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Nitrogen fertilization decreases forest soil fungal and bacterial biomass in three long-term experiments

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Abstract

We examined the effects of N fertilization on forest soil fungal and bacterial biomass at three long-term experiments in New England (Harvard Forest, MA; Mt. Ascutney, VT; Bear Brook, ME). At Harvard Forest, chronic N fertilization has decreased organic soil microbial biomass C (MBC) by an average of 54% and substrate induced respiration (SIR) was decreased by an average of 45% in hardwood stands. In the pine stand, organic soil MBC was decreased by 40% and SIR decreased by an average of 35%. The fungal:bacterial activity ratio was also decreased in the hardwood stands from an average of 1.5 in the control plot to 1.0 in the High-N plot, and in the pine stands from 1.9 in control plot to 1.0 in the High-N stand. At Mt. Ascutney, MBC was reduced by an average of 59% and SIR by 52% in the High N plots relative to the unfertilized plots, and the fungal:bacterial activity ratio was only slightly decreased. The Bear Brook watershed is in an earlier stage of N saturation (Stage 0–1) and did not exhibit significant fertilization effects on microbial biomass. Across all three sites, MBC and SIR had negative relationships with total N inputs in both mineral soils and organic soils, though the effect was much stronger in organic soils. Both MBC and SIR were positively correlated with dissolved organic C, total soil C, and bulk soil C:N ratios. These results are consistent with the N saturation hypothesis, but do not indicate a strong role for microbial N immobilization in preventing N loss.

Keywords: Nitrogen fertilization; Microbial biomass; Nitrogen saturation; Selective inhibition

1. Introduction

Increasing N deposition from human activities has altered biogeochemical cycling in many regions throughout the United States (Fenn et al., 1996; Peterjohn et al., 1996; Williams et al., 1996; Lovett et al., 2000) and Europe (Kuylenstierna et al., 1998). In an effort to understand and predict forest response to increased N deposition, several long-term forest fertilization experiments have been initiated (Aber et al., 1993; McNulty and Aber, 1993; Moldan et al., 1995; Peterjohn et al., 1996; Tietema et al., 1998; Magill et al., 2000). The results of these ongoing experiments have led to the N-saturation hypothesis (Ågren and Bosatta, 1988; Aber et al., 1989; Aber et al., 1998), which predicts a series of plant and soil responses as N supply exceeds demand. These changes include altered rates of important microbial processes such as net N mineralization and nitrification. While N input has been shown to impact the rates of microbially-driven C and N cycling, very little is known about how N deposition impacts the soil microbial communities that control these processes.

Nitrogen mineralization often responds strongly to N amendments. Mineralization rates generally increase in N-limited systems, but decrease as those systems become N saturated (Aber et al., 1998). A broad community of bacteria, fungi, and other soil organisms is involved in the mineralization of organic substrates. Therefore, N mineralization rates are likely to correlate with the biomass and activity of the overall microbial community. Nitrogen fertilization in forests typically increases microbial biomass shortly following initial fertilization (Hart and Stark, 1997; Zhang and Zak, 1998) but over the longer term, biomass generally decreases (Soderstrom et al., 1983; Nohrstedt et al., 1989; Smolander et al., 1994; Arnebrant

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et al., 1996; Fisk and Fahey, 2001; Corre et al., 2003; Lee and Jose, 2003; DeForest et al., 2004). This pattern roughly follows the trends in net N mineralization observed in several long-term fertilization experiments, suggesting that mineralization rates are closely coupled with the microbial biomass. However, the effect of the multiple changes associated with N-saturation on microbial communities remains unclear.

Although microbial biomass is a useful index in ecological research, more specific investigations of the microbial community will improve our understanding of these systems. The fungal:bacterial ratio is one such index that can improve our understanding of microbial community responses to N input. Though there is some overlap in the physiological capabilities of fungi and bacteria, there are important differences that are likely to affect their response to environmental changes. For example, the average C:N ratio of fungi is ~15, versus ~6 for bacteria, suggesting that bacteria are able to use substrates of a lower C:N ratio than fungi.

Increased N inputs may directly and indirectly alter microbial biomass and activity. Increased inorganic N might result in direct immobilization by microbes either by decreasing their C:N ratio, or increasing their overall biomass. However, N immobilization by microbes is often limited by C availability, and N inputs can have varying effects on soil C supply. Changes in N inputs can also affect the nutrient status of plants that could affect root exudation, leaf litter chemistry, and plant-microbial competition for nutrients. Changes in root biomass could also affect microbial biomass through indirect effects on the physical soil environment (Wardle, 2002) and on soil moisture. In the long-term, changes in forest species composition would strongly affect microbial biomass (Smolander and Kitunen, 2002; Templer et al., 2003). Long-term excess N deposition can also lead to base cation loss and related changes in soil pH, which can also affect microbial biomass (Anderson and Joergensen, 1997; Baath and Anderson, 2003).

We examined microbial biomass using both chloroform fumigation-extraction (FE) and substrate induced respiration (SIR) in three long-term N fertilization experiments. At each of these sites, many of the important biogeochemical processes have been well studied; however, the belowground community has received relatively little attention. We hypothesized that treatment effects on N mineralization rates observed by others would be reflected in the microbial biomass. Selective inhibitors were used to separate SIR into fungal and bacterial components. We hypothesized that fungi would be more sensitive to N additions.

2. Study sites

Three long-term N fertilization experiments were selected, located throughout the northeastern United States (Fig. 1). These experiments were independently established to evaluate the effect of chronic N additions on biogeochemical cycling and forest health. They represent a range of soils, forest types, and climate found in this region. The experimental designs and fertilization treatments also vary by site.

2.1. Bear Brook watershed, Maine (44°52′N, 68°06′W)

Nitrogen additions to the West Bear watershed were initiated in 1989 and consisted of additions of dry $(NH_4)_2SO_4$ at a rate of 25.2 kg N ha^{-1} $year^{-1}$ (Norton et al., 1994; Rustad et al., 1996; Norton et al., 1999). The unfertilized East Bear watershed receives only ambient deposition, estimated at 8.4 kg N ha^{-1} $year^{-1}$ (Norton et al., 1999). The vegetation includes both hardwoods and softwoods, with hardwoods (including American beech (*Fagus grandifolia* Ehrh.), sugar maple (*Acer saccharum* Marsh.), red maple (*Acer rubrum* L.)), dominating the lower ~60% of the watersheds. The upper areas of the watersheds are nearly pure softwood stands, 80–120 years old, predominantly red spruce (*Picea rubens* Sarg.), with minor occurrences of balsam fir (*Abies balsamea* L.) and hemlock (*Tsuga Canadensis* L. Carr). The soils are acidic and have low base saturation, cation exchange capacity, and sulfate adsorption capacity (Norton et al., 1999). Soils are predominantly Typic and Aquic Haplorthods. Bedrock geology consists of metamorphosed quartzites and calc-silicate gneiss.

Within each watershed, four 10 m × 10 m plots were established with two of the four plots in each watershed in hardwoods and two in softwoods (Jefts et al., 2004). Plots were chosen to have comparable slopes, dominant tree species, and proximity to stream/stream bed between watersheds. In situ N mineralization and nitrification assays were also conducted on these samples in an independent study (Jefts et al., 2004). This site was sampled on May 28, July 15, and October 13 of 2002.

2.2. Mt. Ascutney, VT (43°26′N, 72°27′W)

Ten paired 15 m × 15 m plots were established in 1988 in high elevation (>725 m) red spruce (*P. rubens* Sarg.) stands (McNulty et al., 1996). For this study, we examined six plots that have been continuously subjected to three levels of N inputs. Fertilizer was applied as NH_4Cl to two of the plots at 15.7 kg N ha^{-1} $year^{-1}$; two plots received 31.4 kg N ha^{-1} $year^{-1}$, and two plots were not fertilized. Ambient deposition at this site is estimated at 5.4 kg N ha^{-1} $year^{-1}$ (McNulty and Aber, 1993). The soils are well-drained frigid Typic Haplorthods. This site was sampled on June 3, July 2, and September 28 of 2002. In situ N mineralization and nitrification assays were also conducted on these samples in an independent study (Steve McNulty, pers. comm.).

2.3. Harvard Forest, MA (42°30′N, 72°10′W).

The Harvard Forest chronic N amendment study was initiated in 1988. The experiment consists of three 30 m × 30 m plots in a mixed hardwood stand and three plots in a red pine (*Pinus resinosa*) stand. The hardwood stand is dominated by black and red oak (*Quercus velutina* Lam.; *Quercus rubra* L.) with significant amounts of black birch (*Betula lenta* L.), red maple (*A. rubrum* L.) and American beech (*F. grandifolia* Ehrh.). The dominant soil types are stony-to sandy-loams formed from glacial till, and are classified as Typic Dystrochrepts of the Canton or Montauk series. Each set of

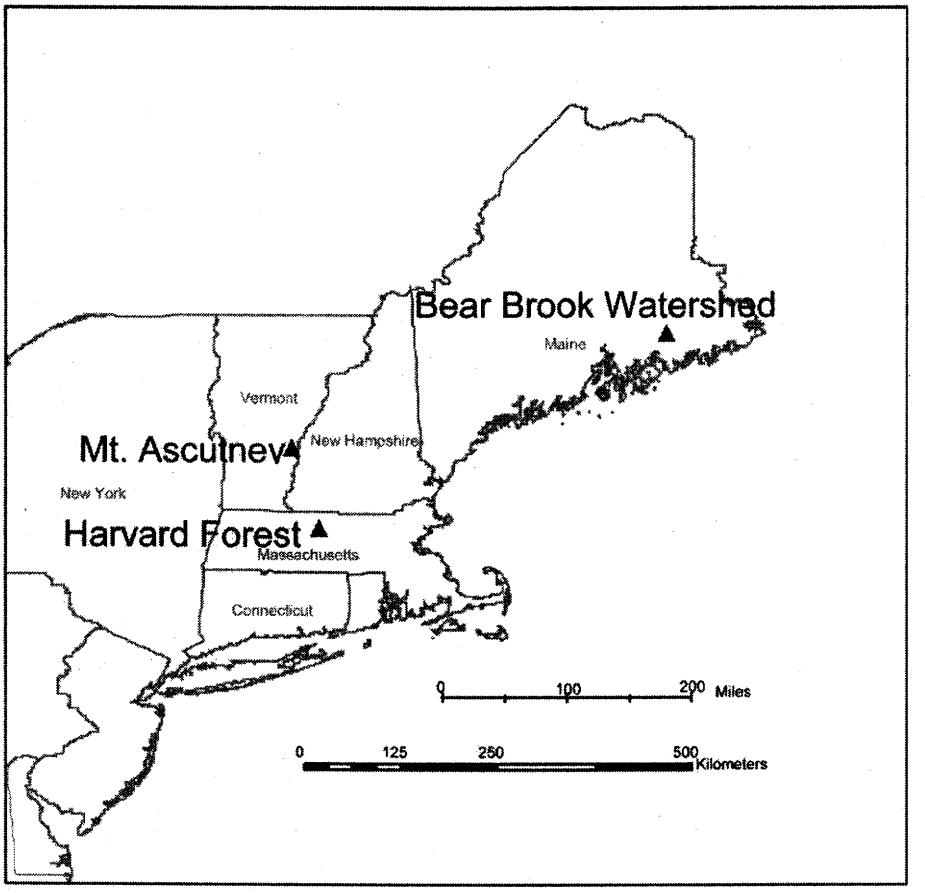

Fig. 1. Map of study sites.

plots includes an unfertilized control plot, one plot that receives 50 kg N ha^{-1} $year^{-1}$, and one plot that receives 150 kg N ha^{-1} $year^{-1}$, applied as NH_4NO_3 since 1988. This site was sampled on June 12 and July 26 of 2002. Soils collected on June 12 were also used to measure potential N mineralization and nitrification rates (Allison Magill, pers. comm.).

3. Methods

3.1. Soil sampling

In each plot, we collected five random samples that were composited and homogenized. Organic soils, excluding fresh surface litter, were sampled with bulk corers to the bottom of the organic horizon. The upper 10-cm of the mineral soil was also sampled at the Bear Brook and Harvard Forest sites. Mineral soil was not collected at Mt. Ascutney because the soil is not as well developed and mineral soil is only found in scattered microsites. Soils were stored on ice before returning to the lab, and stored at 4 °C for 12–36 h before sieving. Organic soils were sieved using 6-mm mesh and mineral soils with 2-mm mesh. Microbial biomass fumigation-extractions and SIR measurements were initiated within 36 h of sieving. A subsample of each soil was oven-dried at 70 °C for gravimetric water determination. Total C and N were analyzed using a Carlo Erba Elemental Analyzer.

3.2. Microbial biomass fumigation-extraction and dissolved organic C

Chloroform-labile microbial biomass C (MBC) was determined by the chloroform fumigation-extraction method (Vance et al., 1987). Six 3-g (approximate dry weight) subsamples of soil were weighed into 50-ml centrifuge tubes for each replicate. Three subsamples were extracted with 30-ml of dH_2O (Haney et al., 2001) with shaking for 1 h. Following centrifugation at 3200 rpm for 5 min, the extracts were filtered through pre-rinsed Whatman #1 filters. The extracts were acidified using HCl to pH 2.0. Cotton balls were placed in the remaining three subsamples, and 3 ml of $CHCl_3$ were pippetted onto the cotton balls. The vials were capped, and stored in darkness at 22 °C for 7 days before they were extracted following the same procedure as above. The samples were analyzed for dissolved organic C using a Shimadzu TOC-5000A. Chloroform-labile MBC was calculated as the difference between fumigated and non-fumigated samples. The concentration of organic C in the non-fumigated samples is hereafter referred to as dissolved organic C (DOC).

Table 1
Amounts of glucose, fungicide (Captan), and bactericide (Bronopol) added to soils for SIR assays and fungal bacterial respiration assays

Site	Soil horizon	Veg type	Glucose (mg/g)	Fungicide (mg/g)	Bactericide (μg/g)
BBWM	O	HW	10	8	1000
BBWM	O	SW	10	8	2000
BBWM	M	HW	4	4	500
BBWM	M	SW	1	4	500
HF	O	HW	2	16	1000
HF	O	SW	4	32	1000
HF	M	HW	4	8	500
HF	M	SW	1	4	500
AS	O	SW	4	4	1000

3.3. Substrate induced respiration and microbial inhibition

Soils (approximately 3 g dry weight) were weighed into 40-ml glass vials fitted with septa. For each organic soil sample, three replicates of each of the following treatments were used (Bailey et al., 2003):

(A) Glucose only; equivalent to substrate-induced respiration (SIR).
(B) Glucose + bactericide (Bronopol).
(C) Glucose + fungicide (Captan).
(D) Glucose + bactericide + fungicide.

Microbial biomass C was calculated according to the equation of Anderson and Domsch (1978) where:

$$\text{Biomass C}\,(\mu\text{g g}^{-1}\,\text{soil}) = (\mu\text{l CO}_2\,\text{g}^{-1}\,\text{soil h}^{-1}) \times 40.04$$

The ratio of fungal respiration to bacterial respiration was calculated as:

$$F:B = \frac{A-C}{A-B}.$$

Amounts of glucose, bactericide, and fungicide used for each soil were determined experimentally in preliminary assays (captions Table 1) using soils collected in May 2002. For mineral soils, we measured SIR but did not use biocides to distinguish between fungal and bacterial respiration. Distilled water was added to bring the soils to 50% of WHC, and bactericide and fungicide were added. Approximately ten 5-mm sterile glass beads were added to each vial, and the vials were shaken vigorously by hand for 10 s to mix the soil. Following storage in the dark for 1 h, glucose was added to the samples (see Table 1 for amounts used). The samples were then incubated in the dark at 22 °C for 6 h. Gas samples (20 ml) were collected with glass syringes and injected into pre-evacuated glass blood vials. Gas samples were analyzed for respired CO_2 on a Varian 3700 gas chromatograph equipped with a flame ionization detector.

3.4. Statistical analyses

Differences among soil horizons, forest types, treatments, and sample dates were analyzed with multiple analysis of variance (ANOVA) measures. Separate MANOVA analyses were conducted for each site. Cross-site correlations were analyzed by linear regressions. Differences are reported as significant when $p < 0.05$.

Table 2
MANOVA table for MBC, SIR, and the fungal:bacterial ratio for all three sites

	d.f.	MBC		SIR		F:B ratio	
		F-value	*p*	*F*-value	*p*	*F*-value	*p*
Harvard Forest							
Vegetation	1	0.02	0.87	14.45	0.001	0.49	0.49
Soil horizon	1	36.2	<0.0001	125.9	<0.0001	1.20	0.28
Treatment	2	2.41	0.11	8.89	0.002	0.49	0.62
Date	1	3.46	0.079	1.76	0.20	3.92	0.06
Mt. Ascutney							
Treatment	2	18.67	0.0001	22.24	<0.0001	4.62	0.03
Date	2	3.24	0.07	26.51	<0.0001	0.20	0.82
Bear Brook							
Vegetation	1	1.48	0.23	1.89	0.18	0.29	0.59
Soil Horizon	1	205.0	<0.0001	149.2	<0.0001	2.63	0.11
Treatment	1	0.01	0.90	2.46	0.12	0.02	0.89
Date	2	4.53	0.017	1.32	0.28	0.60	0.55

4. Results

Chloroform-labile microbial biomass C and SIR were strongly affected by fertilization treatment and SIR also differed between hardwood and softwood stands for organic soils (Table 2). MBC and SIR varied seasonally in organic soils at all sites, though the patterns of seasonal trends varied.

4.1. Harvard Forest

At Harvard Forest, organic soil MBC and SIR were decreased in the fertilized plots relative to the unfertilized plots for both hardwood stands and pine stands, though there was a significant overall treatment effect only for SIR (Table 2). In the hardwood stands, organic soil MBC was decreased by an average of 54% and SIR was decreased by an average of 45% in the High-N plots relative to the control plots (Fig. 2). In the pine stand, organic soil MBC was decreased by 40% and SIR decreased by an average of 35% in the High-N plots relative to the control plots (Fig. 2). The fungal:bacterial activity ratio was also decreased in the hardwood stands from an average of 1.5 in the control plot to 1.0 in the High-N plot, and in the pine stands from 1.9 in control plot to 1.0 in the High-N stand. These results are consistent with the patterns observed by Frey et al. (2004). Overall, there were few differences between the June and July sampling dates, except that MBC was lower in the pine soils in July than in June.

In the mineral soil, MBC and SIR were decreased in both fertilized pine plots relative to the control in June (Fig. 3). In July, the MBC of the High-N Pine plot was less than the control and Low-N plots, while SIR was lower in both fertilized plots relative the control plot. In the hardwood plots, MBC was not affected by fertilization, though SIR was significantly lower in the High-N plot than control and Low-N on both sample dates.

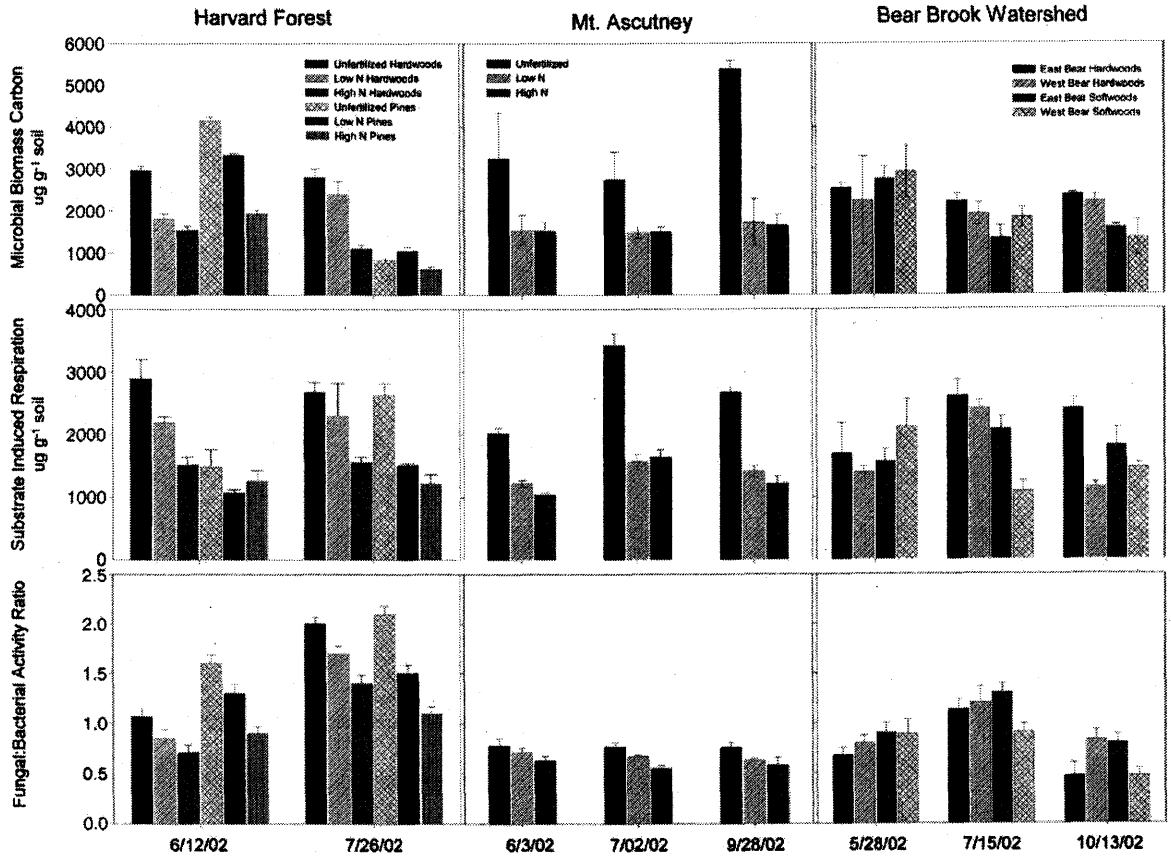

Fig. 2. Fertilization effects on chloroform extractable carbon, substrate induced respiration, and the fungal: bacterial ratio for organic soils from three long-term nitrogen fertilization experiments. Bars represent standard errors.

4.2. Mt. Ascutney

At Mt. Ascutney, MBC was reduced by an average of 59% and SIR by 52% in the High N plots relative to the unfertilized plots. There were no significant differences in MBC or SIR between the low N and High N plots on any sample date. The fungal:bacterial activity ratio was also slightly decreased in fertilized plots relative to unfertilized plots. There was relatively little seasonal variation at this site in MBC or SIR.

4.3. Bear Brook watershed in Maine

At BBWM, there were no differences between the fertilized and unfertilized watersheds in organic or mineral soil MBC and SIR in May or July. In October, SIR was decreased in the fertilized West Bear watershed in both hardwood and pine stands. Across samples dates, MBC and SIR did not differ between hardwood and softwood soils, though the depth of the organic horizon in softwood stands was much greater than in hardwood stands (Jefts et al., 2004), suggesting much lower microbial density in softwood organic horizons. The fungal:-bacterial ratio also did not differ between forest stand type or watershed in May or July. In October the fungal:bacterial ratio of active biomass was increased in fertilized hardwood stands, but decreased in fertilized pine stands relative the control watershed.

5. Discussion

Despite important differences in soil type, forest type, climate, land-use history, and fertilization application, a general trend of decreasing biomass and decreasing fungal:bacterial ratio with N fertilization was observed in this cross-site comparison. It is important to consider these results in the context of each individual site before attempting to draw some broad conclusions from this work.

5.1. Harvard Forest

The N amendments at Harvard Forest have dramatically reduced total and active biomass in both the hardwood and red pine stands. In the hardwood stand, MBC, SIR, and the fungal:bacterial activity ratio did not differ between the two levels of N in June. However, in July the High-N plot had lower MBC, SIR, and fungal:bacterial ratio than the low-N plot. These results are consistent with recently measured declines in N mineralization and soil respiration at this site (Bowden et al., 2004; Magill et al., 2004). While the declines in microbial biomass at this site are dramatic, the level of fertilization at this site, even in the Low-N plots, exceeds the range of current ambient deposition in the United States. On the other hand, maximum annual N addition at this site (and Mt. Ascutney) is roughly double the annual N mineralization rate, and a doubling

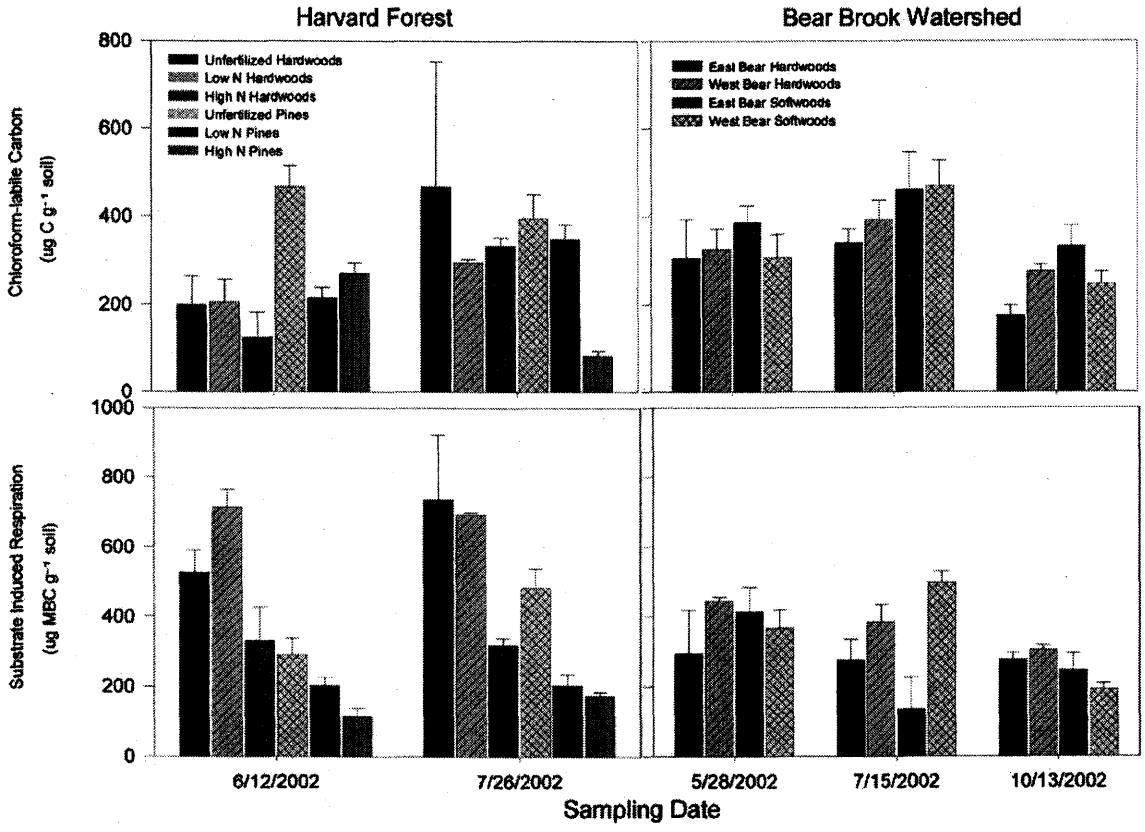

Fig. 3. Fertilization effects on chloroform extractable carbon and substrate induced respiration for mineral soils from three long-term nitrogen fertilization experiments. Bars represent standard error.

of the rate of inorganic N supply is possible at many sites due to elevated N deposition.

The fungal:bacterial activity ratio declined in the organic soils of both the hardwood and pine stand at this site. The technique used in this study to measure fungal biomass is unlikely to assay mycorrhizal activity, as their connection to root C exudates was severed during soil processing. This suggests that the decline observed in this study is primarily due to a decrease in saprotrophic biomass or activity. Both red pine and several of the dominant hardwood species (*Quercus* sp., *B. lenta*, and *F. grandifolia*) form ectomycorrhizal associations. It is likely that the decline in fungal biomass is largely due to decreases in mycorrhizae. In the unfertilized Pine plots, thick mycelia networks are easily visible in organic soils, while in the High-N Pine plots they appear to be entirely absent. However, Other studies have observed a decline in lignolytic enzyme activity (associated primarily with saprotrophic fungi) in N-amended soils (DeForest et al., 2004), consistent with this observation.

5.2. Mt. Ascutney

At Mt. Ascutney, our findings suggest that microbial biomass is decreased significantly in plots that have been fertilized at a rate of 16 kg N ha^{-1} $year^{-1}$, which is within the range of current ambient deposition across parts of the United States and much of Europe. This implies that under current air quality regulations, N deposition may be contributing to changes in microbial communities that could have important implications for ecosystem functioning such as reduced tree N uptake, reduced root biomass, and increased susceptibility to drought and windthrow. The High-N plots, which are fertilized at double the rate of the Low-N plots did not exhibit further decreases in microbial biomass, which is consistent with stage 2 N saturation in the Low-N plots. The High-N plots have also experienced significant tree mortality which is consistent with stage 3 N saturation (Boggs et al., in review). Declines in N mineralization rates, increases in nitrification rates and NO_3-leaching, and decreased basal area growth were also observed in these plots (Boggs et al., in review).

5.3. Bear Brook watershed in Maine

We were unable to detect a treatment effect on MBC, SIR or the fungal:bacterial ratio at BBWM in May or July of 2002. However, in October, we did observe a negative fertilization effect on SIR and the fungal:bacterial ratio. The fertilized West Bear watershed receives the smallest amount of total (ambient + fertilization) N inputs among the treated sites included in this study, and the West Bear watershed appears to be less N saturated (i.e. stage 0–1) than the other sites (Aber et al., 1998). Our results are somewhat inconsistent with recently measured declines in N mineralization and increased net nitrification rates in the fertilized watershed observed

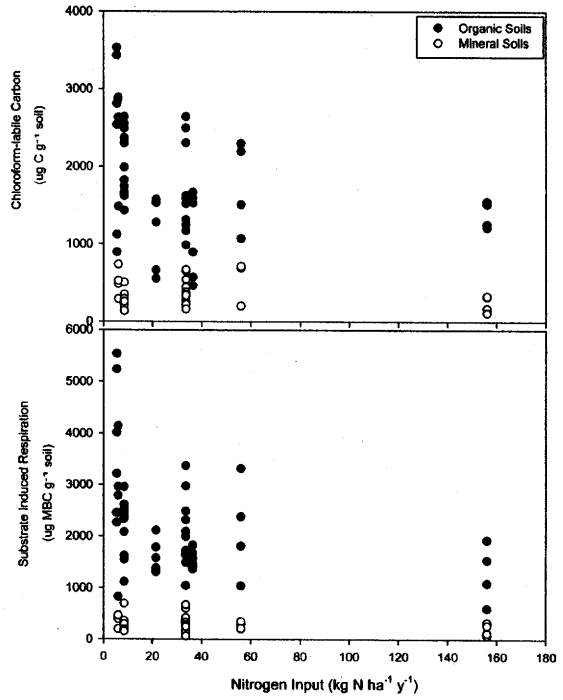

Fig. 4. Relationships of total annual nitrogen inputs (ambient deposition + - fertilization) with chloroform-labile carbon and substrate induced respiration in three long-term N fertilization experiments.

throughout the year (Jefts et al., 2004). This suggests that initial responses of N cycling processes to N fertilization in early stages of N saturation are not controlled by the volume of the microbial biomass, but rather are due to changes in activity, regulated by changes in soil pH and associated chemical changes, temperature, and moisture.

5.4. Cross-site correlations

Across all three sites, MBC and SIR had negative relationships with total N inputs in both mineral soils and organic soils, though the effect was much stronger in organic soils (Fig. 4). This relationship is strongly influenced by the High-N plots at HF, which receive more than double the amount of N of any other plots in this study. Nonetheless, this suggests a robust and consistent effect of N inputs with microbial biomass, regardless of forest type.

The C/N ratio of bulk soil was positively correlated with both MBC and SIR (Fig. 5). This relationship was much stronger for MBC than SIR suggesting that the total fungal and bacterial biomass responds to more closely to general changes in the C/N ratio of the litter, while the active (SIR) portion is more affected by short-term environmental conditions. Both MBC and SIR were positively correlated with dissolved organic C and total soil C (Fig. 5). Total C explained roughly 50% of the variation for both measures of microbial biomass. Dissolved organic C was much more closely related to MBC than to SIR. This may suggest that the active portion of the microbial biomass is more strongly influenced by other environmental factors. However, this may also be a methodological artifact since MBC is partially based on the same extraction procedure as DOC.

5.5. Methodological considerations

The techniques used in this study measure standing-pools of active (SIR) or total (MBC) biomass. However, the same standing mass could be present in soils with differing microbial turnover rates. Microbial turnover rates can be affected by substrate availability, temperature, pH, soil moisture, and predation. The abundance of bacterial and fungal feeding

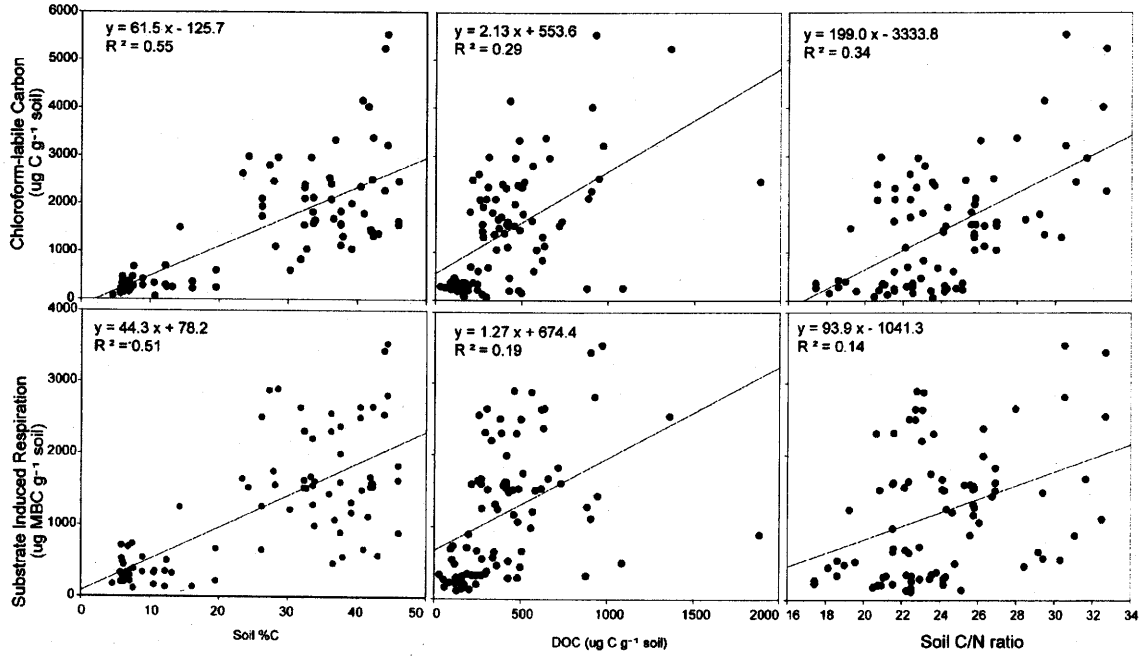

Fig. 5. Relationships of chloroform-labile carbon and SIR with bulk soil %C, water-extractable DOC, and bulk soil C/N ratio.

microfauna can be affected by N additions (Maraun et al., 2001) and thus may play a role in regulating microbial activity and biomass (Mamilov et al., 2000). In a study by Fisk and Fahey (2001), 8 years of N fertilization resulted in a 20–30% decrease in biomass, but did not result in a decreased N mineralization rate. They attributed this finding to increased microbial turnover and activity. This is not likely to be the case in this study, as N mineralization rates were lower in fertilized plots compared to control plots at all three study sites.

The chloroform fumigation-extraction technique may not be equally sensitive to changes in fungal and bacterial biomass, due to varying extraction efficiencies for different organisms (Ingham and Horton, 1987; Eberhardt et al., 1996). The changes in fungal:bacterial ratio observed in this study suggest that the extraction efficiency of the FE technique was not equivalent for all samples used in this study, and may affect the patterns observed within sites. The SIR technique may also underestimate fungal biomass, as mycorrhizal respiration decreases substantially when the fungi are separated from their supply of root exudate C due to soil sieving and processing.

The use of selective inhibitors to distinguish between fungal and bacterial respiration was pioneered by Anderson and Domsch (1973) and has since been applied to a variety of soils. We used novel antibiotics that were reported to have less non-target inhibition compared to other commonly used antibiotics (Bailey et al., 2003). The specificity of the antibiotics can be assessed empirically using the inhibitor additivity ratio (IAR) to calculate the degree to which the activities of the antibiotics overlap (Beare et al., 1990). The IAR is calculated as (see methods for description of variables):

$$\mathrm{IAR} = \frac{(A-B)+(A-C)}{(A-D)}.$$

We calibrated the amounts of glucose, bactericide, and fungicide using soils collected in May of 2002 prior to initiating these experiments. Using these values, we obtained a mean IAR across all samples of 1.06, though there was a relatively high degree of variability (standard deviation = 0.84). While the optimal amounts of biocides required for this assay almost certainly varied by sampling date, we believe that the using the same conditions throughout the year allowed for more reasonable comparisons between dates. In addition, we were able to conduct the assays within 48 h of field collection using pre-calibrated amounts of glucose and biocides which minimized storage effects.

5.6. Possible causes of decreasing biomass

The results of this study add to a growing body of evidence for negative long-term effects of ecosystem N enrichment on soil microbial biomass (Wardle, 1992). The underlying mechanisms governing this cause and effect relationship are not well defined, despite the emerging evidence for this N effect on soil microbial communities across a wide range of ecosystem conditions. Several mechanisms are plausible.

First, as we observed here, N deposition leads to a decreasing soil C:N ratio (Table 3). Theoretically, the lower C:N ratio of bacteria relative to fungi implies that they are capable of utilizing organic matter of a lower C:N ratio. However, this is a simplified interpretation of the energy costs associated with exoenzyme production (Schimel and Weintraub, 2003).

A second possible cause of decreased MBC is soil acidification resulting from NH_4^+ uptake by plants, nitrification of NH_4^+ in soils, and NO_3^- leaching. In a study of forested sites representing a pH gradient, Baath and Anderson (2003) found that pH was positively correlated to SIR and negatively correlated to the fungal:bacterial ratio. At all of the sites used in this study, fertilization has resulted in decreased soil pH (Table 3), thus pH and the associated soil chemical changes that occur as pH changes, may be an important factors controlling soil microbial communities.

Most temperate forests in regions with low deposition rates are thought to be N-limited (Schlesinger, 1997; Fenn et al., 1998). As

Table 3
Soil chemistry data for experimental plots

Site	Plot-treatment	pH	%C	%N	C:N
Harvard Forest	Hardwood Unfertilized	3.16[a]	17.29 (2.17)	0.77 (0.09)	22.09 (1.70)
	Hardwood Low N	2.91[a]	19.65 (2.64)	0.85 (0.11)	22.60 (0.92)
	Hardwood High N	2.99[a]	18.02 (1.99)	0.79 (0.10)	22.14 (1.47)
	Pine Unfertilized	3.16[a]	21.14 (3.02)	0.84 (0.13)	23.59 (4.30)
	Pine Low N	2.95[a]	20.37 (1.53)	0.8 (0.17)	23.65 (3.27)
	Pine High N	2.91[a]	17.17 (1.44)	0.72 (0.12)	22.98 (1.91)
Mt. Ascutney	Unfertilized	2.66 (0.13)[b]	44.04 (1.42)	1.39 (0.08)	31.66 (1.06)
	Low N	2.71 (0.05)[b]	38.46 (4.08)	1.43 (0.17)	27.09 (2.09)
	High N	2.80 (0.01)[b]	39.77 (2.85)	1.53 (0.18)	26.20 (2.23)
Bear Brook	East Pine	3.54[c]	26.55 (3.12)	1.01 (0.11)	25.70 (2.47)
	West Pine	3.69[c]	25.75 (2.73)	1.08 (0.19)	23.59 (2.15)
	East Hardwood	4.01[c]	21.72 (2.14)	0.98 (0.07)	22.67 (1.19)
	West Hardwood	3.97[c]	17.58 (2.59)	0.85 (0.10)	19.82 (1.94)

Values are means across sample dates with standard error in parentheses.
[a] pH data is from Allison Magill (unpublished data).
[b] pH data is from Boggs et al. (in review).
[c] pH data is from Jefts et al. (2004).

N limitation is alleviated, plants may reduce their allocation of resources belowground by decreasing root production and exudation. There is some evidence at these study sites of decreased belowground productivity (Lindsey Rustad, pers. comm.).

5.7. Possible consequences of decreased microbial biomass and changing community structure

The implications of decreased microbial biomass and changes in the fungal:bacterial ratio are unknown, but are likely to be important. Processes that involve diverse groups of heterotrophic organisms such as N mineralization and denitrification are likely to decline with decreasing biomass. Autotrophic microorganisms such as methanotrophs and NH_4^+ oxidizers may not follow the same response, since they will not be directly affected by changes in litter quality and C supply.

The loss of fungi, particularly mycorrhizal fungi, is of special concern for plant productivity and forest species composition. If soil environmental factors such as changes in soil chemistry are causing a loss of mychorrizal biomass, tree hosts may suffer from a loss of nutrient availability and water uptake. This effect would be consistent with evidence of N additions altering fungal community composition and abundance (Wallenda and Kottke, 1998; Lilleskov et al., 2001; Peter et al., 2001; Lilleskov et al., 2002; Avis et al., 2003). There is a pressing need in future research to identify the specific groups of fungi that are being affected by N additions in order to better define the potential future consequences of N deposition for forested ecosystems.

5.8. Implications for the nitrogen saturation hypothesis

One of the surprising results of many long-term N fertilization experiments has been the ability of soils to retain a high percentage of added N (Aber et al., 1998). One proposed mechanism for incorporating this N into soil organic matter is increased N immobilization by microbes (Aber, 1992). The absence of increases in heterotrophic soil respiration has cast some doubt on this mechanism (Aber et al., 1998). The results of this study provide additional evidence to reject the hypothesis that ecosystem N retention is driven by microbial immobilization during chronic, long-term N enrichment. Though we did not measure the C:N ratio of microbial biomass in this study, the ~50% decrease in microbial biomass would preclude the possibility of increased N immobilization unless the C:N ratio of microbial biomass was also decreased by greater than 50%. The decreasing fungal:bacterial ratio suggests that the C:N ratio has likely decreased, but the magnitude of this change is unlikely to indicate a 50% decrease.

6. Conclusions

The results of this study support evidence in the literature that declines in soil microbial biomass result with chronic elevated N inputs to forest soils. We propose that these findings are a component of the N saturation hypothesis. The magnitude of the decline in microbial biomass (~50%) resulting from just over a decade of chronic N fertilization at the Mt. Ascutney and Harvard Forest experiments is likely to have important implications for the future form and function of these ecosystems. Similar results did not occur at BBWM due to the lower historical N deposition for northern New England that presumably leaves over a decade of N additions at this site still in the early stages of N saturation. Future work should examine the implications of decreased biomass for ecosystem processes. Changes in microbial function should also be examined by examining the response of specific functional communities to long-term N fertilization. This work suggests that elevated N deposition is altering soil microbial communities by mechanisms that remain poorly understood. Understanding these mechanisms will be essential to predict long-term responses of ecosystems to elevated N deposition.

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