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The role of monoterpenes in soil nitrogen cycling processes in ponderosa pine

Results from laboratory bioassays and field studies

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Abstract. The effects of select monoterpenes on nitrogen (N) mineralization and nitrification potentials were determined in four separate laboratory bioassays. The effect of increasing monoterpene addition was an initial reduction in NO_3^-–N production (nitrification inhibition), followed by a reduction in the sum of NH_4^+–N and NO_3^-–N (inhibition of net N mineralization and net immobilization at high monoterpene additions. Monoterpenes could produce this pattern by inhibiting nitrification, reducing net N mineralization, enhancing immobilization of NO_3^-–N relative to NH_4^+–N, and/or stimulating overall net immobilization of N by carbon-rich material.

Initial monoterpene concentrations in the assay soils were about 5% of the added amount and were below detection after incubation in most samples.

Potential N mineralization-immobilization, nitrification, and soil monoterpene concentrations were determined by soil horizon for four collections from a ponderosa pine (*Pinus ponderosa*) stand in New Mexico. Concentrations of monoterpenes declined exponentially with soil depth and varied greatly within a horizon. Monoterpene content of the forest floor was not correlated with forest floor biomass. Net N mineralization was inversely correlated with total monoterpene content of all sampled horizons. Nitrification was greatest in the mineral soil, intermediate in the F–H horizon, and never occurred in the L horizon. Nitrification in the mineral soil was inversely correlated with the amount of monoterpenes in the L horizon that contain terminal unsaturated carbon–carbon bonds ($r^2 = 0.37$, $P \leqslant 0.01$). This pattern in the field corresponded to the pattern shown in the laboratory assays with increasing monoterpene additions.

Introduction

A considerable amount of research has been performed on factors that control decomposition (Swift et al. 1979; Horner et al. 1988), nitrogen (N) mineralization (Keeney 1980; White & Gosz 1987), and nitrification (Prosser 1986) in terrestrial ecosystems, where the supply of N often limits primary production. Temperature and moisture are major factors controlling N mineralization and nitrification (Keeney 1980; Schmidt 1982), and fluctuations in these factors in the field lead to temporal variation in rates of N mineralization and nitrification. Laboratory assays for potential N mineralization and nitrification maintain temperature and moisture at near-optimal conditions, yet temporal variation has been reported in N mineralization and nitrification potentials for many

forest soils incubated in the laboratory (Nadelhoffer et al. 1983, 1984; Carlyle & Malcolm 1986; Gosz & White 1986; White & Gosz 1987; White et al. 1988). White & Gosz (1987) suggested that temporal changes in the relative quality of the organic substrate play an important role in determining within-year variation in N mineralization and nitrification potentials for soils from ponderosa pine and other forests in New Mexico.

White (1986b) proposed that monoterpenes act as inhibitors of N mineralization and nitrification in a ponderosa pine (*Pinus ponderosa* Laws.) forest. This hypothesis was based, in part, upon the results of laboratory bioassays that introduced either

—water-soluble or volatile constituents of organic soil horizons (forest floor), or
—vapors from a mixture of five monoterpenes found in ponderosa pine resin to subsamples of the upper mineral soil horizon.

Net N mineralization and nitrification were reduced by the additions in all assays. Monoterpenes may have been the active agents since they are highly volatile and partially water-soluble (Massaldi & King 1973; Smyrl & LeMaguer 1980); however, water extracts and vapors from the forest floor were not analyzed for their monoterpene content (White 1986b).

Monoterpenes are major constituents of ponderosa pine resin oils (Smith 1977). Although monoterpenes are well recognized for their role as plant defense compounds in a wide variety of plants (Mabry & Gill 1979), very little is known about their distribution in soils and their effects on soil microorganisms. Monoterpene concentrations in forest floor and mineral soil from a single collection in two pinyon pine (*Pinus monophylla* [Torr.] Frem.) woodlands (Wilt et al. 1988) and from three samples from ponderosa pine (White 1986b) are the only known data on soil monoterpenes.

A mechanistic explanation for the inhibition of nitrification by monoterpenes was suggested by White (1988). He proposed that monoterpenes may directly inhibit ammonia monoxygenase activity and that inhibitory activity may be dependent upon certain molecular structures that bind with the enzyme. Based upon structures that were common to other known inhibitors, White predicted that the highest inhibitory activity should be displayed by monoterpenes with: a 6-carbon (C) ring, and terminally-located unsaturated C–C bonds (terminal C=C used in this article). The inhibitory activity may vary according to the basic monoterpene types, with monocyclic > acyclic > bi-tricyclic (according to the monoterpene classification system used by Dev 1982). Monocyclic monoterpenes (Fig. 1), which are rather planar in shape and contain a 6-C ring structure, are predicted to have the highest inhibitory activity. Bi- and tricyclic monoterpenes contain a 6-C ring structure, but are more spherical; thus, their inhibitory activity should be lower than the monocyclic compounds. Acyclic monoterpenes are planar, but lack true ring structures; thus, their inhibitory activity is predicted to be intermediate. Activity should increase with the number of terminal C=C within each type of monoterpene, analogous to the increased inhibitory activity of acetylenic compounds with terminal unsaturated bonds compared to other compounds of similar molecular weight (McCarty and Bremner 1986).

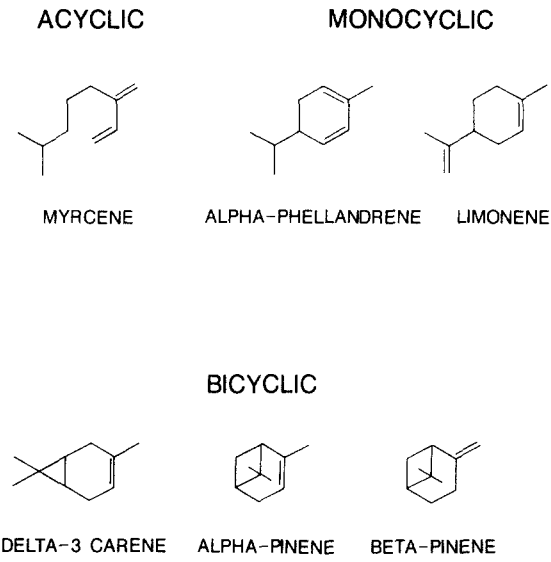

Fig. 1. Structures of selected monoterpenes (grouped according to structural-type).

Bremner & McCarty (1988) tested the effects of six monoterpenes on nitrification in laboratory bioassays of three Iowa agricultural soils. Additions of monoterpenes resulted in N immobilization with no inhibition of ammonium oxidation. Although acknowledging that Bremner and McCarty's experiments indicated immobilization rather than inhibition, White (1990) questioned whether assays with 200 μg/g NH_4^+ -N added to agricultural soils were adequate to invalidate the hypothesis.

This article evaluates the effect of monoterpenes on N mineralization and nitrification in a ponderosa pine forest based upon the results from four laboratory bioassays and four field collections. The objectives of the laboratory bioassays were:

—to determine if monoterpenes inhibited N mineralization and/or nitrification;
—whether inhibition of nitrification was based upon the molecular structure of the individual monoterpene; and
—to determine the persistence of monoterpenes in the assay soil.

The objectives of the field study were:

—to identify variation (both qualitative and quantitative) in monoterpene concentrations within the organic and 0–10 cm mineral soil horizon; and
—to identify the relationship between the amount of monoterpene and N mineralization and nitrification potentials for these soil horizons.

Methods

Laboratory bioassays

The activity of the following monoterpenes was assayed in one or more of these experiments: two monocyclic (limonene, Aldrich Chemical Company, Inc.; and a-phellandrene, ICN Pharmaceutical, Inc., Plainview NY); one acyclic (myrcene, Aldrich Chemical Company, Inc.); and three bicyclic monoterpenes (Δ^3-carene, a-pinene, and b-pinene, Aldrich Chemical Company, Inc.). Commonly referred to as essential oils, monoterpenes are liquids at room temperature. Each monoterpene was tested individually by application as the pure liquid to a subsample of the assay soil.

Experimental design

Four aerobic incubation assays were performed. In each assay, sufficient water to adjust a moist subsample of the assay soil to 50% of its water-holding capacity (WHC, as determined in White & McDonnell 1988) was added to a pint canning jar. A monoterpene was added to the water surface, creating an oil-like monoterpene film on the water. The assay soil was added to the jar, allowing the water-monoterpene mixture to moisten the sample. All jars were sealed with new canning lids and bands, and incubated in the dark at 20 °C. Previous experiments determined that oxygen within the jars was not depleted to a level that inhibited nitrification during the incubation period. After incubation, analysis for monoterpene content, NH_4^+-N and NO_3^--N was performed on subsamples from separate jars, or by splitting the soil from one jar into separate portions for each analysis.

Assay 1. This assay was designed to:

— simulate the range of naturally occurring monoterpene concentrations in the organic and mineral soil horizons of a ponderosa pine stand;
— identify the monoterpene concentration needed to alter N mineralization and nitrification; and
— determine if individual monoterpenes had different effects on these processes.

The assay soil consisted of a mixture of 90% sandy loam collected in October 1987 from the ponderosa pine stand sampled in field collections by White (1986a) and 10% clay loam from a Douglas-fir (*Psuedotsuga menziesii* (Mirb.) Franco) stand that exhibited high rates of nitrification (site characterized by White et al. 1988). The purpose of adding the soil from the Douglas-fir stand was to increase the rate of nitrification to allow detection of inhibition. Each monoterpene was tested by adding 0 (control), 0.6, 3.0, or 30.0 μl to three replicate jars (six replicate jars for control) containing 2.8 ml deionized water. A total of 17.2 g of moist assay soil was added to each jar (20 g total wet weight). Initial NH_4^+-N and NO_3^--N, concentrations were determined by extracting three control jars with KCl. The rest of the jars (including three control jars) were analyzed for NH_4^+-N, NO_3^--N, and monoterpenes after 25-d incubation.

Assay 2. This assay followed the same basic design as Assay 1, but used a narrow range of monoterpene additions and unaltered soil from the same ponderosa pine stand. The assay soil was collected in March 1989, six months after the site was treated with prescribed fire. This assay soil had lower NH_{4+}-N concentrations and slower rates of nitrification than the composite assay soil in Assay 1. Each monoterpene was applied at a rate of 3, 6, 12, or 24 μl of pure monoterpene to 20 g wet weight mineral soil as described above. Triplicate samples of each level of monoterpene addition were subsampled immediately for monoterpene and inorganic-N analyses, and another set of triplicate samples were subsampled for all analyses after 28-d incubation.

Assay 3 and 4. Assays 3 and 4 were designed to test the effects of a fairly narrow concentration gradient of a monocyclic (limonene) and a bicyclic (a-pinene) monoterpene on soils from a site other than ponderosa pine. In Assays 3 and 4, the assay soil (a Turney loamy sand, Johnson 1988) was from a desert grassland area within the Sevilleta National Wildlife Preserve, located in central New Mexico. This soil was selected because:

— it was not continually exposed to monoterpenes in the field, avoiding potential effects of preconditioning by high monoterpene exposure;
— nitrification rates were similar to ponderosa pine, as determined in separate experiments (C. White unpublished data);
— texture and organic matter content were similar to the ponderosa pine soil;
— select research plots were fertilized with ammonium-nitrate, which provided two assay soils with inorganic N concentrations similar to soils used in the first two assays.

Assay 3 used the unamended soil (control) and Assay 4 used the soil from a fertilized plot. In Assays 3 and 4, either 5, 10, or 20 μl of limonene or a-pinene was added as the pure liquid to 2.9 ml of water, followed by the addition of 30.1 g of field-moist soil. This procedure resulted in 33 g wet weight of soil at 50% WHC. Two replicate jars of each monoterpene addition were subsampled immediately for NH_4^+-N, NO_3^--N, and monoterpene analyses. Two replicates were subsampled and analyzed for all parameters after 7, 14 and 21-d incubation.

In Assays 1 and 2, 10 g subsamples were immediately extracted with 100 ml of 2 *N* KCl, and the extracts were analyzed for NH_4^+-N and NO_3^--N + NO_2^--N (NO_2^--N was never detected) as described below. In Assays 3 and 4, the extraction was modified by using 50 ml of 2 *N* KCl to increase concentrations in the extract. Subsamples for monoterpene analysis were placed in separate plastic scintillation vials and kept at −80 °C until analyzed by gas chromatography (GC) by the method described below.

Field collections

The research site of White (1986a) in north-central New Mexico was used for the field portion of this study. The site was on a small knoll of pumice, which

provided uniform soils between plots. The overstory was a pure stand of ponderosa pine with scattered seedlings and saplings of pinyon pine (*Pinus edulis*) and various species of junipers (*Juniperus* sp.). Plots within the site were chosen to avoid heavy fuel deposits and favor areas with approximately equal accumulations of forest floor and woody debris. Samples were collected from four plots that measured 4 × 9 m with a 5-m perimeter buffer zone. Collections were made on 7 October 1987, 12 November 1987, 15 March 1988, 17 May 1988, and 27 October 1988 (referred to as Oct. '87, Nov., March, May, and Oct. '88 collections, respectively). The Oct. '87 collection was used to develop methods and verify procedures; thus, only limited data are reported from this collection.

Forest floor and mineral soil samples were collected along a 4-m transect in each plot. For each collection, two samples of forest floor material and mineral soil were collected at intermediate points along the line transect, except in Oct. '88 when only one sample per plot was collected.

From each sample location, forest floor (O horizon) beneath a 0.5 × 0.5 m template was harvested. The template was placed on the forest floor and the surrounding forest floor scraped away after cutting along the template border with a knife. The forest floor was separated into litter (L) and duff (F-H) horizons, except for the Oct. 1988 collection. Freshly fallen needles were collected in Oct. '87 from the four plots. The needles were weighed and analyzed for monoterpenes and moisture content. A 10-cm diameter core of mineral soil was collected to 10-cm depth at the center of the area from which the forest floor was harvested. For the May collection, mineral soils were sieved and sorted (as described below) in the field, and subsamples were placed in jars, sealed, and kept refrigerated for determination of "field" monoterpene content. The rest of the May mineral soil collection was handled by normal procedures.

All samples were kept in the dark on ice during transport to the laboratory, where they were kept refrigerated at 4 °C. All material larger than 6.4 mm diameter was removed by sieving. Roots greater than about 1 mm diameter were removed by hand sorting. Needles too long to fit into the incubation cup were cut in half or thirds.

N mineralization and nitrification potentials

For all collections, N mineralization and nitrification potentials were measured by aerobic incubation of each horizon. After adjusting a portion of each sample to 50% of determined WHC, a total of 22 subsamples were apportioned into plastic cups. Cups contained approximately 10 g dry-weight (DW) mineral soil, 2 g DW L, or 3 g DW F-H. One subsample was immediately extracted with 100 ml 2 *N* KCl for NO_3^--N and NH_4^+-N analyses, and another subsample was frozen for monoterpene analysis. The remainder of the cups were covered with plastic wrap, sealed with a rubber band, and incubated in the dark at 20 °C. The plastic wrap minimized water loss during incubation, yet exchange of CO_2 and O_2 was sufficient to keep the subsamples aerobic during incubation. Moisture content was monitored by mass loss and replenished as needed. At intervals of 2, 4, 7, and 10 weeks, two subsamples of each horizon were removed, one for

NO_3^--N and NH_4^+-N analyses and the other for monoterpene analyses. After extraction with KCl for 18–24 h, the clarified KCl was filtered through a Kimwipe® and analyzed for NH_4^+-N and NO_3^--N + NO_2^-N (NO_2^--N was never detected) on a Technicon AutoAnalyzer as described in White (1986a).

Monoterpene analyses

Each subsample for the analysis of monoterpenes was immediately frozen. Mineral soil subsamples were transferred to plastic scintillation vials and stored at −80 °C. The L, F-H, and total forest floor subsamples were ground separately under liquid N using a mortar and pestle, then transferred with liquid N to a Tecator® centrifugal grinder fitted with a 1.0-mm screen. The ground sample was transferred to a vial, sealed, and stored at −80 °C until monoterpene analyses could be performed. Subsamples (10–15 g for mineral soil, 2–3 g for F-H or total forest floor, and 1–2 g for L) were extracted with 10 ml ether containing a known amount of fenchyl acetate for use as an internal standard in a 50-ml Erlenmeyer flask that was covered with paraffin film and aluminium foil. After 1-h extraction at room temperature, samples were centrifuged (mineral soils did not require centrifugation), and the clarified supernatant was pipetted into a ground-glass stoppered culture tube, sealed with paraffin film, and refrigerated at 4 °C. A 4-μl portion of the refrigerated ether extract was injected into a gas chromatograph (Shimadzu GC-9) fitted with a split injector (split ratio was 50:1), a bonded methyl silicone capillary column (25 m length, 0.25 mm inside diameter, 0.25 micron film thickness), and a flame-ionization detector. The injector temperature was 270 °C, flow rate was 4 cc min^{-1}, and initial oven temperature was 60 °C. Oven temperature was increased by 4 °C min^{-1} to 109 °C, then by 40 °C min^{-1} to 250 °C. Monoterpenes were identified by co-chromatography by adding individual monoterpene standards that had been verified with GC-MS to sample extracts. Peak area was converted to mass of individual monoterpenes using calibration curves generated with standards. An average calibration factor was used to convert the peak area of unknowns to relative mass.

The following compounds, listed by type (Dev 1982), were identified and directly quantified (compounds included in types with terminal C=C indicated by *). Acyclic; myrcene*; linalool*, citronellal, citronellol, nerol. Monocyclic; p-cymene, a-phellandrene, limonene*, limonene oxide*, g-terpinene. Bicyclic; d,l-camphene*, a-pinene, b-pinene*, sabinene*, Δ-3-carene, verbenone, bornyl acetate.

Statistical design

Assays. The following criteria were used to assess the effects of a monoterpene addition on the N-cycling processes (differences accepted as significant at the $P \leqslant 0.05$ level).

— An increase in net N mineralization occurred when the amount of inorganic-N (NH_4^+-N + NO_3^--N) in treated soil samples was greater than the

amount of inorganic-N in the control samples after incubation.

— Inhibition of N mineralization occurred when the amount of inorganic-N in treated soil samples was less than the amount of inorganic-N in the control samples after incubation and was greater than or equal to the initial amount of inorganic N.
— Nitrification occurred when the amount of NO_3^--N increased during incubation.
— Inhibition of nitrification occurred when the amount of NO_3^--N in treated soil samples after incubation was less than the amount of NO_3^--N in the control samples after incubation.
— Net N immobilization occurred when the amount of inorganic-N in treated soil samples after incubation was less than the initial amount of inorganic-N.
— Immobilization of NO_3^--N occurred when the amount of NO_3^--N in the treated samples after incubation was less than the initial amount of NO_3^--N.
— Immobilization of NH_4^+-N occurred when the amount of NH_4^+-N in treated samples after incubation was less than the amount of NH_4^+-N and there was no increase in NO_3^--N in the treated samples during incubation.

Field collections. Samples were nested by plot within a collection (2 samples per plot, 4 plots per collection); thus, the field data were analyzed by a two-factor analysis of variance testing for plot and collection effects. Significant plot or plot-collection interaction factors are reported when they occurred. Monoterpene concentrations were log transformed for within-collection analyses. All concentration data were converted to mass per unit area for comparison of collections. Net N mineralization was defined as the increase in the amount of inorganic-N over the entire 10-week incubation. Net immobilization was defined as the decrease in inorganic-N over 10-weeks. Relative nitrification was defined as the fraction of the total inorganic-N pool comprised by NO_3^--N at the end of the incubation. Although the arithmetic means and standard deviations are reported, relative nitrification data were arcsin transformed prior to statistical analyses.

All statistical analyses were performed with either SAS-PC© (Statistical Analysis System, SAS Institute Inc.) or StatView SE© (BrainPower, Inc.). ANOVA followed by Duncan's Multiple Range Test were used to analyze for the effect of each monoterpene addition for each assay.

Results

Laboratory bioassays

Monoterpene concentrations

Assay 1. All monoterpenes in all of the 0.6 and 3.0-μl additions were below detection after the 25-d incubation. In the 30-μl addition, no limonene or

myrcene could be detected, and all other monoterpenes were present at less than 0.4% of the original addition (Table 1).

Assay 2. All soil monoterpene concentrations were less than 10% of the actual quantity added to the jar when extracted immediately after addition, with most concentrations between 2 and 5% of the added amount (Table 1). Following the 28-d incubation, only the 24-μl additions had detectable amounts of extractable monoterpenes, with all concentrations less than 0.04% of the original addition.

Assays 3 and 4. In the 20-μl additions, extractable soil concentrations varied from 1.1 to 3% of actual additions on Day 0 (similar to Assay 2) (Table 2). For both monoterpenes in both assays, extractable soil concentrations declined dramatically within the first week of incubation, followed by a gradual decline over the last 14-d incubation (Table 2). This pattern appeared to be independent of initial inorganic-N levels. Less than 0.4% of the added monoterpene was extracted from the assay soils at the end of both incubations. Extractable concentrations of a-pinene were generally greater than limonene at comparable levels of addition and incubation times, regardless of initial inorganic N concentration.

Inorganic-N

Assay 1. During the 25-d incubation, significant net N mineralization and nitrification occurred in the control samples (Fig. 2). In the 0.6-μl additions, only two monoterpenes showed significant effects relative to the control after incubation; Δ^3-carene significantly increased nitrification and net N mineralization, while limonene significantly increased the amount of NH_4^+-N remaining in the soil (NO_3^--N was not significantly different from control). In the 3.0-μl additions, both monocyclic (limonene and a-phellandrene) and the acyclic monoterpene (myrcene) significantly inhibited nitrification and N mineralization (NO_3^--N and sum of NH_4^+-N + NO_3^--N were lower than control after incubation, see definitions given above); however, net N immobilization did not occur with any monoterpenes. In the 30-μl additions, all monoterpenes (except limonene) resulted in significant immobilization of initial inorganic-N, with immobilization of NO_3^--N greater than NH_4^+-N. The 30-μl addition of limonene immobilized NO_3^--N, but did not inhibit net N mineralization. At the end of incubation, NH_4^+-N concentrations in all 30-μl additions were equal to or greater than the control, indicating that NH_4^+-N concentrations in the monoterpene additions were sufficient for the nitrifying bacteria to utilize this resource if monoterpenes were not present.

Assay 2. The assay soil contained only NH_4^+-N at the beginning of incubation (Fig. 3), which was typical of soil from that ponderosa pine stand. In the control samples, net N mineralization and nitrification occurred during the 28-d incubation. All of the 3-μl monoterpene additions significantly inhibited nitrification, net N mineralization, and lowered NH_4^+-N (limonene significant for

Table 1. Percent recovery of added monoterpenes ((actual conc./predicted conc.) × 100) in Assays 1 and 2. Values are means (standard deviations in parentheses) of three samples. Other additions within Assay 1 did not yield recoverable monoterpenes.

Incubation Period (d)	Addition (μl)	Monoterpene					
		Limonene	a-Phellandrene	Myrcene	Δ-3 Carene	a-Pinene	b-Pinene
			Assay	1			
25	30	0.00 (0.00)	0.14 (0.17)	0.00 (0.00)	0.31 (0.19)	0.11 (0.11)	0.02 (0.02)
			Assay	2			
0	3	1.01 (1.03)	0.70 (0.31)	2.68 (0.53)	2.77 (0.31)	1.04 (0.36)	1.95 (0.42)
	6	4.86 (0.86)	1.23 (0.29)	3.07 (0.83)	2.86 (1.03)	1.93 (0.74)	4.28 (1.49)
	12	5.76 (0.79)	3.23 (0.36)	3.72 (0.43)	4.03 (0.13)	3.92 (0.43)	9.71 (3.34)
	24	6.15 (0.55)	4.01 (1.05)	5.28 (1.18)	5.09 (1.23)	3.97 (0.37)	9.38 (1.68)
28	3	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.03 (0.06)	0.00 (0.00)
	6	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	12	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.002 (0.004)	0.00 (0.00)
	24	0.03 (0.006)	0.01 (0.01)	0.00 (0.00)	0.03 (0.03)	0.01 (0.002)	0.00 (0.00)

Table 2. Percent recovery of added monoterpenes ((actual conc./predicted conc.) × 100) and standard deviations for soils in Assays 3 and 4 (n = 3). *indicates only one sample analyzed.

Incubation Period (d)	Addition (ml)	Monoterpene			
		Limonene		a-pinene	
		% Recovery	Std Dev	% Recovery	Std Dev
		Assay 3			
0	5	0.23	*	0.39	*
	10	1.10	*	1.10	*
	20	1.66	*	1.33	*
7	5	0.00	0.00	0.04	0.08
	10	0.12	0.02	0.61	0.04
	20	0.29	0.15	0.84	0.38
14	5	0.00	0.00	0.05	0.11
	10	0.13	0.25	0.14	0.07
	20	0.30	0.39	0.40	0.08
21	5	0.00	0.00	0.00	0.00
	10	0.00	0.00	0.10	0.18
	20	0.08	0.03	0.20	0.08
		Assay 4			
0	5	0.42	*	0.37	*
	10	0.89	*	0.89	*
	20	1.16	*	3.05	*
7	5	0.02	0.04	0.09	0.18
	10	0.24	0.10	0.42	0.02
	20	0.32	0.15	0.85	0.87
14	5	0.11	0.06	0.00	0.00
	10	0.09	0.18	0.03	0.01
	20	0.31	0.05	0.44	0.36
21	5	0.15	0.29	0.02	0.04
	10	0.00	0.00	0.20	0.06
	20	0.06	0.04	0.36	0.39

nitrification only) compared to the control after 28-d incubation. The 3-μl limonene addition significantly inhibited nitrification with no change in the amount of NH_4^+-N with respect to the control. The 3-μl additions did not lower the net amount of inorganic-N with respect to the starting levels (no net immobilization). Increasing monoterpene additions to 6, 12, and 24 μl further inhibited nitrification, resulting in almost no NO_3^--N production with NH_4^+-N present as the dominant form. Immobilization of initial inorganic-N was significant ($P < 0.05$) in all the 24-μl additions.

Assay 3. In the control soil, newly mineralized N was converted readily to NO_3^--N, resulting in relatively constant NH_4^+-N concentrations (Fig. 4). Ad-

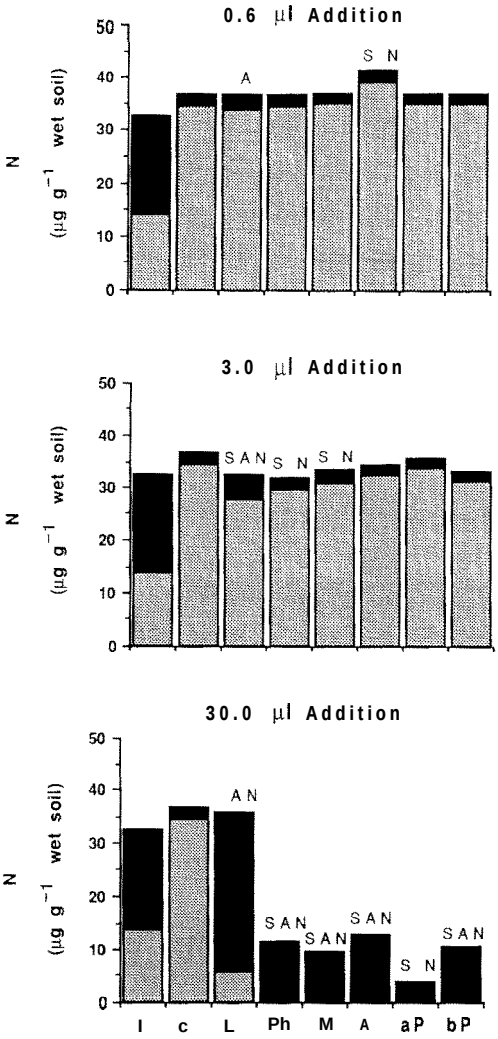

Fig. 2. Concentrations of soil NO_3^--N (shaded) and NH_4^+-N (solid) at the initiation of Assay 1 (I) and after 25-d incubation following addition of water (control C), limonene (L), a-phellandrene (Ph), myrcene (M), Δ^3-carene (A), a-pinene (aP), or b-pinene (bP) at the indicated volumes to 20g of moist soil. Letters above a bar indicate that NH_4^+-N (A), NO; -N (N), or their sum (S) in the respective treatments were significantly different ($P < 0.05$) from the control values after incubation.

dition of limonene or a-pinene inhibited net N mineralization and nitrification in nearly direct proportion to the amount added (Fig. 4). No additions caused immobilization of the initial inorganic N. Concentrations of NO; -N and NH_4^+-N remained unchanged over the incubation in the highest additions of both monoterpenes.

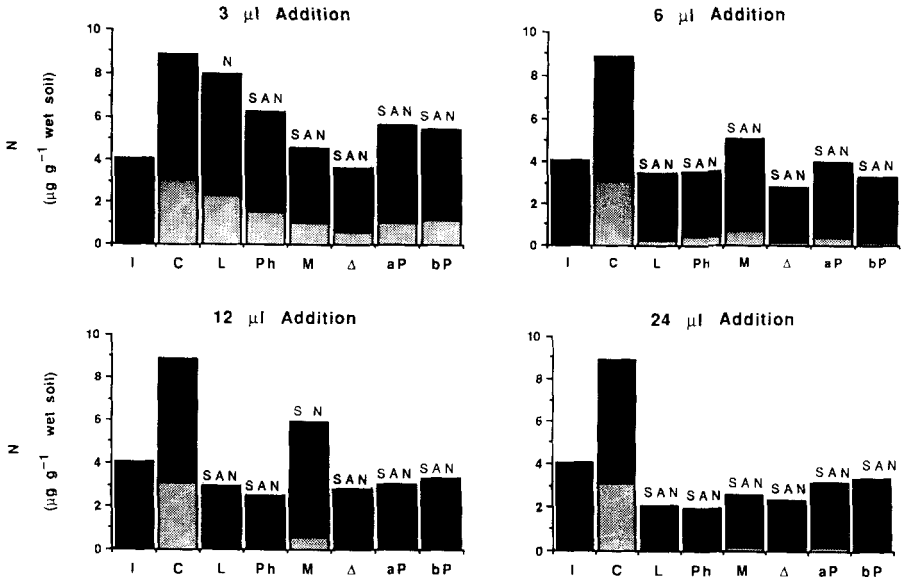

Fig. 3. Concentrations of soil NO_3^--N (shaded) and NH_4^+-N (solid) at the initiation of Assay 2 (I) and after 28-d incubation following addition of water (control C), limonene (L), a-phellandrene (Ph), myrcene (M), Δ^3-carene (Δ), a-pinene (aP), or b-pinene (bP) at the indicated volumes to 20 g of wet soil. Letters above a bar indicate that NH_4^+-N (A), NO_3^--N (N), or their sum (S) in the respective treatments were significantly different ($P < 0.05$) from the control values after incubation.

Assay 4. Due to fertilization, initial NO_3^--N and NH_4^+-N concentrations in Assay 4 soil were about 10 times higher than in Assay 3 soil (Fig. 4). After the 21-d incubation, net N mineralization occurred in the control samples with most of the inorganic-N present as NO_3^--N. Limonene inhibited N mineralization in all additions and inhibited nitrification in the highest addition. Limonene additions tended to increase the amount of NH_4^+-N, but this trend was not significant with 2 replicates. Similar effects were shown with a-pinene, except the lowest addition caused no significant changes. Limonene appeared to be more effective than a-pinene at inhibiting nitrification, but the difference was not statistically significant. Immobilization did not occur, even with the highest additions of limonene or a-pinene.

Field collections

Monoterpenes

Total monoterpene concentrations differed by more than three orders of magnitude between the fresh litter and the mineral soil (Table 3). Declining monoterpene concentration with soil depth was a consistent pattern during all collections. Within a horizon, total monoterpene concentration or total monoterpene

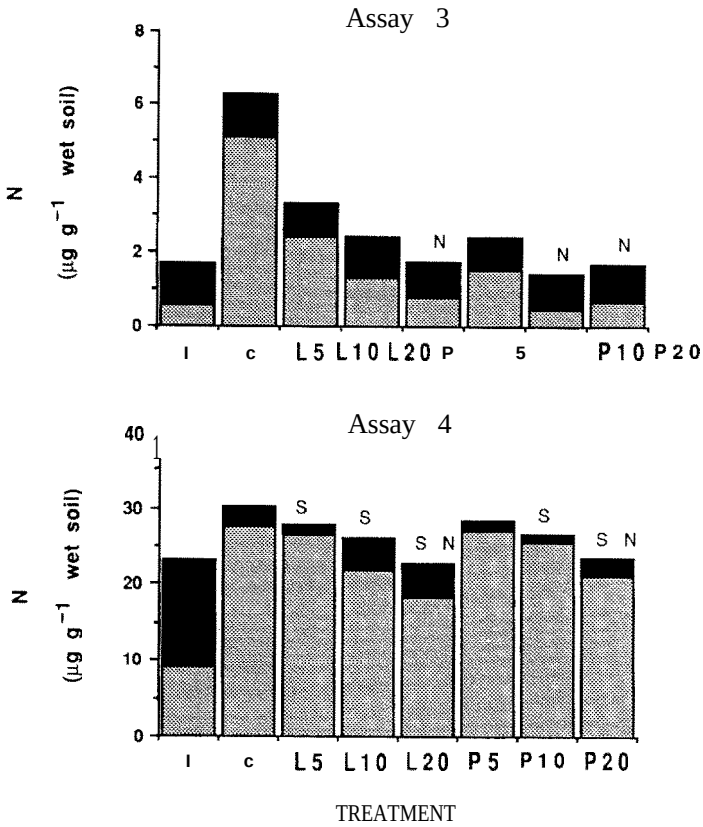

Fig. 4. Concentrations of soil NO_3^--N (shaded) and NH_4^+-N (solid) at the initiation of Assay 3 (I, upper graph) or Assay 4 (I, lower graph) and after 21-d incubation following the addition of 5, 10, or 20 μl limonene (L5, LIO, L20, respectively) or a-pinene (P5, P10, P20, respectively) to 30g wet soil. Letters above a bar indicate that NO; -N (N), NH_4^+-N (A), or their sum (S) in the respective treatments were significantly different from the control values at the end of the incubation.

mass did not show significant seasonal change. Total monoterpene concentration was independent of total forest floor biomass (r^2 = 0.08, not significant). When expressed as the total amount of monoterpene per unit area ($mg\,m^{-2}$), the L horizon had higher absolute quantities of monoterpenes than the F-H horizon for the Nov. and March collections (Table 3), even though the F-H horizon had greater biomass. In the May collection, the F-H horizon contained more monoterpenes than the L horizon.

Live roots in the mineral soil samples of the March collection were quantified and analyzed for monoterpenes. Root monoterpene concentrations were about 2.5 times higher than in L horizon samples for the March collection (Table 3). Expressed on an area1 basis, roots represented a potential source of monoterpenes nearly three times greater than the total forest floor. This study did not measure the quantity of monoterpene retained in senescent root material;

Table 3. Concentration and mass of total extractable monoterpenes, net N mineralization, and relative nitrification (NO_3^- N/(NO_3^- N + NH_4^- N)) within a horizon for field collections from a ponderosa pine stand. Values are means (standard deviation in parentheses) of eight samples for each horizon, except October 1988, which had four samples. Within a column, values within a collection followed by different letters are significantly different ($P \leq 0.05$, Fresh litter was compared to November 1987 for monoterpene concentration). For N mineralization, horizons in different collections followed by different letters in parentheses are significantly different ($P \leq 0.05$).

Collection Date	Horizon/sample	Monoterpene concentration ($\mu g\, g^{-1}$ DW)	Monoterpene mass ($mg\, m^{-2}$)	N Mineralization ($g\, m^{-2}$)	Relative nitrification (fraction)
October 1987	Fresh litter	1360 (472) a			
November 1987	L	172 (68.5) b	176 (95.7) a	−0.19 (0.13) a (a)	0.0 (0.0) a
	F–H	41.8 (46.5) c	117 (109) a	0.25 (0.5) b (b)	0.36 (0.30) b
	Mineral soil	0.03 (0.09) d	2.3 (6.4) b	0.35 (0.43) b (b)	0.64 (0.31) c
March 1988	L	140 (65.5) a	122 (58.3) a	−0.01 (0.01) a (b)	0.0 (0.0) a
	F–H	11.6 (5.47) b	32.2 (12.2) b	1.61 (1.02) b (a)	0.38 (0.27) b
	Mineral soil	0.17 (0.44) c	12.1 (28.7) b	1.46 (0.93) b (a)	0.85 (0.2) c
	Roots	350 (263) d	373 (446)		
May 1988	L	138 (73.5) a	117 (103) a	0.02 (0.01) a (b)	0.0 (0.0) a
	F–H	35.0 (46.3) b	131 (177) a	0.69 (0.63) b (b)	0.26 (0.31) b
	Mineral soil	0.19 (0.38) c	15.5 (28.2) a	0.61 (0.30) b (b)	0.77 (0.16) c
	Mineral soil (field)	2.23 (4.56)	183 (371)		
October 1988	O (L + F–H)	88.7 (37.2) a	391 (151) a	−0.03 (0.02) a	0.0 (0.0) a
	Mineral soil	0.23 (0.37) b	19.4 (27.4) b	0.39 (0.37) b (b)	0.76 (0.16) b

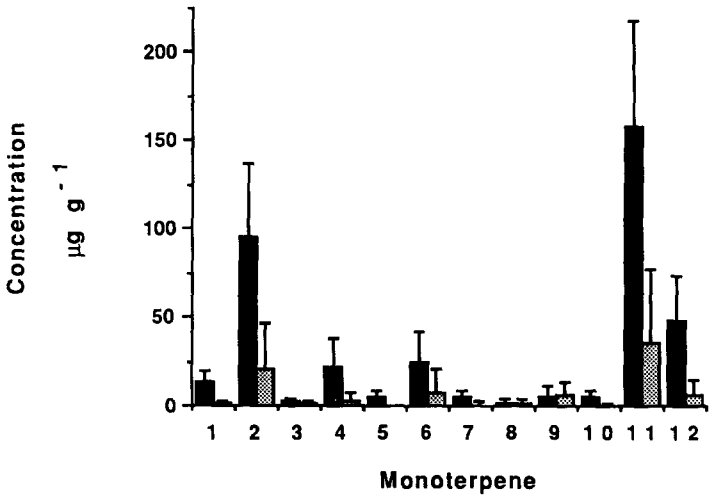

Fig. 5. Mean monoterpene concentration (bars indicate one standard deviation of the mean, n = 8) per g dry weight of the L (solid) and the F-H (shaded) horizon from the November 1987 collection. Monoterpenes are: (1) d- and l-camphene, (2) a-pinene, (3) sabinene, (4) b-pinene, (5) myrcene, (6) Δ^3-carene, (7) limonene, (8) verbenone, (9) sum of all others, (10) sum of monocyclics, (11) sum of bicyclics, and (12) sum of compounds with terminal C=C (see methods for monoterpene classification).

therefore, the actual contribution of roots to monoterpenes in the soil is unknown.

The monoterpene content (mg m^{-2}) of the field-prepared mineral soil samples from the May collection was over 10 times greater than the content of the laboratory-prepared samples (Table 3). The major difference between the field and laboratory-prepared samples was the loss of compounds having longer GC retention times from the laboratory-prepared samples, including limonene, verbenone and a number of unidentified compounds (probably oxidized monoterpenes, data not shown). This suggests that these compounds were loosely held and readily lost to the atmosphere, and/or that these compounds reacted very rapidly within the soil. The organic horizon samples did not appear to be susceptible to substantial loss of monoterpenes. Even during the 10-week incubation, a 5-fold reduction in concentration was the greatest seen for all monoterpenes in the forest floor for all collections.

Monoterpene composition changed between horizons (Fig. 5) and among collections (Fig. 6). The differences between the L and F-H horizons in the Nov. collection (Fig. 5) showed that the F-H horizon was not simply a diluted version of the L horizon. Concentrations of most monoterpenes were lower in the F-H than in the L horizon, but some L horizon monoterpenes could not be detected in the F-H (myrcene, Fig. 5), whereas concentrations of some monoterpenes were the same in both horizons (verbenone, Fig. 5). In general, monoterpenes with the lowest concentrations in the L horizon were absent in the lower horizons. The major exception to this pattern was limonene, which increased in relative importance with increasing soil depth.

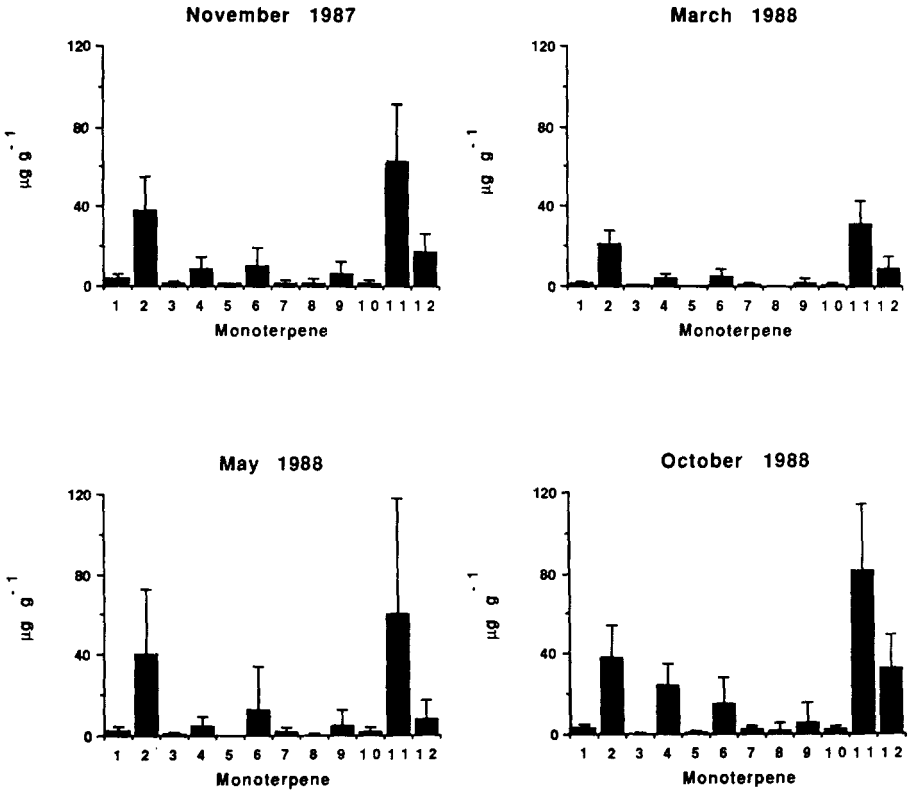

Fig. 6. Weighted mean monoterpene concentration per g dry weight of the forest floor (L plus F-H horizons; bars indicate one standard deviation of the mean; n = 8; October 1988, n = 4) for the four collections. Monoterpenes are: (1) d- and l-camphene, (2) a-pinene, (3) sabinene, (4) b-pinene, (5) myrcene, (6) Δ^3-carene, (7) limonene, (8) verbenone, (9) sum of all others, (10) sum of monocyclics, (11) sum of bicyclics, and (12) sum of compounds with terminal C=C (see methods for monoterpene classification).

Expressed as mass-corrected concentration within the total forest floor ($\mu g\, g^{-1}$ DW), a-pinene concentrations were highest, Δ^3-carene was second highest (except Oct. '88), and limonene or the isomers of camphene were the next most abundant monoterpenes in all collections. Monoterpenes that showed significant among collection variation (Fig. 6) were sabinene, b-pinene, myrcene, verbenone, limonene oxide (not shown), and the sum of monoterpenes with terminal C=C (Fig. 6). Myrcene, verbenone, and limonene oxide also had significant plot-collection interaction factors.

N mineralization and nitrification potentials

Potential N mineralization varied between horizons and among collections (Table 3). Net immobilization occurred in the L horizon in all collections except May. Net N mineralization occurred in the F-H and mineral soil horizons.

Nitrate concentrations were at or below detection in nearly all samples from

all horizons at the beginning of the incubations. Since the amount of NH_4^+-N (representing substrate) would limit potential NO_3^--N production, the fraction of the total inorganic N that was NO_3^--N (NO_3^--N/NH_4^+-N + NO_3^--N) was termed relative nitrification. Expressed in this manner, relative nitrification was greater in the mineral soil than in the F-H horizon ($P < 0.01$, Table 3). Nitrification never occurred in the L horizon (significantly different than mineral soil and F-H, $P < 0.001$; Table 3), even when NH_4^+-N was produced during incubation.

Discussion

Soil monoterpene concentrations: an interpretation

Monoterpenes were evaluated with respect to the amount added to the soil because actual soil concentration were two to three orders of magnitude less than the concentrations added, and actual concentrations varied between replicates (Tables 1 and 2). Thus, the additions represented the total amount of monoterpene that could have acted to control N mineralization and nitrification and should not be regarded as actual soil concentrations.

In the field, mineral soil may act as a sink for monoterpenes transported from upper horizons. Monoterpenes may move via gas exchange or by infiltrating water. Laboratory analyses identify the amount of unbound monoterpene in a soil, but they do not identify monoterpenes contributed to the soil prior to collection. Thus, monoterpenes could control N mineralization and nitrification potentials without being detected in the sample.

Effects of monoterpenes on N cycling processes

I proposed that monoterpenes controlled N mineralization and nitrification in soils of ponderosa pine (White 1986a). Instead of an alternative hypothesis that excluded the interaction of other factors known to control these processes, this hypothesis was presented as a complementary factor that is significant when forest soils accumulate monoterpene-rich litter. The results from the assays and field collections provide an array of evidence that supports this hypothesis.

The addition of monoterpenes to mineral soil samples in all assays produced a consistent result, even though each assay used soils that had different initial NO_3^--N and NH_4^+-N levels and different net mineralization and nitrification rates. A conceptual model for this general pattern is displayed in Fig. 7. Net N mineralization (an increase in NO_3^--N + NH_4^+-N) and nitrification occurred in the control samples in all assays, with concentrations of NO_3^--N usually greater than NH_4^+-N after incubation. At very low monoterpene additions (as in Assay 1), there was a slight (not significant) increase in net N mineralization. Increasing monoterpene additions reduced NO_3^--N concentrations (interpreted as nitrification inhibition) and total inorganic-N concentrations (interpreted as inhibition of net N mineralization), resulting in higher NH_4^+-N concentrations

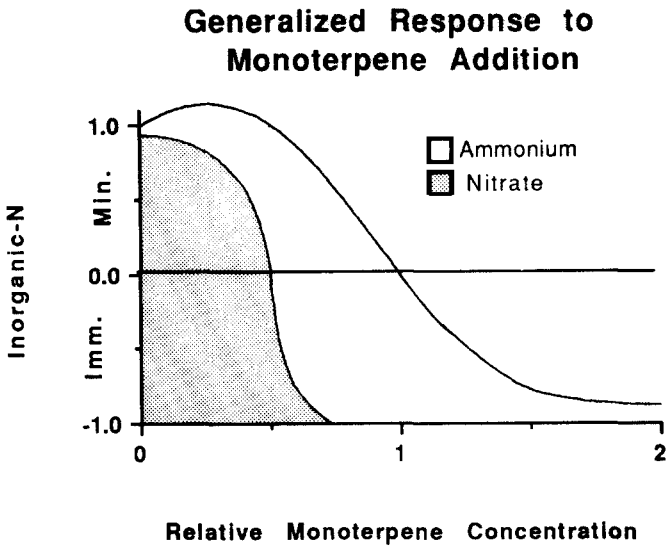

Fig. 7. Generalized response to increasing monoterpene addition for relative concentrations of soil inorganic-N after incubation for potential mineralization. Mineralization (Min.) occurs when total inorganic-N increases during incubation. Immobilization (Imm.) occurs when total inorganic-N falls below the initial concentrations. Monoterpene additions are relative values, not actual concentrations.

relative to NO_3^--N at the end of incubation. The highest additions (30 μl) immobilized the initial inorganic-N, so that NH_4^+-N was the only form of inorganic-N. In summary, the net effect of increasing monoterpene additions to soils is to inhibit net N mineralization and nitrification with eventual immobilization of initial inorganic-N at the highest additions.

Bremner & McCarty (1988) questioned whether monoterpenes acted to inhibit the oxidation of NH_4^+-N to NO_3^--N, or whether they simply acted to promote immobilization of N during the processing of a C source by the microbial populations. My results also show strong immobilization with high monoterpene additions; however, the lower additions significantly inhibited net mineralization and nitrification with no immobilization of the initial inorganic N (the 3-μl additions in Assay 1 and 2). In Assays 3 and 4, net mineralization and/or nitrification was significantly inhibited yet no immobilization of the initial inorganic N occurred in all additions. When immobilization occurred, NH_4^+-N was the dominant or only form of inorganic N at the end of the incubation, whereas only NO_3^--N was present in the soils examined by Bremner & McCarty. Aeration at 2-d intervals by Bremner & McCarty could have removed the highly volatile monoterpenes, which would have ceased immobilization and allowed the NH_4^+-N that was not immobilized to be converted to NO_3^--N with no apparent inhibition.

It appears that the response of any given soil to the addition of monoterpenes may depend upon the interaction of three factors:

Factor 1. Inherent rate of nitrification in the soil. The degree of nitrification inhibition by monoterpenes was inversely proportional to the inherent rate of nitrification in the assay soil. When rates of nitrification were low in Assay 2 (unaltered ponderosa pine soil), all of the 3-μl monoterpene additions significantly inhibited nitrification, whereas only three of six monoterpenes significantly inhibited nitrification at the same level of addition in Assay 1 when rates of nitrification were high (Figs. 2 & 3). Thus, monoterpenes may be more effective inhibitors of nitrification in soils with low rates of nitrification.

Factor 2. Initial NH_4^+-N concentration. Nitrification inhibition was inversely proportional to the initial NH_4^+-N concentration. In Assay 3 with low initial NH_4^+-N concentration, no significant production of NO_3^--N occurred in the 10 or 20-μl additions of either a-pinene or limonene, whereas most of the original NH_4^+-N was converted to NO_3^--N in the comparable additions in Assay 4 with high initial NH_4^+-N levels. The pattern of decreasing inhibition with increasing levels of ammonium (substrate) would be compatible with the hypothesis that monoterpenes inhibit ammonium monooxygenase (White 1988). Thus, it may be possible to overcome inhibition of nitrification by monoterpenes by adding high NH_4^+-N concentrations.

Factor 3. Initial NO_3^--N concentration. With high initial NO_3^--N concentrations and high monoterpene additions, NO_3^--N appeared to be preferentially immobilized rather than NH_4^+-N. As shown in Assay 1, NO_3^--N concentrations in the 30-μl monoterpene additions were lower than initial NO_3^--N concentrations even though NH_4^+-N concentrations were greater than in the control samples after incubations (Fig. 2). Depletion of the initial NO_3^--N was probably due to assimilatory pathways since the samples were maintained at 50% WHC and well aerated.

Evidence for assimilatory uptake of NO_3^--N was reported for soils from other conifer forests in the Southwest (White & Gosz 1987). Rice & Tiedje (1989), however, report that assimilation of NO_3^--N was effectively inhibited at low NH_4^+-N concentrations. There may be two possible explanations for the apparent conflict: (1) differing soil communities prefer different forms of inorganic N, or (2) preference for inorganic N may change with the C-source being used by the soil community. In this study and the study by White & Gosz (1987), monoterpenes were the major source of available-C, whereas Rice & Tiedje (1989) exposed their assay soil to glucose prior to incubation.

In the field collections, monoterpene concentrations were highest in the fresh litter and declined exponentially through the organic horizons to the mineral soil (Table 3). There was an inverse relationship between net mineralization and monoterpene content of a horizon (Table 3). Immobilization occurred in the L horizons which had the highest monoterpene concentrations. Net mineralization with NH_4^+-N as the major form of inorganic-N occurred in the F-H horizons that had intermediate monoterpene concentrations, and net N mineralization with high relative nitrification rates occurred in the mineral soil horizons which had the lowest monoterpene concentrations. This pattern is

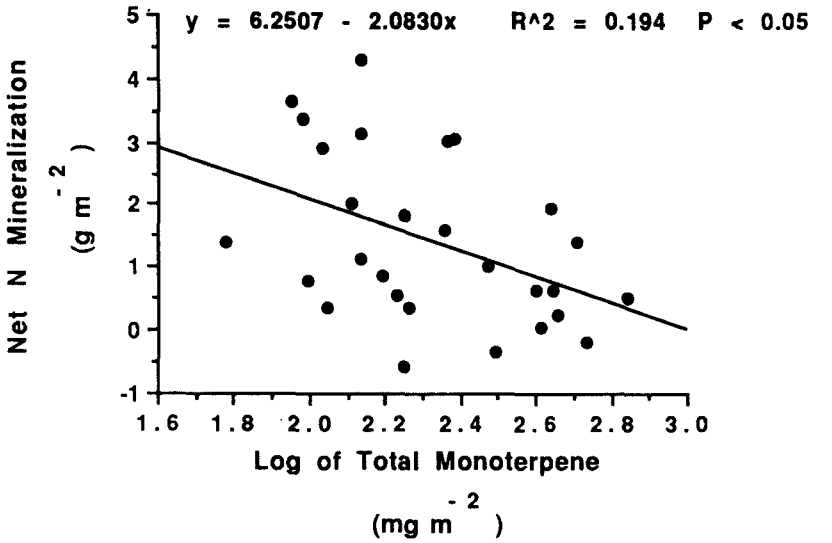

Fig. 8. The relationship between the log total monoterpene content and net N mineralization summed for all horizons. Each point indicates an individual sample (n = 28).

nearly identical to the response of N mineralization and nitrification to increasing monoterpene addition in the assays (Fig. 7). Thus, the range of N mineralization and nitrification potentials shown in different soil horizons was reproduced in the assay soils by increasing the monoterpene additions.

For each field sample, net N mineralization potential and total monoterpene content for the entire soil profile were determined by summing the respective values from all sampled horizons. There was a significant inverse relationship between net N mineralization and the log of total monoterpene content for each field sample (Fig. 8). Increasing monoterpene content of field samples appeared to constrain or restrict potential N mineralization at that site. At low monoterpene content, a wide range of N mineralization occurred. The variation in N mineralization at low monoterpene content may be due to variation in other factors that control this process (Keeney 1980).

There was an inverse relationship between the concentration of all monoterpenes with terminal C=C and net N mineralization in all L horizon samples ($r^2 = 0.27$, $P < 0.01$). Within the entire forest floor, there was a significant negative correlation between the weighted concentration of all monoterpenes with terminal C=C and net N mineralization for all samples ($r^2 = 0.15$, $P < 0.05$). In light of factors that could cause variation in monoterpenes and N mineralization, I consider explaining 15–27% of the total variation in potential N mineralization a surprising result.

Effectiveness of nitrification inhibition based upon molecular structures

If monoterpenes directly inhibit ammonia monooxygenase activity (White 1988), then increasing the monoterpene additions should increase concentrations of NH_4^+-N relative to NO_3^--N without a decline in their sum with

respect to the initial concentrations, and an increase in NH_4^+-N relative to NO_3^--N should occur at lower monoterpene additions for compounds with higher activity. An increase in NH_4^+-N relative to NO_3^--N occurred in the 3-μl additions of limonene, a-phellandrene and myrcene in Assay 1, in all of the 3-μl additions in Assay 2, and in most additions in Assay 3 and Assay 4. The increase in NH_4^+-N relative to NO_3^--N resulted from lower concentrations of NO_3^--N, while NH_4^+-N was unchanged relative to the control. Evidence for differential inhibitory activity based upon molecular structures is not as clear in my assays. In Assay 1, limonene was most effective at increasing NH_4^+-N relative to NO_3^--N (apparent nitrification inhibition) at all levels of addition. In the 3-μl additions of Assay 1, the results closely fit the predicted pattern of greatest inhibition with additions of monocyclic monoterpenes (limonene and a-phellandrene), intermediate with acyclic monoterpenes (myrcene), and the least (no apparent inhibition) with bicyclic monoterpenes (a-pinene, b-pinene, Δ^3-carene). Within a type, apparent inhibitory activity was the highest in compounds with terminal C=C (i.e. limonene > a-phellandrene). In Assay 4, limonene was more effective than a-pinene at inhibiting nitrification, which again is consistent with my prediction. However, the predicted pattern was not seen in either Assay 2 or Assay 3 when initial NH_4^+-N concentrations and nitrification rates were low.

The effects of limonene and a-pinene on NO_3^--N production in all of the Assays are summarized in Fig. 9. Both monoterpenes appeared to have a similar effect on nitrification in Assays 2 and 3 when nitrification rates are low, and in Assays 1 and 4 when nitrification rates were high. Both compounds appeared to have similar inhibitory activity. Thus, the laboratory studies provide limited support for the hypothesis.

Predicted inhibition of nitrification is partially supported by the field collections. The best estimate of relative composition of monoterpenes that could have been contributed to the mineral soil preceding each collection was the monoterpene composition of the L horizon, since the L horizon represented the largest close source of monoterpenes. There was a consistent negative correlation between relative nitrification in the mineral soil and monoterpene concentrations in the respective L horizon. Combining all collections, relative nitrification in the mineral soil was negatively correlated with total monoterpene concentration of the L horizon ($r^2 = 0.17$, $P < 0.05$ with degree of freedom (df) = 22) and highly negatively correlated with the total concentration of monoterpenes with terminal C=C (Fig. 10). Within a collection, significant negative correlations existed between individual monoterpenes or groups of monoterpenes and relative nitrification (Table 4). That relative nitrification was significantly correlated with a monoterpene or groups of monoterpenes is not strong evidence in support of my hypothesis, simply because one or more significant correlations might be expected by pure chance when many comparisons are made between a large number of variables. However, it is important to note that all significant correlations were negative and occurred between relative nitrification and monoterpenes that have terminal C=C (limonene,

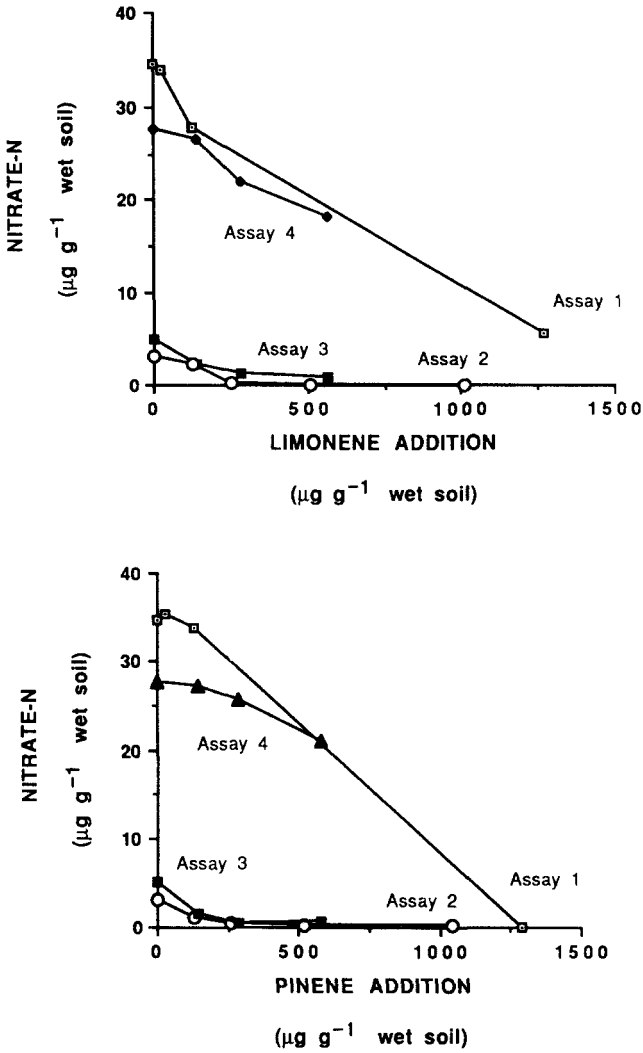

Fig. 9. Response in NO_3^--N concentrations at the end of the incubation following addition of limonene (upper graph) or a-pinene (lower graph) in each assay. Monoterpene additions are expressed in equivalent soil concentrations, assuming all added monoterpene was extractable.

b-pinene, and camphene). Terminal C=C may be more important than ring structure (monoterpene type) for inhibition of nitrification. Thus, my results support the hypothesis that monoterpenes inhibit nitrification, but only partially support the hypothesis that inhibitory activity is dependent upon molecular structures of the various monoterpenes.

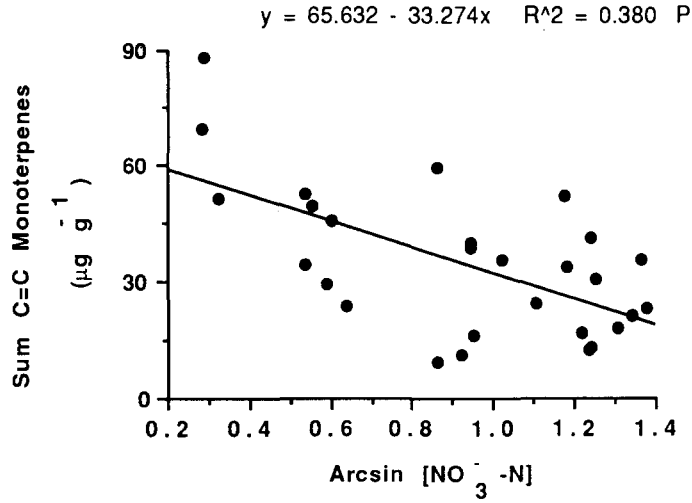

Fig. 10. The relationship between relative nitrification (NO_3^--N/NH_4^+-N + NO_3^--N) after arcsin transformation and the sum concentration of monoterpenes with terminal C=C in the horizon. Each point indicates an individual sample (n = 28).

Table 4. Relationship between relative nitrification (NO_3^- N/NO_3^- N + NH_4^+ – N) in mineral soil samples after 10-week incubation and initial monoterpene(s) concentration in the respective L horizon for three different collections. Listed are the correlation coefficient (r) and probability level (P) for each comparison. No significant correlations occurred for the May collection. For the November 1987 and March 1988 collections, n = 8; n = 4 for October 1988.

Collection	Monoterpene(s)	r	P
November 1987	b-Pinene	0.774	0.05
	Σ b-Pinene, Limonene	0.780	0.05
March 1988	b-Pinene	0.767	0.05
	Σ b-Pinene, Limonene	0.775	0.05
	Σ b-Pinene, Limonene, Camphene	0.873	0.01
October 1988	Camphene	0.960	0.05
	Σ Camphene, Limonene	0.970	0.05

Conclusion

— In the field, monoterpenes place an upper constraint on N mineralization and nitrification. In the absence of monoterpenes, these processes reach higher rates and are constrained by other factors (such as C/N ratio, lignin-cellulose, pH, etc.).
— Monoterpenes react very rapidly in soils and represent extremely labile organics that simultaneously influence a number of soil processes.
— The net effect of increasing monoterpene addition was to reduce the sum of NH_4^+-N and NO_3^--N (inhibition of net mineralization at low additions progressing to net immobilization with high monoterpene additions) and to

increase the amount of NH_4^+-N relative to NO_3^--N (apparent inhibition of nitrification).

— Monoterpenes could produce the pattern described in (3) by altering the rates of four processes: (a) reducing net N mineralization, (b) inhibiting nitrification, (c) enhancing immobilization of NO_3^--N relative to NH_4^+-N, and (d) stimulating overall net immobilization of N during breakdown of high C content material. The relative importance of each process cannot be determined with certainty from these results.

— Nitrification inhibitory activity appeared to be related to molecular structures of the monoterpenes, with highest inhibition by monoterpenes with terminal C=C. However, differences in activity were small and further experiments are necessary to test the effects of molecular structure on inhibition of nitrification.

Acknowledgements

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