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INHIBITION OF *Nitrosomonas europaea* BY MONOTERPENES FROM COASTAL REDWOOD (*Sequoia sempervirens*) IN WHOLE-CELL STUDIES

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Abstract—Inhibition by allelochemicals, including monoterpenes, has been suggested as a factor in the extremely low nitrification rates observed in coastal redwood forests. Similarities between the molecular structure of known nitrification inhibitors and some conifer monoterpenes have been suggested as one reason for the inhibition of autotrophic nitrifiers by conifer monoterpenes. The effect of monoterpenes on nitrification rate and growth of *Nitrosomonas europaea* was examined in whole-cell pure culture experiments using the five most abundant monoterpenes in coastal redwood needles. These are (in order of decreasing concentration in the needles) limonene, α-pinene, sabinene, myrcene, and γ-terpinene. Four of the five compounds significantly inhibited growth of *N. europaea* in batch culture experiments. Short-term kinetic studies of the two most inhibitory monoterpenes, limonene and α-pinene, were performed on whole cells to evaluate the mode of interaction between these chemicals and nitrification rates. Inhibition constants (K_i) of limonene (38 μM) and α-pinene (95 μM) were determined. Lineweaver-Burk plots of nitrification in the presence of monoterpenes appear to fit a noncompetitive inhibition model; however, the mechanisms of inhibition may be more complex.

Key Words—*Nitrosomonas europaea*, nitrification, inhibition, kinetics, monoterpenes, *Sequoia sempervirens*, conifers, nitrogen cycling.

INTRODUCTION

The forest floor (the combined fresh litter, fermentation, and humic horizons) has a profound effect on nitrogen cycling in forest ecosystems (Hart and Fire-

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stone, 1991; Killham, 1990; White, 1986, 1991, 1994). The effects of secondary chemicals in leaf litter on nutrient cycling have not been thoroughly investigated, but have been suggested as a factor that might control nutrient cycling in forests (Horner et al., 1988; Lohdi and Killingbeck, 1980). Monoterpenes have been shown to inhibit nitrification in a variety of ecological and laboratory settings. Foliar monoterpenes inhibit nitrification in soil nitrification potential bioassays, and field studies suggest that monoterpenes inhibit nitrification beneath California bay [*Umbellularia californica* (Hook & Arn) Nutt.] in coastal woodlands (Wood, 1996). Monoterpenes from needle resins are implicated in the inhibition of nitrification in a ponderosa pine (*Pinus ponderosa* Laws) ecosystem on the basis of soil bioassay experiments (White, 1988, 1990, 1991).

White (1988) proposed that monoterpenes inhibit ammonia monooxygenase (AMO, the first enzyme in the ammonia oxidation pathway in autotrophic nitrifying bacteria) based on chemical similarities between some conifer monoterpenes and known nitrification inhibitors that act on AMO. AMO is inhibited by a number of *n*-alkanes, *n*-terminal alkenes and alkynes, and by some cyclic compounds (Bedard and Knowles, 1989; Hooper and Terry, 1973; Hyman et al., 1985; Lees, 1952; McCarty and Bremner, 1991; Ward, 1990).

Oxidation products, which are produced by ammonia-oxidizing bacteria, of some of the cyclic inhibitory compounds, such as benzene, phenol, and Nitrapyrin, suggest that these compounds are alternative substrates for AMO (Hyman et al., 1985; Vannelli and Hooper, 1992). The hypothesis that conifer monoterpenes are competitive inhibitors for AMO is consistent with observations from White's (1991) study in which nitrification inhibition by ponderosa pine monoterpenes was inversely proportional to initial concentrations of ammonium in soil bioassays. A noncompetitive mode of inhibition for monoterpenes would be consistent with the study by Keener and Arp (1993), in which the mode of inhibition for a series of hydrocarbons and halogenated hydrocarbons was investigated. In these whole cell experiments, it was inferred that inactivation of AMO was caused by binding of large hydrocarbons at a site other than the active site of the enzyme (Keener and Arp, 1993). Regardless of the mode of inhibition, it is evident that structure and relative concentration of inhibitor and substrate are primary factors controlling the magnitude of the inhibition (White, 1994).

The multiple effects of monoterpenes on edaphic microbes have not been thoroughly investigated. Monoterpenes have antimicrobial properties, disrupt electron transport, and uncouple oxidative phosphorylation (Bedard and Knowles, 1989; Knobloch et al., 1986; Rice, 1984; Uribe et al., 1985). They may also serve as a carbon source for some heterotrophic bacteria (Shukla et al., 1968; Harder and Probian, 1995). Many factors could influence the fate of monoterpenes in the soil solution, thus influencing their efficacy as inhibitors of nitrification. For example, at high concentrations, nonspecific toxicity to microbes

by monoterpenes could prevent decomposition and mineralization. A reduced pool size of nitrate in soil bioassays would appear to result from inhibition of nitrification.

Soil bioassays do not precisely determine the roles of monoterpenes in the forest floor. Therefore, the present study was designed to determine whether monoterpenes, and in particular coastal redwood monoterpenes, are specific inhibitors of the autotrophic soil nitrifier, *Nitrosomonas europaea*. This investigation focused on the low concentration range for both the primary substrate, ammonium, and for the inhibitors, in an attempt to test substrate concentrations likely to be encountered on the forest floor. Nitrapyrin, a commercial nitrification inhibitor, was used as a positive control for inhibition. Although its exact mechanism of inhibition is unknown, Nitrapyrin is oxidized by ammonia monooxygenase and is a good model with which to compare the monoterpenes (Vanelli and Hooper, 1992). Growth rates in batch culture were used to determine the degree of inhibition caused by each monoterpene. Short-term kinetic experiments were also performed to investigate the mode of action by the two most inhibitory monoterpenes, limonene, and α-pinene. AMO has proven difficult to purify in active form, so these experiments were performed on whole cells. An additional advantage of using whole cells is that they may be more applicable than purified enzymes for predicting the behavior of whole cells in the environment. A disadvantage is the difficulty in distinguishing multiple possible interactions between potential inhibitors and multiple sites of action in the cell.

The needle resin of coastal redwood is composed of 15 monoterpenes, several sesquiterpenes, and other minor oxygenated terpenoids (Okomoto et al., 1981; Hall and Langenheim, 1986a). For this study, we chose the five most abundant monoterpenes whose combined concentration comprises over 90% of the total yield of coastal redwood needle resin. These five, limonene, sabinene, α-pinene, myrcene, and γ-terpinene, are all hydrocarbon monoterpenes. One additional (structurally similar) monoterpene, β-pinene, was also included.

METHODS AND MATERIALS

Strains and Culture Conditions. Nitrosomonas europaea (ATCC No. 19718) was maintained in liquid culture at room temperature (approximately 25°C) in the dark in a freshwater medium derived from Soriano and Walker (1968). Phenol red was used as a pH indicator, and pH was maintained near neutral by the addition of sterile K_2CO_3 (5% solution in distilled water) as required. The culture was ascertained to be pure at the beginning of the set of experiments by spreading on rich organic medium (5 g peptone per liter) and by periodic inoculations into liquid rich medium.

Growth Inhibition Experiments. Experiments were carried out in the above

medium (Soriano and Walker, 1968) with 100 ml of culture in 250-ml flasks and an initial ammonium concentration of 50 mM. The flasks were inoculated with 1 ml of exponentially growing stock cultures and incubated statically in the dark at room temperature.

Monoterpenes (technical grade, >90% purity) were obtained from Aldrich Chemical Company, Inc., and Nitrapyrin (2-chlor-6-trichloromethyl-pyridine) was obtained from Dow Chemical Corp. Their chemical structures are shown in Figure 1. Potential inhibitors, including Nitrapyrin, were emulsified in 0.5% ethanol in distilled water and added to the cultures at the time of inoculation. Nitrapyrin served as a positive control for inhibition and was used at the prescribed field concentration of 1 μg/ml. Monoterpenes were used at final concentrations of 1 and 10 μg/ml, and each treatment was performed in duplicate or triplicate. One set of flasks received only 0.05% ethanol as a control for solvent effects. Growth was monitored by approximately daily measurement of nitrite concentration with a colorimetric method (Strickland and Parsons, 1972) in 1-ml aliquots that were removed from the growth flask with sterile technique.

Growth rate (approximated by nitrification rate) was measured during the initial portion of the exponential phase of the batch cultures. Growth rates were compared statistically by regression analysis of the log-transformed data in a two-tailed *t* test (Systat).

Kinetics of Monoterpene Inhibition of Ammonia Oxidation. A semicontinuous batch culture was initiated by inoculating 500 ml of a 1-week-old batch culture of *N. europaea* into 2 liters of medium containing 50 mM ammonium. The 4-liter culture bottle was equipped with sterile sampling and medium-addition ports but was not stirred. It was incubated in the dark at room temperature and growth was monitored by periodic determination of nitrite concentration. The culture was maintained in exponential phase by periodic dilution (removal of 2 liters of culture for experimentation and replacement with 2 litres fresh medium).

For kinetic experiments, cells from 2 liters of semibatch culture were harvested on 0.3-μm-pore size 47-mm-diameter polycarbonate filters by filtration under gentle vacuum. Cells were resuspended (at a concentration of approximately 10^5 cells/ml, see below) in ammonium-free medium and transferred into experimental flasks (50 ml experimental volume in 125-ml flasks). Experimental medium was identical to culture medium except that ammonium concentrations varied according to experimental design (0.1–100 μg/ml, equivalent to 0.0056–5.6 mM as NH_4^+). Monoterpenes (1 μg/ml for α-pinene and 10 μg/ml for limonene) were added as described above. Samples were removed from each experimental flask at the time of inoculation for measurement of initial ammonium concentration (Grasshoff et al., 1983) and cell number (epifluorescence enumeration using DAPI) (Porter and Feig, 1980). Each experimental treatment was performed in triplicate. Each flask was sampled using sterile pipets five to

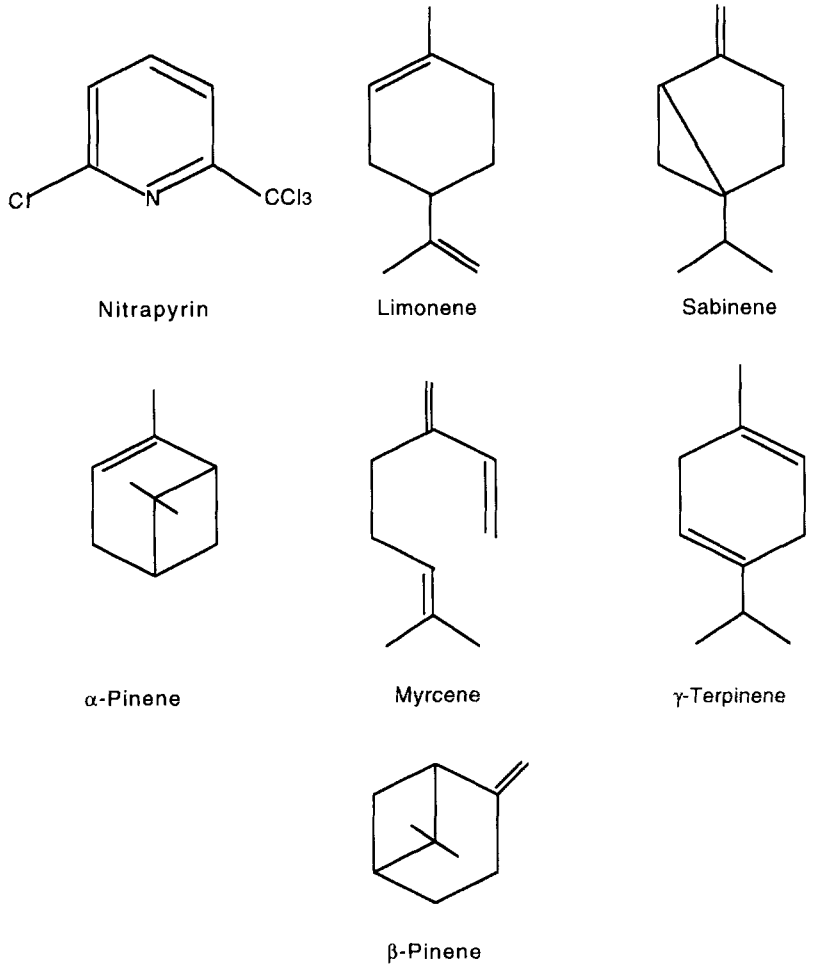

FIG. 1. Molecular structures of the potential inhibitory compounds tested in batch culture experiments.

seven times over the course of 4–5 hr for measurement of nitrite concentration (Strickland and Parsons, 1972).

Initial velocities of nitrite production (μmol NO_2^-/cell/hr) were calculated by linear regression from the increase in nitrite concentration with time. Double-reciprocal plots (Lineweaver-Burk) of initial rate and substrate concentration were used to calculate kinetic parameters. Regressions and probabilities were determined using Systat.

RESULTS

Growth Inhibition. Growth of *N. europaea* in batch culture was estimated as net nitrite production rate and was measured by daily changes of the NO_2^- concentration. Growth rates were calculated from the semi-log plot of nitrite concentration versus time during exponential phase and then normalized to the control. In the absence of inhibitors, *N. europaea* grew in a normal exponential fashion following a short lag phase after inoculation (Figure 2). Ethanol is an inhibitor of ammonia oxidation at 0.09 M concentration (Hooper and Terry 1973), but the 0.05% (0.011 M) ethanol treatments (used here as an addition control) were not significantly different from the controls (Table 1). The monoterpene treatments also exhibited logarithmic growth curves (i.e., with slopes significantly different from zero; Figure 2).

The growth rate of *N. europaea* in batch culture was significantly reduced by 1 μg/ml of the commercial nitrification inhibitor, Nitrapyrin ($P < 0.05$). Most of the monoterpenes significantly inhibited the growth of *N. europaea*, at least at the 10 μg/ml level (Table 1). Myrcene and limonene were inhibitory at the 1 μg/ml level and limonene at the 10 μg/ml level; the large variability for the 10 μg/ml myrcene treatments precluded detection of a significant effect. α-Pinene and γ-terpinene had a significant effect only at 10 μg/ml. Sabinene and β-pinene did not produce significant inhibition at either concentration, and in fact, β-pinene appeared to be stimulatory at the higher concentration.

Kinetics of Monoterpene Inhibition of Ammonia Oxidation. Ammonium oxidation rate (NO_2^- μmol/cell/hr) in the absence of inhibitors increased with increasing substrate concentration up to at least 5.6 mM (as NH_4^+) and exhibited apparently normal first-order kinetics as described by the Michaelis-Menten equation:

$$v = \frac{V_{\max} \cdot [S]}{K_m + [S]}$$

Double reciprocal plots of initial rates of nitrite production versus substrate concentration were linear and consistent with Michaelis-Menten kinetics (Figure 3). Kinetic parameters were calculated from the Lineweaver-Burk rearrangement of the above equation:

$$\frac{1}{v} = \frac{K_m}{V_{\max} \cdot [S]} + \frac{1}{V_{\max}}$$

Ammonia, rather than ammonium, is the substrate of ammonia monooxygenase, the enzyme that catalyzes the first step in the oxidation of ammonia to nitrite. Therefore, K_m is expressed as the combined concentration of $NH_4^+ + NH_3$. In the absence of inhibitors at pH 7.6, the K_m for $NH_4^+ + NH_3$ was 532

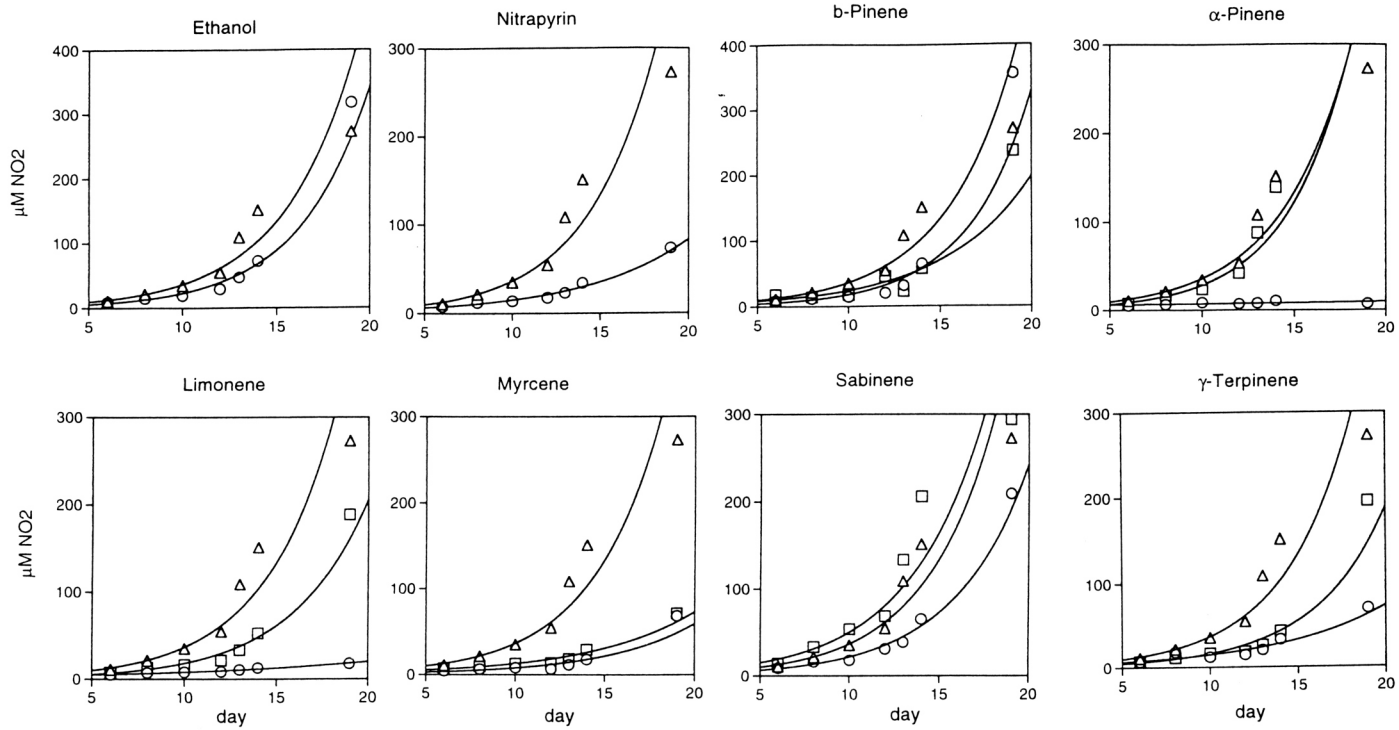

FIG. 2. Growth curves from batch cultures of *N. europaea*. Growth was monitored by nitrite accumulation in the medium over a period of 19 days; Δ, nitrite concentration in the absence of inhibitor. □, nitrite concentration in the presence of 1 μg/ml potential inhibitor; ○, nitrite concentration in the presence of 10 μg/ml of potential inhibitor.

TABLE 1. EFFECT OF POTENTIAL ON GROWTH (NITRITE PRODUCTION) OF *N. europaea* IN BATCH CULTURE[a]

Treatment	Relative nitrite production rate (mean ± SD)	*P*
control	1.00 ± 0.011	
ethanol (0.05%)	1.06 ± 0.009	0.424
nitrapyrin (1 μg ml^{-1})	0.43 ± 0.024	0.041*
γ-terpinene (1 μg ml^{-1})	0.87 ± 0.032	0.486
γ-terpinene (10 μg ml^{-1})	0.62 ± 0.004	0.018*
β-pinene (1 μg ml^{-1})	0.93 ± 0.043	0.782
β-pinene (10 μg ml^{-1})	1.15 ± 0.003	0.062
limonene (1 μg ml^{-1})	0.74 ± 0.007	0.047*
limonene (10 μg ml^{-1})	0.35 ± 0.013	0.014*
sabinene (1 μg ml^{-1})	0.92 ± 0.018	0.449
sabinene (10 μg ml^{-1})	0.96 ± 0.001	0.645
α-pinene (1 μg ml^{-1})	1.07 ± 0.022	0.645
α-pinene (10 μg ml^{-1})	0.11 ± 0.009	0.001**
myrcene (1 μg ml^{-1})	0.56 ± 0.010	0.023*
myrcene (10 μg ml^{-1})	0.45 ± 0.030	0.062

[a]Results are reported as the rate of nitrite production (mean of two or three replicates) in the experimental flasks as a fraction of the rate in the uninhibited control flasks. The standard deviation is that associated with the mean nitrite production rate in the replicate flasks. *$\alpha < 0.05$ and **$\alpha < 0.001$ denote rates significantly different from rates in the control flasks.

μM (the average of two control experiments; see Figure 3A and B). These results compare well to those of Suzuki et al. (1974) who reported a K_m for $NH_4^+ + NH_3$ of 480 μM at pH 8. The V_{max} we observed averaged 1.32×10^{-8} μmol/cell/hr.

In batch cultures, α-pinene inhibited growth in *N. europaea* more strongly than did limonene at the 10 μg/ml level (Figure 2, Table 1). Both α-pinene and limonene inhibited ammonia oxidation in short-term kinetic experiments. The lower concentration of α-pinene, which was not inhibitory in the growth experiment, exhibited significant inhibition in the kinetic experiments. Double-reciprocal plots again were linear, but differed in both slope and *y* intercept between the control and monoterpene treatments. In the presence of 10 μg/ml limonene, the apparent K_m for $NH_4^+ + NH_3$ was 1220 μM and in the presence of 1 μg α-pinene, the apparent K_m was 344 μM. The double-reciprocal plots for limonene are consistent with a noncompetitive mode for the interaction between limonene and ammonia; both the slope and the *y* intercept of the double-reciprocal plots (Figure 3A) are significantly different ($P < 0.001$). In the case of

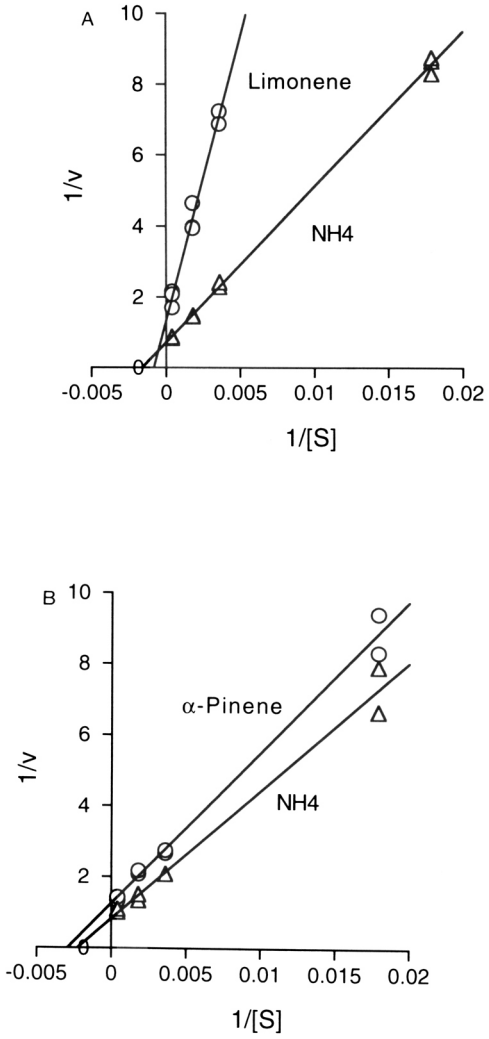

FIG. 3. Double-reciprocal plots ($1/v = 1/(\text{mol/cell/hr} \times 10^{-8})$; $1/[S] = 1/\mu M$) of the effect of limonene (A) and α-pinene (B) on ammonia oxidation by *N. europaea*. Δ, nitrite production rate in the absence of inhibitor; ○, nitrite production rate in the presence of inhibitor. Data derived from the regression equations are shown in Figure 2. Curve fits: (A) limonene: $R^2 = 0.987$, $N = 9$; ammonium: $R^2 = 0.999$, $N = 12$; (B) α-pinene: $R^2 = 0.991$, $N = 8$; ammonium: $R^2 = 0.984$, $N = 8$.

α-pinene, the slopes of the double-reciprocal plots (Figure 3B) are different ($P < 0.025$) but the y intercepts are not significantly different ($P > 0.05$). Therefore, these data do not clearly distinguish between noncompetitive and competitive modes of inhibition.

In both the competitive and noncompetitive inhibition model, the apparent half saturation constant for inhibition can be computed from:

$$K_i = \frac{K_m(1 + [I])}{K'_m}$$

where K'_m is the apparent half saturation constant of the reaction rate in the presence of inhibitor and [I] is the concentration of the inhibitor. The inhibition constant, K_i, corresponds to the concentration of inhibitor that reduces the V_{max} of the primary substrate reaction rate by 50%. K_i's computed from the data in Figure 3 were 38 μM for limonene and 95 μM for α-pinene. These values imply strong inhibition by both monoterpenes.

DISCUSSION

Growth of *N. europaea* in batch culture exhibited conventional logarithmic growth. The lag phase (not shown in Figure 2), was short, if present, probably because the cultures were all inoculated from rapidly growing cultures from the same medium and temperature conditions. The effects of the putative inhibitors were easily discernible during the early exponential phase of growth in batch culture, and clear differences in the strength of inhibition were demonstrated among the monoterpenes.

The inhibition of growth and nitrification by *N. europaea* in batch culture by redwood monoterpenes is consistent with the hypothesis that monoterpenes specifically inhibit ammonia oxidation and may be responsible for the slow rates of nitrification in coniferous forests (White, 1990, 1991, 1994). The different degrees of inhibition indicating that each compound's inhibitory capability is specific to its molecular structure demonstrates that these are specific inhibitors of *N. europaea*. In contrast, general toxicity by monoterpenes requires high concentrations (5 mM) to disrupt electron transport and uncouple oxidative phosphorylation in cell free extracts of bacterial membranes (Uribe et al., 1985; Knobloch et al., 1986).

The most inhibitory monoterpenes in batch cultures and kinetic experiments with *N. europaea*, limonene, and α-pinene were also the most active monoterpenes in soil bioassays (White, 1991). The degree of inhibition differed with molecular structure but not in consistent ways related to degree of terminal bond saturation or cyclicity. Thus, although inhibition by these terpenes appears to

be compound-specific, the variation in inhibition depending on structure of the inhibitor may imply that more than one mode of inhibition is occurring.

Direct measurements of monoterpene concentrations in the water layer, which is the actual site of bacterial colonization, of natural litter under redwood canopies have not been reported, so it is not possible to compare the concentrations found to be inhibitory in this study with those occurring naturally. Monoterpene yields in needles and litter, however, can provide a basis for predicting whether natural levels could be inhibitory. White (1986, 1991, 1994), in studies on ponderosa pine stands, reported total monoterpene levels in the upper, relatively fresh litter layer of 1.4 mg/g, decreasing to 0.15 mg/g in year-old litter. Wilt et al. (1993) reported a similar pattern for pinyon pine (*Pinus monophylla* Torf. & Frem.), with total monoterpenes decreasing from 3.6 mg/g in the fresh litter to less than 40% of that level in senescent needles. Total yield of monoterpenes in redwood litter ranged from over 6 mg/g dry wt in the fall to 1 mg/g dry wt in the late spring months following the wet season (Wood et al., 1995), indicating that most of the monoterpenes were lost during the winter (wet) months. As in redwood needles (Hall and Langenheim, 1986a, b), α-pinene and limonene were major constituents of the total monoterpene yield in the pinyon pine needles and litter (Wilt et al., 1993). Limonene and α-pinene each contribute more than 20% of the total monoterpene yield in both redwood and pinyon pine needles and litter. The observed decrease in monoterpene content upon senescence of the needles and decay of the litter implies major loss by volatilization or leaching. Field studies showed that *Umbellularia* litter lost between 65% and 85% of the monoterpene total yield in one rainy season (Wood et al., 1995). Laboratory studies corroborated the importance of water leaching in the loss of monoterpenes from both *Umbellularia* and *Sequoia* litter and further substantiated the importance of monoterpene water solubility (Wood, 1996).

Even though monoterpenes are volatile hydrocarbons, they are more water soluble than previously recognized (Weidenhamer et al., 1993) and are found at detectable levels in fjords and river water bordering forested areas (Button, 1984; Bertsch and Anderson, 1975). The exposure levels used in the present study (1 and 10 μg/ml) are less than the solubilities reported by Weidenhamer et al. (1993) for all monoterpenes tested except myrcene (whose solubility is less than 10 μg/ml). Fischer (1991) and Fischer et al. (1994) also showed that monoterpenes not only are water soluble, but in many cases the compounds are biologically active at concentrations well below the limits of their aqueous water solubilities. The monoterpene content of redwood litter (see above) suggests that significant levels of monoterpenes could be present in the soil solution during the rainy season. With a K_i of 38 μM for limonene (equivalent to 5.2 μg/ml), and 2.6% inhibition of ammonia oxidation by 1 μg/ml limonene (Table

TABLE 2. KINETIC PARAMETERS FOR NITRITE PRODUCTION IN SHORT-TERM KINETIC STUDIES OF *N. europaea* WITH AND WITHOUT MONOTERPENE INHIBITORS

Substrate or inhibitor	V_{max} (μmol/cell/hr)	K_m (μM)	K_i (μM)
$NH_4^+ + NH_3$	$1.32 \times 10^{-8} \pm 0.33 \times 10^{-8}$	532 ± 127	
NH_3		2.65 ± 0.63	
Limonene	7.58×10^{-9}		38
α-Pinene	8.08×10^{-9}		95

2), it seems likely that limonene may affect nitrification in situ as the monoterpenes in litter percolate through the soil. The K_i of 95 μM for α-pinene (equivalent to 13 μg/ml) suggests that α-pinene at naturally occurring concentrations could also be a factor influencing litter nitrification rates.

Molecular aggregation may be a mechanism that increases monoterpene activity when combined with other terpenoids or unknown additional soil components (Tanrisever et al., 1981). Monoterpenes are found aggregated with soil colloids into droplets (Muller and Del Moral, 1996). Aggregation may explain the large standard deviations observed in some of the treatments (e.g., 10 μg/ml limonene and 1 μg/ml sabinene), due to droplets formed in solution. The effects of synergistic compounds should also be considered as potentially important factors regulating the activity and availability of monoterpenes in nature.

Mechanisms of Monoterpene Inhibition. Ammonia monooxygenase (AMO) is a mixed function oxidase, and its mechanism is hypothesized to be similar to that of tyrosinase, including the role of copper in the active site (Lerch, 1981; Shears and Wood, 1985). Some inhibitors of AMO are effective because they are substrate analogs, and many can be partially oxidized by the enzyme (Hyman and Wood 1985; Bedard and Knowles, 1989; Hyman et al., 1988; Hooper and Terry, 1973; Bremner and McCarty, 1991; Ward, 1987). Metal chelators constitute a second set of inhibitors for AMO, probably because they bind the copper ions in AMO (Lees, 1946, 1952; Hooper and Terry, 1973). Based on studies of the kinetics of ammonium and hydrocarbon oxidation, hydrocarbon inhibitors have been described as competitive inhibitors of AMO (benzene: Hyman et al., 1985; methane: Hyman and Wood, 1983; Ward, 1987; ethylene: Hyman and Wood, 1985; Vanelli and Hooper, 1992). Chelating agents, such as allylthiourea, guanidine, and L-histidine, which inhibit by binding to the copper ions, exhibit noncompetitive inhibition (Lees, 1946). Copper binding inhibitors, which fit a classical noncompetitive model of inhibition, are thought to inhibit

by binding copper at the active site (Lees, 1952). It is counterintuitive that this chelation results in noncompetitive inhibition, since chelation of the copper ion would seem likely to affect binding by the substrate at that site.

Larger hydrocarbons, such as monoterpenes, may exhibit a third mechanism of inhibition. Although Keener and Arp (1993) did not investigate monoterpenes directly, a logical extension of their argument is that monoterpenes should inhibit noncompetitively; they hypothesized that nonpolar compounds would bind with AMO at a hydrophobic region separate from the active site of the enzyme. Like the copper-binding inhibitors (above), monoterpenes acting as noncompetitive inhibitors should have no effect on substrate binding and should not affect the K_m because the enzyme and enzyme-inhibitor complex have the same affinity for the substrate (Segel, 1976).

Any organic molecule with unshared or π electrons may chelate metal ions. Therefore, inhibitors with unsaturated bonds (i.e., C=C) may bind copper at the active site of AMO. The apparent similarity in kinetic behavior between monoterpenes and metal chelators could imply that a common mechanism is at work and that the monoterpenes inhibit by chelating copper at the active site. An equally viable interpretation is that the monoterpenes bind or obstruct at a site removed from the active site, in such a manner as to inhibit substrate oxidation but not substrate binding. This is the mechanism proposed by Keener and Arp (1993).

In the experiments reported here, inhibition by both limonene and α-pinene was consistent with a noncompetitive inhibition model, although competitive inhibition, in which part of the α-pinene molecule competes for substrate binding site with the primary substrate, may also be occurring to some extent.

Ammonia oxidizers are also sensitive to the usual cell poisons and uncouplers of phosphorylation, although these effects are not directly related to AMO. We therefore expected that monoterpenes might exhibit two kinds of inhibitory effects: a specific inhibition of AMO by competitive or noncompetitive inhibition at low concentrations, and a general toxicity at high concentrations. Benzene and phenol are also inhibitors of autotrophic nitrifiers and are oxidized by ammonium monooyxgenase to phenol and hydroxyquinone, respectively (Hyman et al., 1985). However, kinetic studies of benzene at low concentrations do not fit either competitive or noncompetitive inhibition models, indicating a more complex behavior (Hyman et al., 1985). In the same study, higher concentrations of benzene (4 mM) added to cell-free extracts with 500 μM hydroxylamine and 10 μM allylthiourea inhibited oxygen uptake. These results suggest that at high concentrations, benzene is inhibitory at a site other than AMO. Results from kinetic studies may not have entirely illustrated the true mechanism of benzene inhibition because of these two potential modes of inhibition. Because the consumption of monoterpenes and the increase of possible oxidation products were

not monitored in these experiments, it is possible that the inhibitory monoterpenes were also oxidized by AMO and thus fail to fit a simple competitive inhibition model for the same reasons.

This investigation focused on the low concentration range for both the primary substrate, ammonium, and for the inhibitors, choosing substrate concentrations likely to be encountered on the forest floor. The results imply that levels of redwood monoterpenes that occur in forest litter are capable of direct specific inhibition of *N. europaea*-like ammonia oxidizers in forest soils. Several of the compounds were very inhibitory, both in batch culture and in short-term kinetic experiments. These observations are consistent with the hypothesis that direct inhibition by monoterpenes could be a factor in regulating nitrification rates in some forest soils. The whole-cell experiments reported here clearly identify a physiological basis for direct inhibition of nitrification by monoterpenes. The net effect of monoterpenes on soil nitrogen cycling may not be easy to detect or quantify in natural systems, however, due to the multitude of chemicals and chemical–microbial interactions possible in such a complex system.

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