID) Porapak Q 80/100 column. After sampling, jars were flushed with CO_2-free air and resampled to obtain a baseline CO_2 concentration. Respiration was measured at intervals (1–21 days) sufficient to prevent CO_2 concentration from exceeding 2% (Santruckova and Simek 1994, 1997).

On days 1, 10, 30, 60, 120, 210, and 300, a single vial from each site/substrate combination was removed from the jar. The substrate in the vial was removed, thoroughly mixed, and then allocated to tests for extractable N, active bacterial and fungal biomass, extra-cellular enzyme activity (data not presented), and gravimetric water content. Nitrogen was extracted with 0.5 M K_2SO_4 at a 1:5 (w:v) ratio of substrate to solution. Extracts were stored frozen at −20 °C until analysis, when they were thawed and shaken. Nitrate was measured on an Astoria Pacific Analyzer (Astoria Pacific Int'l, Astoria, OR) and ammonium on an Alpkem Flow Solution (OI Corporation, College Station, TX). This ammonium and nitrate represented net accumulation over the entire incubation and will henceforth be referred to as 'mineralized N' $\left(NH_4^+ + NO_3^-\right)$ or 'nitrate' $\left(NO_3^-\right)$. Active microbial biomass was obtained through direct microscopy (Soil Food Web, Inc., Corvallis, OR). Substrates were diluted and stained with fluorescein diacetate, allowing active bacteria and fungi to be viewed and measured with an epifluorescence microscope (Ingham and Klein 1984a, b; Stamatiadis et al. 1990).

Data management and analysis

Carbon remaining in the substrate at the time of each respiration measurement was calculated by subtracting the cumulative respiration to that point from the initial C in the substrate. For each time point, the g C respired kg^{-1} remaining C day^{-1} was

then calculated as

$$\text{g C respired kg}^{-1}\text{ remaining C day}^{-1} = \frac{\text{g C respired}_i/\text{kg remaining C}_i}{\text{day}_i - \text{day}_{i-1}},$$

where i corresponds to the time point at which the respiration measurement was taken, and g C respired$_i$ refers to the C respired since the last time point ($i-1$).

Cumulative respiration, mineralized N, and nitrate data were analyzed using a split-plot model, that is, a general linear mixed model. Potential fixed effects were the effect of elevated nitrogen (the main plot), the effect of substrate type (the split plot) and interaction effects between substrate type and N level. Sources of random variation were site to site variation, variation among the large plots at each site and variation among replicate soil fractions.

Prior to accepting statistical results, plots of residuals were examined for constant variance. Most of the datasets required natural logarithm transformations to stabilize variance. When an analysis of variance (ANOVA) resulted in a P-value <0.1, pre-planned comparisons of interest were made using orthogonal contrasts. The number of contrasts conducted was equal to the unused degrees of freedom. Specifically, three substrates (Oe + a, LF, HF) were compared, as were whole soil and reconstituted soil (WS, RF). Significance for the contrasts was set at 0.05, although P-values up to 0.1 were also discussed.

Microscopy data and respiration rate were compared at each time point and through time. Although different vials were sampled each time, the same site and substrate in each jar were repeatedly measured through time, necessitating the use of a repeated-measures ANOVA. Repeated-measures analysis involves multivariate calculations that use the estimated covariances between the repeated measurements on the same experimental units (von Ende 2001).

Means and confidence limits generated by the ANOVAs were back-transformed and provided in several figures and tables to illustrate trends and variability in the data. The reader is cautioned, however, that the 95% confidence intervals for the estimated means are not the same as the 95% confidence intervals for the differences between treatments. The latter differences are adjusted for blocking error that was not accounted for in the confidence intervals plotted in the figures. Most data manipulation, and all statistical analysis, used SAS version 8.1 (SAS Institute, Inc.).

Results

Overview of main effects

The split-plot analyses gave no convincing evidence of interaction between N treatment (long-term elevated N v.s. control) and substrate for any measured variable (Table 3). Elevated N had significant effects on C:N ratio and nitrate, a marginally significant effect on total cumulative respiration, and no effect on mineralized N (Table 3). All variables differed significantly among substrates (Table 3). The split-plot repeated-measures analysis of respiration and microbial biomass

Table 3. *F*-ratios and *P*-values for main effects and interactions on soil variables arranged and tested according to split-plot designs.

Variable	Elevated-N × substrate		Elevated-N		Substrate	
	F^a	*P*	F^b	*P*	F^a	*P*
C:N ratio	1.2	0.12	29.8	0.002	115	<0.0001
Total cumulative respiration	0.2	0.9	5.4	0.059	52.9	<0.0001
Mineralized N	0.9	0.5	1.9	0.2	19.4	<0.0001
Nitrate	0.7	0.6	6.5	0.04	8.8	<0.0001

[a]Degrees of freedom = 4, 48.
[b]Degrees of freedom = 1, 6.

Table 4. *F*-ratios and *P*-values (df = degrees freedom) for soil variables arranged and tested according to repeated-measures split-plot designs.

Variable	Time × elevated-N × substrate			Time × elevated-N			Time × substrate		
	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Rate of respiration	2, 200	0.5	0.97	530	1.9	0.12	20, 200	5.0	<0.0001
Fungal biomass	24, 204	0.3	0.99	636	0.2	0.98	24, 204	2.1	0.0037
Bacterial biomass	24, 204	0.3	0.99	636	0.3	0.95	24, 204	4.7	<0.0001

showed temporal trends differed among substrates, but did not reveal other interactions (Table 4).

Pre-incubation analyses

Pre-incubation C:N ratios were tested in the WS to ensure that the fundamental design of the study, elevated N in one of the paired plots, was statistically viable. The Oe + a and density fractions were likewise tested and compared with each other and the WS to determine if the N was elevated differently in the soil components. Samples from N-treated plots gave about 20% lower C:N ratios for all substrates (Tables 1 and 2). Light fraction had about double the C:N ratio of HF ($t_{48} = 15.0$, $P < 0.0001$). The Oe + a C:N ratio was 12% higher than the LF ratio ($t_{48} = 2.74$, $P < 0.0001$). Finally, there was weak evidence that the RF had a C:N ratio about 8% higher than the WS ($t_{48} = 1.87$, $P = 0.07$). There was no convincing evidence that the N treatment affected C:N ratios differently for different substrates (Table 3), although the *P*-value of 0.12 was low enough to warrant a closer look. Any suggestion of an interaction between N treatment and substrate derived largely from the HF, which appeared to have responded somewhat less to the N treatment than did the other substrates.

The higher C:N ratios for the RF than the WS (Table 2) could be an artifact of density fractionation: unequal N and C loss during repeated suspensions and rinses.

This loss of C and N could lead to spurious estimates of C:N ratios for the different fractions if N were lost at unequal rates from the LF and HF. The loss of N in the our study was not large enough to change the ordering of mean C:N ratios across fractions. This would have been unclear, however, had we calculated C and N values for the HF by difference from the values for the WS and LF. Although calculating HF C and N by difference has been a common practice (Boone 1994; Alvarez et al. 1998; Fierro et al. 1999), it effectively allocates all C and N lost during the fractionation process to the HF. If we calculate HF values by difference, C and N concentrations in the HF are overestimated by as much as 28 and 24%, respectively. We thus recommend strongly that researchers analyze the WS and all fractions for C and N.

There was no evidence for a substantial effect of urea on pH at the time of sampling our sites, which was about 10 years following the last urea application. The mean pH for the Oe + a from the elevated-N plots was 4.97, and for control plots the pH was 5.05 (paired *t*-test, $t_6 = 1.18$ $P = 0.3$). The mean pH for the whole mineral soil (0–5 cm) from the elevated-N plots was 5.22, and for control plots the mineral soil pH was 5.34 (paired *t*-test, $t_6 = 2.27$, $P = 0.06$).

Respiration rate through time

The repeated measures analysis gave no indication of an interaction between time, N treatment, and substrate (Table 4). Further, there was no convincing evidence of differing temporal patterns of respiration with N treatment (Figure 1(a)). Averaged across all time periods, however, the rate of respiration was 16% lower in the elevated-N substrates ($F_{1,6} = 4.4$, $P = 0.08$).

The substrates showed strong differences in temporal patterns of the rate of respiration (Table 4). These differences were particularly apparent in the first 120 days (Figure 1(b)). Oe + a respiration rate dropped quite rapidly, whereas respiration rates of the other substrates declined more slowly. Differences were less obvious, but still distinct, in the latter half of the incubation. Orthogonal contrasts showed that by 210 days the LF had a higher rate of respiration than the HF ($t_{40} = 2.1$, $P = 0.04$), and still had a higher respiration rate at 300 d ($t_{40} = 3.0$, $P = 0.005$).

Cumulative respiration

There was evidence for lower cumulative respiration from elevated N regardless of substrate type (Table 3). The split-plot analysis (Table 3) showed a marginally significant decrease in total cumulative respiration for elevated-N samples (Table 3). Moreover, there was evidence of a decrease at each of the harvest dates (Figure 2), with *P*-values ranging from 0.04 to 0.08 and an average drop of 15%.

Strong differences emerged between substrates. Cumulative respiration (per kg C) averaged 35% higher for WS than for the RF (Table 5). The cumulative

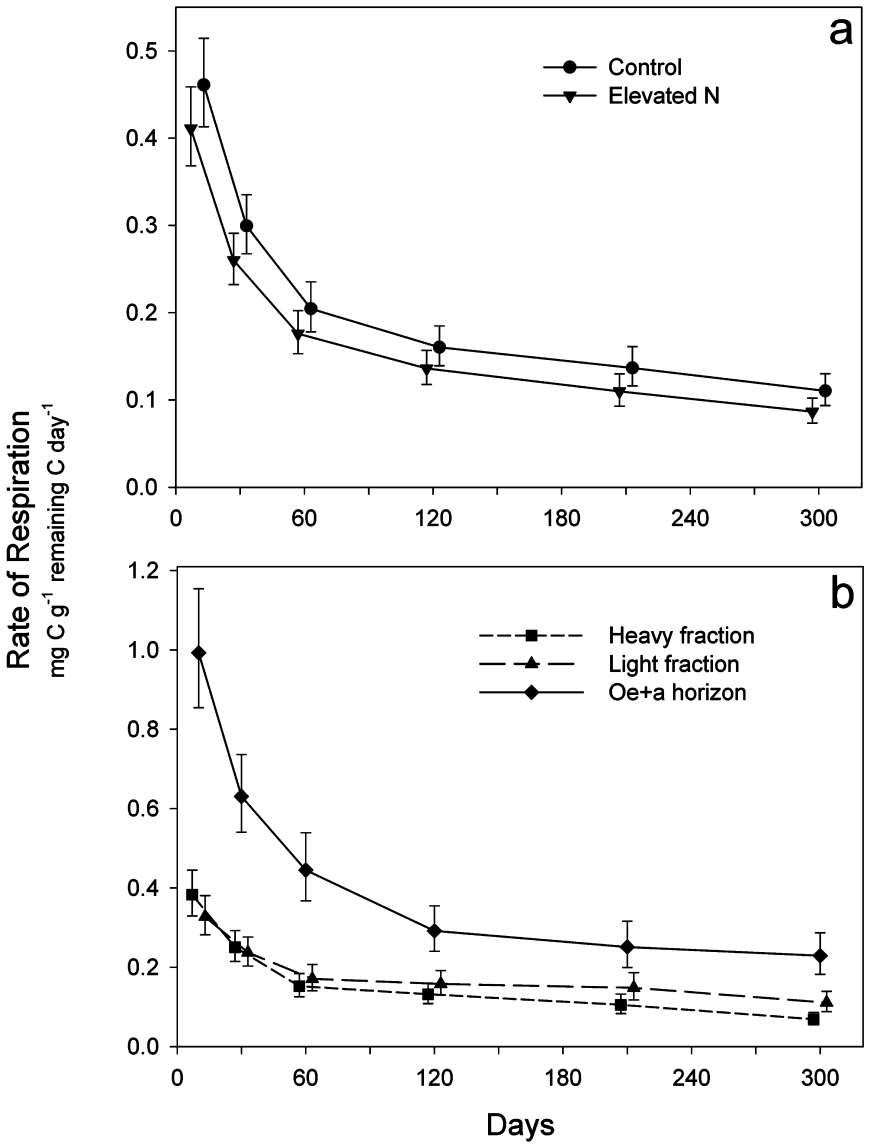

Figure 1. Rate of respiration through time in (a) the control and elevated-N treatments, and (b) the Oe + a, light fraction, and heavy fraction substrates. Mean values and 95% confidence intervals were back-transformed from the natural logarithm scale.

respiration from the Oe + a was twice that from the LF and HF, and cumulative respiration from the LF was slightly higher than from the HF (Table 5). A clear difference between cumulative respiration from the LF and HF only emerged during the last third of the incubation (Figure 1(b)). For the first 210 days, orthogonal

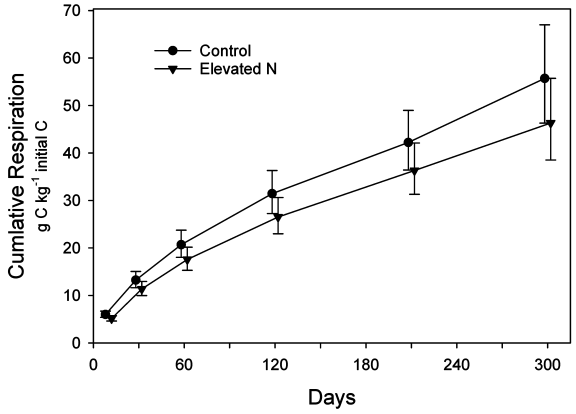

Figure 2. Cumulative respiration through time. Means and 95% confidence intervals for control and elevated-N treatments were back-transformed from the natural logarithm scale.

Table 5. Orthogonal contrasts of total cumulative respiration ($g\,C\,kg^{-1}$ initial C), mineralized N ($g\,N\,kg^{-1}$ initial N), and nitrate (g nitrate kg^{-1} initial N) means in litter, soil fractions, and whole soil after 300 days of incubation. Means (Oe + a, light fraction, and heavy fraction, or whole soil and recombined fractions) in the same row are not significantly different ($p > 0.05$) when followed by the same letter. Means were back-transformed from the natural-logarithm scale, in which the analyses were conducted.

	Soil components			Soil	
	Oe + a horizon	Light fraction	Heavy fraction	Whole soil	Recombined fractions
Total respiration ($g\,C\,kg^{-1}\,C_i$)	119.2a	48.6b	39.4c	48.4a	30.6b
Mineralized N ($g\,N\,kg^{-1}\,N_i$)	48.1a	25.7b	21.6b	43.3a	19.0b
Nitrate (g nitrate $kg^{-1}\,N_i$)	4.3b	19.7a	3.6b	36.0a	2.4b

contrasts did not reveal convincing evidence of a difference between these fractions (*P*-values >0.1).

Extractable N at end of incubation

Mineral N was extracted at the end of the 300-day incubation. This N represented net accumulation over the entire incubation, referred to here as 'mineralized N' $\left(NH_4^+ + NO_3^-\right)$ or 'nitrate' $\left(NO_3^-\right)$, and analyzed as $mg\,N\,g^{-1}$ initial N. There was no indication of an interaction between N treatment and substrate in either mineralized N or nitrate, nor was mineralized N in the elevated-N samples different from that in the controls (Table 3). Yet, nitrate for elevated-N samples was more than twice that for controls (control mean = 4.8; N-treatment mean = 12.0; $F_{1,6} = 6.5$, $P = 0.04$). There were distinct differences in mineralized N among the substrates (Table 3). The differences between the rinsed density fractions and non-rinsed WS and Oe + a should be interpreted with caution, however, as the repeated

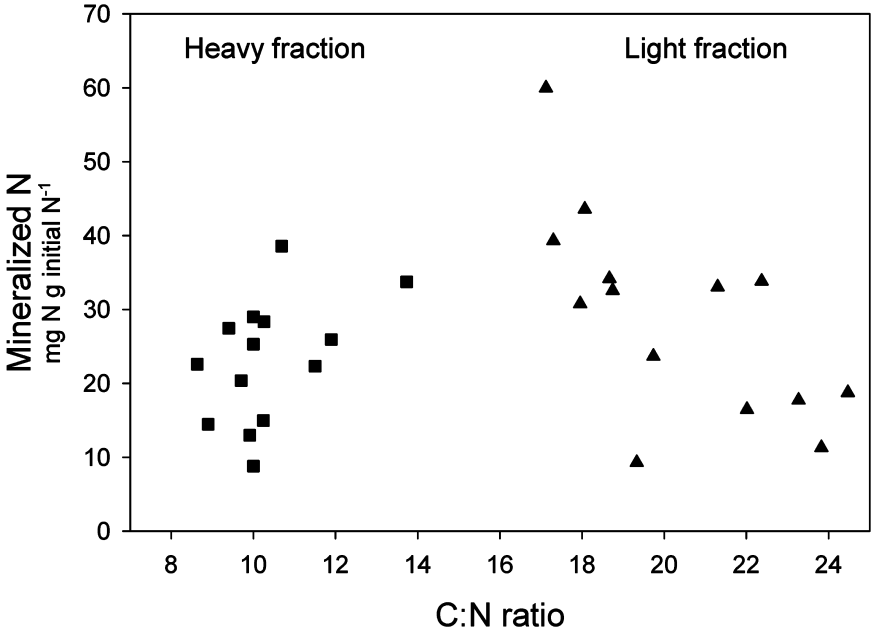

Figure 3. Mineralized N of the light fraction and heavy fraction plotted against the C:N ratios of those fractions. A simple regression was significant for the light fraction ($F_{1,11} = 9.8$, $P = 0.009$, adj $R^2 = 0.45$), but not the heavy fraction ($F_{1,11} = 2.9$, $P = 0.11$, adj $R^2 = 0.20$).

rinsing of the density fractions almost certainly resulted in lower initial K_2SO_4-extractable mineral N (mean (SE) at day 1: Oe + a = 10.2 (0.15), LF = 4.75 (0.04), HF = 6.7 (0.01); WS = 9.35 (0.15), RF = 5.65 (0.26)). At day 300, the Oe + a yielded mineralized N values nearly twice those of the LF and HF, which did not differ significantly (Table 5). Mineralized N was much higher for the WS than the RF (Table 5). Though quite variable, nitrate was much higher for LF than for Oe + a and HF, which did not differ (Table 5). The bulk of mineral N in the WS at 300 days was NO_3^-, thus nitrate for the WS nearly equaled mineralized N.

Plotting mineralized N for LF and HF against C:N ratio (Figure 3) and respiration rate (Figure 4) gave interesting relationships that were certainly suggestive, if not always significant. Mineralized N decreased with increasing C:N ratios for the LF, but showed no pattern or perhaps even increased with increasing C:N ratio for the HF (Figure 3). Similarly, mineralized N from the LF may have decreased with increasing respiration rate (Figure 4(a)), while mineralized N clearly increased with increasing respiration rate for the HF (Figure 4(b)).

Active microbial biomass

Estimates of active fungal biomass (mg biomass kg^{-1} C) were extremely variable and contained numerous zero values (Table 6), decreasing statistical power. There

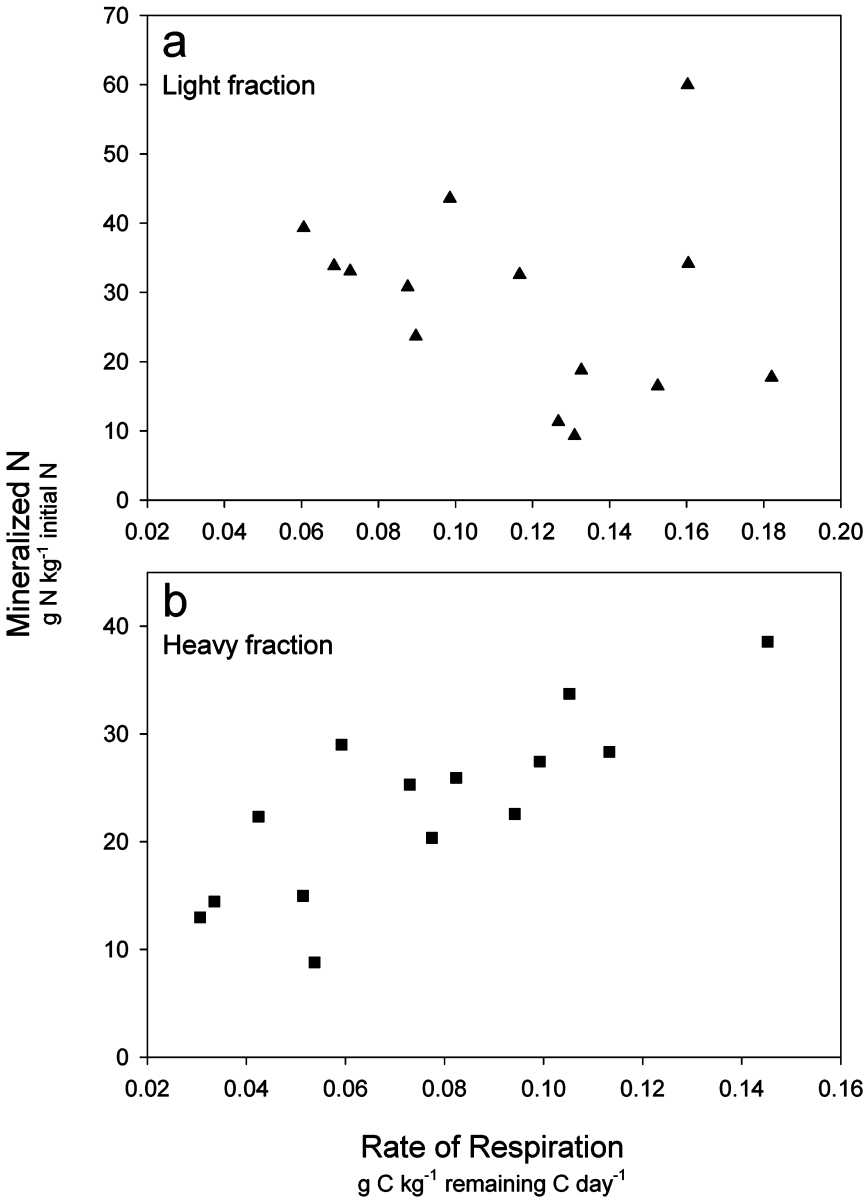

Figure 4. Mineralized N plotted against rate of respiration in (a) light fraction and (b) heavy fraction. A simple regression was significant for the heavy fraction ($F_{1,11} = 24$, $P = 0.0003$, adj $R^2 = 0.67$), but not the light fraction ($F_{1,11} = 0.5$, $P = 0.48$, adj $R^2 = 0.04$). Note, however, that if the outlier in the upper right quadrant is dropped from the light fraction the regression is significant ($P = 0.04$, adj $R^2 = 0.32$).

Table 6. Mean active fungal biomass (mg kg^{-1} C) in whole soil (WS), recombined fraction (RF), light fraction (LF), and heavy fraction (HF) from control and N-fertilized treatments. Values in parentheses are 1 SE, $n = 7$.

Day	Treatment	Whole soil	Recombined fraction	Oe + a	Light fraction	Heavy fraction
1	Control	37.2 (20.0)	0.6[a] (0.6)	5.7 (2.8)	0	3.8[a] (3.8)
	Elevated-N	13.9 (5.1)	0	34.3 (28.4)	6.6[a] (6.6)	141.5 (100.1)
10	Control	27.0[b] (9.7)	73.3 (29.1)	167.8 (87.9)	170.4 (94.7)	160.0 (69.0)
	Elevated-N	96.7 (71.4)	70.7 (40.9)	183.7 (71.2)	160.3 (104.4)	213.8 (139.0)
30	Control	99.7 (42.7)	170.3 (61.0)	118.0 (55.8)	26.2 (25.5)	120.9 (91.7)
	Elevated-N	100.2 (29.8)	134.4 (24.8)	141.5 (44.0)	13.8 (9.0)	319.6 (204.7)
60	Control	120.1 (40.4)	93.2 (53.5)	134.3 (34.6)	100.0 (27.6)	325.2 (71.4)
	Elevated-N	81.8 (71.8)	96.9 (18.4)	151.1 (70.9)	62.7 (23.7)	492.8 (113.9)
120	Control	179.3 (53.7)	58.9 (18.8)	9.6 (5.1)	62.7 (38.9)	127.5 (43.8)
	Elevated-N	67.0 (28.5)	70.3 (13.2)	78.2 (57.0)	72.1 (20.1)	156.5 (43.5)
210	Control	24.9 (13.1)	4.4[b] (3.1)	86.0 (22.6)	45.1 (16.1)	27.0 (14.3)
	Elevated-N	27.7 (10.5)	0.3[a] (0.3)	71.5 (49.1)	42.0 (10.2)	84.9 (36.2)
300	Control	24.4[b] (22.4)	42.9 (27.8)	31.4 (8.8)	44.3 (17.7)	11.1 (5.4)
	Elevated-N	12.1 (11.9)	12.2 (8.7)	68.3 (40.1)	28.3 (9.1)	15.8 (13.0)

[a]Only one sample contained active fungal biomass.
[b]WS: $n = 6$ at 10 days, $n = 4$ at 300 days; RF: $n = 3$ at 210 days.

Table 7. Mean active bacterial biomass (mg kg^{-1} C) in whole soil (WS), recombined fraction (RF), light fraction (LF), and heavy fraction (HF) from control and N-fertilized soils. Values in parentheses are 1 SE, $n = 7$.

Day	Treatment	Whole soil	Recombined fraction	Oe + a	Light fraction	Heavy fraction
1	Control	179.9 (42.0)	30.1 (10.2)	44.2 (27.8)	68.3 (9.9)	93.9 (34.0)
	Elevated-N	163.3 (27.1)	19.0 (4.5)	23.5 (10.3)	60.8 (9.1)	107.3 (44.2)
10	Control	170.5[a] (44.8)	110.0 (43.2)	215.2 (71.4)	511.1 (118.7)	147.3 (56.6)
	Elevated-N	175.3 (29.7)	57.6 (20.3)	169.5 (61.8)	345.6 (75.6)	126.9 (45.6)
30	Control	193.6 (35.2)	193.4 (53.9)	325.5 (115.4)	455.3 (150.8)	269.4 (78.1)
	Elevated-N	201.9 (43.1)	180.2 (43.0)	233.0 (51.6)	407.8 (165.6)	532.1 (243.8)
60	Control	105.6 (25.5)	236.3 (57.1)	335.7 (51.4)	608.7 (144.9)	588.6 (211.3)
	Elevated-N	122.0 (34.0)	251.1 (78.3)	314.5 (66.0)	430.0 (81.2)	454.9 (144.4)
120	Control	197.5 (30.2)	413.7 (142.6)	570.7 (37.9)	1352.5 (330.3)	1025.3 (358.1)
	Elevated-N	203.4 (56.0)	278.2 (93.2)	530.0 (104.3)	1454.2 (280.0)	891.3 (255.1)
210	Control	90.3 (30.3)	79.5[a] (6.2)	512.4 (133.0)	439.8 (159.3)	513.0 (194.0)
	Elevated-N	91.2 (28.5)	50.8 (32.6)	450.6 (116.0)	415.8 (92.1)	414.9 (168.1)
300	Control	34.0[a] (17.9)	399.4 (249.5)	301.6 (123.7)	515.5 (137.3)	78.1 (45.9)
	Elevated-N	68.4 (44.6)	198.3 (111.0)	267.4 (72.3)	496.5 (117.4)	58.7 (38.1)

[a]WS: $n = 6$ at 10 days, $n = 4$ at 300 days; RF: $n = 4$ at 21.

was no evidence that elevated-N samples and controls changed differently through time (Table 4), nor was there evidence of a difference between elevated-N samples and controls at any single time during the incubation (P-values >0.2). There was strong evidence, however, that values changed differently through time for the five substrates (Table 4). Orthogonal contrasts at each time period comparing the Oe + a, LF, and HF, or the WS and RF, showed very few significant differences. The WS, of course, contained more active fungal biomass than the RF on the first day of the incubation, but it ceased being higher by day 10. The significance of the overall interaction was likely the result of especially high active fungal biomass values in the HF at 30 and 60 days.

Active bacterial biomass (mg biomass kg^{-1} C) was more consistent than fungal biomass, but still quite variable (Table 7). As with fungal biomass, there was no evidence for an effect of N treatment on bacterial biomass through time (Table 4) or at any specific time (P-values >0.2). The biomass in the substrates showed different patterns through time, however (Table 4). The WS values were much higher on day 1, but not different from the RF thereafter. Bacterial biomass in the HF, while not different from that in the LF on day 1, was much lower by the termination of the incubation at 300 d. The bacterial biomass of the Oe + a often fluctuated between that of the LF and HF.

In general, the LF and HF tended to show the extremes in biomass among the five substrates. Fungal biomass was higher in the HF, whereas bacterial biomass appeared somewhat higher in the LF throughout the course of the incubation. The WS showed the highest biomass initially and fewest extremes, probably reflecting its lack of disturbance relative to the other substrates.

Discussion

Organic matter stabilization

Long-term elevated N decreased cumulative respiration for whole soil, Oe + a, LF, HF, and RF by an average of 15%. We interpret these decreases in respiration as indicating an increase in the stability of the organic matter caused by long-term elevated N. By stability we mean the tendency of the organic matter to resist further transformation or degradation. The stability of SOM may be viewed as a result of three characteristics, discussed in depth by Sollins et al. (1996): (i) accessibility, which refers to the location of organic substances with respect to microbes and enzymes, (ii) interactions, which refer to inter-molecular interactions between organics and other substances, (iii) recalcitrance, which comprise molecular-level characteristics. Our soils were from paired plots and the density fractions were thoroughly dispersed; therefore, a difference in accessibility due to N treatment is unlikely. We selected sites in which the paired plots did not differ substantially in the amount mineral soil organic C; therefore, it is not likely that elevated N created differences in interactions between soil organic matter and clay plus silt, which can stabilize SOM (Sollins et al. 1996). Increased recalcitrance due to formation of

more complex N compounds remains as a potential mechanism for increasing stability.

Decreased respiration may have resulted from N-inhibition of microorganisms and production of extracellular enzymes in addition to increased SOM stability (Fog 1988). However, we did not observe an N effect on active bacterial biomass, active fungal biomass, or their relative amounts. Furthermore, in a study that included the soils of this study, elevated N resulted in 20% lower cellulase activity in only one of three soil layers, with no effect on xylanase or phosphatase activity in any of three soil layers (Homann et al. 2001). This does not preclude that specific microbial species, functional groups, or enzymes were influenced by N, but there does not appear to be a general microbial or enzymatic response to elevated N.

Although elevated N resulted in a depression in the respiration of the Oe + a, LF, and HF, it is possible that the N stimulated different stabilization mechanisms in the litter and LF than in the HF. The LF may be substantially more recalcitrant than the Oe + a (Figure 1), but these substrates are nonetheless more visually and chemically similar to each other than to the HF (Spycher et al. 1983; Skjemstad et al. 1986; Golchin et al. 1994a). The negative response of microbial respiration to elevated N in litter has ample precedent. Berg (1988) used ^{15}N to trace the fate of N in labeled litter in a Scots pine forest. He noted that ^{15}N was conserved in the litter in general, and appeared to concentrate in the Klason lignin fraction (KL) as mass was lost from the litter. In addition to an increase in the concentration of ^{15}N in the KL, and an increase in the concentration of KL in the residual litter, the total weight of KL increased. Harmon et al. (1990) detailed slow degradation of several litter types with low lignin:N ratios. Berg (2000b) reviewed numerous instances where litter decomposed less with increasing N concentration, and where the rate of respiration in FH (Oe, Oa) organic layers was depressed with increasing N concentration. The incorporation of N into KL was probably greater in the Oe + a and LF of the elevated-N plots sampled in this study, resulting in slower degradation of the elevated-N organic matter.

The HF might be expected to respond to elevated N in a manner more similar to the bulk mineral soil, which has also shown decreased respiration in response to increased N (Scott et al. 1998). Specifically, turnover of HF in mineral soil appears to be regulated more by accessibility and mineral interactions than by recalcitrance (Dalal and Mayer 1986; Skjemstad et al. 1986; Golchin et al. 1995a; Swanston et al. 2002). Yet, the mechanisms for degradation of complex humic molecules in the HF may in fact be similar to those for degradation of lignin (Gramss 1997), and similarly inhibited by elevated N (Swamy and Ramsay 1999). Fog (1988) proposed that bombardment with •OH radicals is an important mechanism for degradation of recalcitrant and complex organic matter, but that the formation of •OH is inhibited by elevated N (Forney et al. 1982; Kelley and Reddy 1982). Bichat et al. (1999) studied the degradation of ^{14}C and ^{15}N-labeled atrazine, a recalcitrant heterocyclic, in soil and pure culture. With the addition of N, one of the three species of bacteria used in pure culture was severely inhibited. In soil, addition of N inhibited degradation of atrazine unless the soil was inoculated with one of the two species not influenced by elevated N.

Long-term versus short-term experiments

The rate of incorporation of the added N into the forest floor, LF, and HF may have varied with time. Eight years after fertilization, Chappell et al. (1999) reported significantly lower C:N ratios in the litterfall and forest floor of fertilized plots, but no difference in N content ($Kg\,ha^{-1}$) in the forest floor. Based on samples collected 10 years after fertilization ended, we found similar percent decrease in C:N (as a result of the elevated N) for all substrates, which is consistent with the similar percent decrease in respiration for all five substrates. In shorter-term studies, more ^{15}N was incorporated into the LF than into the HF in a few days (Strickland et al. 1992) to nearly 2 years (Swanston and Myrold 1997). In work at 13 sites including the seven used in the our study, Homann et al. (2001) found evidence that the LF still contained slightly more added N than the HF 9–15 year after fertilization ended. If rates of incorporation were variable, analyses conducted immediately after the first fertilization, 25 year prior to our sampling, would have most likely recovered a much higher proportion of added N in the LF than in the HF.

The long-term nature of the present study may not only have influenced the range of C:N ratios across fractions, but also the nature of the C and N: many studies show that amino N added as fertilizer is steadily converted to soil organic N (Heilman et al. 1982; Preston and Mead 1994). An important observation by Fog (1988) was that, whereas short-term addition of N to substrates followed by short-term incubation often yielded increased respiration, longer-term incubations often yielded the opposite. We expected to see initially higher rates of respiration in the elevated-N substrates, but followed eventually by an earlier or greater decrease in elevated-N respiration rates; thus, the control substrates would ultimately have a higher total cumulative respiration. However, the fertilized plots had in a sense undergone 12–15 years of field incubation before the 300-day laboratory incubation. Thus, consistent with Fog's (1988) hypothesis, after a long-term field incubation, the control substrates had higher rates of respiration throughout the 300-day lab incubation.

Net N mineralization

In one of the earliest incubations of LF, Greenland and Ford (1964) observed that net nitrification in LF was negatively related to the C:N ratio. Barrios et al. (1996) confirmed this negative relationship in incubations of LF. The HF, however, appears to behave differently. By adding LF or HF to whole soil and incubating the mixtures, Whalen et al. (2000) determined that whereas LF may be a sink for mineralized N, HF is the main source. After incubations of pure LF and HF, Sollins et al. (1984) reported a strong positive relationship between mineralized N and C:N ratio for the HF, in addition to the negative relationship for LF. Our trends (Figure 3) are not as clear, perhaps partly because of smaller ranges in C:N ratios, but they are consistent with those of Sollins et al. (1984). They suggested that the inverse relation between N mineralization and C:N for the LF resulted from increasing N

immobilization with increasing C:N ratio, whereas the direct relationship for the HF may have resulted from decreasing recalcitrance and physical protection with increasing C:N ratio. Addressing low N mineralization from LF, Boone (1994) also proposed that more N might be immobilized in the LF than HF during incubation, resulting in lower net rates of N mineralization in LF. If correct, these suggestions imply a negative relationship between mineralized N and respiration in the LF, and a positive relationship in the HF, which is what we found in our study (Figure 4).

Hart et al. (1994) suggested that decreasing gross N mineralization throughout the course of a long-term incubation was caused by increasing substrate recalcitrance. The consequent decreases in N demand and immobilization resulted in a net increase in mineralized N pools. Hart (1999) found similar results in fallen tree boles. All substrates in our study, regardless of C:N ratio, produced more mineralized N as the incubation progressed. However, the patterns of the LF and HF as measured at 300 days, and illustrated in Figure 3, had already developed by 10 days (data not shown). This suggests that these contrasting trends were not simply products of the incubation, but were instead related to the nature of the substrate.

Scott et al. (1998) conducted a 340-day incubation on forest floor and soils collected from fertilized and unfertilized *Pinus radiata* D. Don plantations. They showed that, 15 year after heavy fertilization, cumulative respiration was highest in both the unfertilized forest floor and soils. They also observed a negative relationship between the rates of respiration and gross N mineralization in the forest floor, but a positive relationship in the whole soil. They attributed the opposing trends to qualitative differences in the substrates, and suggested that the consequent microbial communities may have been different. Although our ANOVA did not show a difference in mineralized N between the LF and HF at 300 days, the microbial-substrate interactions influencing N mineralization may have been quite different. The lowest mineralized N in HF appeared to coincide with both low C:N ratios and low respiration rate. The lowest mineralized N in LF appeared to occur at high C:N ratios and high respiration. Net N mineralization in HF thus seemed typified by release of N in excess of metabolic demands, whereas net N mineralization in LF was typified by immobilization of N to meet metabolic demands.

Microbial biomass and processes

Measures of active microbial biomass were consistent with differences in respiration and levels of inorganic N in the substrates. At 300 days, Oe + a contained less bacterial biomass than the LF but was still respiring at nearly twice the rate of the LF, indicating a much more metabolically active population. Bacterial biomass in the HF was several times lower than that in the LF, but respired at only a slightly lower rate, also indicating a more metabolically active population in the HF. Associated with the higher metabolic activity in the Oe + a and HF, there were lower levels of nitrate. This is consistent with Hart et al.'s (1994) suggestion that nitrifiers are least competitive when heterotrophic populations are active.

Alternatively, the lower levels of nitrate in the Oe + a and HF could have been from higher rates of nitrate immobilization by the heterotrophs. Yet, the high availability of ammonium, as indicated by the difference between mineralized N and nitrate, favors the nitrification inhibition explanation. That is, if immobilization were dominant, both ammonium and nitrate levels would likely be low. The opposite trend was apparent in the LF, where low microbial metabolic activity coincided with high nitrate and low ammonium levels.

Swanston et al. (2002) suggested that the low respiration in the RF might result from a negative interaction between the LF and HF, or microbial communities best adapted to the LF and HF, when these fractions were recombined with disrupted soil structure. The same negative interactions may have taken place in the RF with elevated N, but did not appear to be especially exacerbated by elevated N. The biomass data presented by Swanston et al. (2002), expanded in our study with values from elevated-N substrates, were still too variable to allow firm conclusions. Yet, it would seem that the RF contained a less metabolically active population than the WS, given the lack of difference in microbial biomass but low rate of RF respiration during most of the incubation.

When considered within the context of possible inhibitory effects of residual NaPT on microbial metabolism (Magid et al. 1996; Swanston et al. 2002), otherwise natural differences between the Oe + a and the LF may seem artificially exacerbated (Figure 1). Damage to the microbial populations from the fractionation process almost certainly led to lower initial rates of respiration in the fractions, and may have persisted well into the incubation. However, the Oe + a also respired at a much higher rate than the WS, which was not rinsed or treated with NaPT. Although the density fractions may have begun with somewhat smaller or less metabolically-active populations due to the fractionation process, the differences between the Oe + a and the fractions were simply too large to attribute solely to biological suppression by NaPT.

Ecosystem relevance

The effects of elevated N on SOM stability in soil and soil fractions during laboratory incubations can be clearly identified, but the significance of such effects for forest biology and management is less readily apparent. The incubation technique we used (Stotzky 1965) is adequate for comparing the relative effects of elevated N on SOM stability in various substrates, which was our primary goal. However, these estimates of respiration are not directly applicable to forest ecosystems. Carbon dioxide evolution measured within our sample jars was almost exclusively heterotrophic respiration, while CO_2 evolution measured from forest soils may include well over 50% root respiration (Andrews et al. 1999; Hanson et al. 2000). Additionally, our substrates did not receive C and N inputs, while typical forest soils in the Pacific Northwest receive frequent inputs of organic C and N from roots, throughfall, and litter degradation (Jandl and Sollins 1997; Prescott et al. 2000). The laboratory incubations have however demonstrated the potential of long-term N elevation to stabilize SOM.

In spite of the demonstrated SOM stabilization in the laboratory, field surveys of soil C at sites within the Stand Management Cooperative, including sites used in our study, have not shown significant soil C sequestration (Canary et al. 2000; Homann et al. 2001). If SOM is more stable in the fertilized plots in the field, then with similar C inputs the fertilized plots should accrue C at a greater rate (several hundred $kg\,C\,ha^{-1}\,year^{-1}$ if our incubation results represented actual soil heterotrophic respiration). Chappell et al. (1999) measured aboveground C inputs at several sites in the Stand Management Cooperative, but did not detect a difference in the amount of litterfall between the paired plots. Canary et al. (2000) reported an average increase of $26.7\,Mg\,C\,ha^{-1}$ in tree biomass due to N fertilization, but were unable to detect statistical differences in litter or soil C. Although aboveground C inputs to soil may have been comparable between the plots, belowground inputs may have been dissimilar. Beets and Whitehead (1996) determined that increased N availability in *P. radiata* plantations in New Zealand resulted in decreased fine root production. If indeed SOM was stabilized by elevated N in the plots studied by Homann et al. (2001) and Canary et al. (2000), the resulting C sequestration may have been offset by lower C inputs from fine root production and turnover. Finally, the inability to detect statistically significant differences in C mass could have been exacerbated by high natural variability or inadequate sampling. Homann et al. (2001) determined that C increases of up to 15%, equivalent to $10\,Mg\,ha^{-1}$, may have been necessary to observe significant differences at the 0.05 alpha level.

Concluding remarks

In this study, we expanded the understanding of the effects of N on soil organic matter stability by examining several distinguishable soil components a decade after massive N fertilization was terminated. This lag time allowed the added N to cycle through the ecosystem and become incorporated into organic matter via chemical, microbial, and higher-plant processes. The Oe + a, mineral-soil light fraction, and mineral-soil heavy fraction differed in their microbial characteristics, but their responses to elevated N were similar: microbial respiration was suppressed by 16%, net nitrification was increased 2.5 times, and net N mineralization and biomass were unaffected. The lack of statistical differences in microbial responses to elevated N were surprising, because previous studies found the light and heavy fractions to have very different physical, chemical, and morphological characteristics. The high variation in the microbial data may have obscured actual differences. Increased recalcitrance of the soil organic matter is a plausible explanation for the observed suppression of microbial respiration, although this does not preclude elevated N from affecting specific microbial species, functional species groups, or enzymes. Indeed, in spite of similar responses to elevated N as measured by respiration, different mechanisms may have been operating in the various fractions. For example, these differing mechanisms may have been expressed as contrasting patterns of mineralized N for LF and HF with changing C:N ratio and rate of respiration.

We have demonstrated that long-term elevated N can increase SOM stability in seven forest soils, raising the possibility of increased C sequestration. Several forest fertilization studies have shown an increase in forest soil C. In other studies, lack of significant differences may result from altered C inputs or the well-known tendency of forest soils to be highly variable spatially. Long-term elevated N may result in greater SOM stability, but any significant net increase in C stores may be obscured or mitigated by variation in factors such as the frequency and types of C inputs, soil texture and structure, hydrology, and mineralogy.

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