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Selective Inhibition of Ammonium Oxidation and Nitrification-Linked N_2O Formation by Methyl Fluoride and Dimethyl Ether

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Methyl fluoride (CH_3F) and dimethyl ether (DME) inhibited nitrification in washed-cell suspensions of *Nitrosomonas europaea* and in a variety of oxygenated soils and sediments. Headspace additions of CH_3F (10% [vol/vol]) and DME (25% [vol/vol]) fully inhibited NO_2^- and N_2O production from NH_4^+ in incubations of *N. europaea*, while lower concentrations of these gases resulted in partial inhibition. Oxidation of hydroxylamine (NH_2OH) by *N. europaea* and oxidation of NO_2^- by a *Nitrobacter* sp. were unaffected by CH_3F or DME. In nitrifying soils, CH_3F and DME inhibited N_2O production. In field experiments with surface flux chambers and intact cores, CH_3F reduced the release of N_2O from soils to the atmosphere by 20- to 30-fold. Inhibition by CH_3F also resulted in decreased $NO_3^- + NO_2^-$ levels and increased NH_4^+ levels in soils. CH_3F did not affect patterns of dissimilatory nitrate reduction to ammonia in cell suspensions of a nitrate-respiring bacterium, nor did it affect N_2O metabolism in denitrifying soils. CH_3F and DME will be useful in discriminating N_2O production via nitrification and denitrification when both processes occur and in decoupling these processes by blocking NO_2^- and NO_3^- production.

Microbial oxidation of NH_4^+ to NO_2^- or NO_3^- (nitrification) provides a link between nitrogen mineralization and nitrogen loss through denitrification (21, 39–42). Coupled nitrification and denitrification results in the loss of soil fertilizer nitrogen and a potential hazard to the stratospheric ozone layer through the release of N_2O (7, 9, 10), contributing to the 0.2 to 0.3% per year increase in tropospheric N_2O since preindustrial times (25). Nitrous oxide is a product of both nitrification and denitrification in soils (9, 15, 17, 29) and in pure cultures of nitrifying and denitrifying bacteria (16, 37, 38, 50). Quantifying the contribution of nitrification or denitrification to the N_2O pool or flux is difficult when both processes occur simultaneously (12, 23). However, if N_2O production from nitrification can be specifically inhibited, then the amount of N_2O production from denitrification alone may be determined. Studies of the origin of N_2O may therefore be facilitated by use of specific inhibitors of nitrification.

Presently, the best known inhibitors of nitrification, nitrapyrin and C_2H_2, each have drawbacks to their general use. Nitrapyrin is insoluble in water and must be added with acetone or an alcohol carrier, resulting in the addition of an unwanted carbon substrate (32). Acetylene is a known gaseous inhibitor of denitrification at levels slightly higher than those used to inhibit nitrification (3), requiring detailed examination of the specificity of C_2H_2 for each system under investigation (23). Acetylene also inhibits a number of other important microbial processes and has additional drawbacks that limit its efficacy as a specific nitrification inhibitor (3, 32).

We investigated the feasibility of using methyl fluoride (CH_3F) and dimethyl ether (DME) as specific inhibitors of ammonia monooxygenase (AMO) to block the production of NO_2^- and N_2O during nitrification. Both gases were previously found to inhibit methane oxidation in soils and cell suspensions of *Methylococcus capsulatus* (33, 34). In a preliminary study (34), both CH_3F and DME blocked NO_3^- production and NH_4^+ loss during soil nitrification without affecting N_2O reductase activity during denitrification. Our results extend this conclusion to additional soils and aquatic sediments and to a pure culture of an ammonium-oxidizing bacterium, demonstrating that these inhibitors are effective agents in discriminating N_2O sources. In addition, these gaseous inhibitors hold promise as tools for evaluating the degree of coupling occurring between nitrification and denitrification in soils and sediments.

MATERIALS AND METHODS

Solubility and purity of inhibitor gases. Because of the high aqueous solubilities of CH_3F (1.7 ml/ml), DME (35 ml/ml), and C_2H_2 (0.9 ml/ml), the characterization of soil moisture conditions is required when these gaseous inhibitors are used (8). In general, higher headspace concentrations of inhibitors were used in experiments with slurries and cultures than in experiments with dry soils. CH_3F (minimum purity, 99.0%) and DME (minimum purity, 99.87%) were obtained in lecture bottles from Matheson (Lyndhurst, N.J.). Acetylene was prepared from CaC_2 and water immediately prior to its use.

Experiments with bacterial cultures. For examination of the effect of CH_3F and DME on ammonium oxidation and N_2O production, washed-cell suspensions of pure cultures were prepared. The ammonium-oxidizing bacterium *Nitrosomonas europaea* was grown in a semicontinuous batch mode in mineral salts medium containing NH_4^+ and harvested by filtration (0.2-μm-pore-size Nuclepore filters) during exponential growth (48). Cell suspensions of *N. europaea* were washed twice with sterile, NH_4^+-free medium, resuspended in NH_4^+-free medium, and dispensed into

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37-ml serum bottles (total volume, 15 ml; 2.0×10^7 to 3.7×10^7 cells ml^{-1}). NH_4Cl or NH_2OH (4.5 μmol; volume, 0.1 ml) was added by use of a pipet prior to stoppering of the bottles. Controls consisted of NH_4^+-free medium without cells and amended with the appropriate substrate. Inhibitor gases (CH_3F and DME) were added by use of a syringe at the levels indicated in Results. Incubation was done at room temperature (22 ± 2°C) with constant shaking (200 rpm) for up to 5 h.

A marine NO_2^--oxidizing bacterium, a *Nitrobacter* sp., was grown in seawater-mineral salts medium in a batch mode, harvested during exponential growth, washed in NO_2^--free medium, and dispensed into serum bottles as described above. $NaNO_2$ (4.5 μmol; volume, 0.1 ml) was added by use of a pipet prior to stoppering of the bottles. CH_3F (10% [vol/vol]) or DME (10 and 25% [vol/vol]) was added by use of a syringe to the headspace of some bottles, and the samples were incubated statically at room temperature.

An anaerobic, selenate-respiring bacterium capable of dissimilatory NO_3^- reduction (strain SES-3 [44]) was grown in mineral salts medium containing NO_3^-. Cells were harvested during exponential growth, washed, and centrifuged twice (8,000 × *g* for 10 min each time) with mineral salts medium lacking NO_3^-, lactate, yeast extract, and vitamins. Final cell suspensions (total volume, 30 ml; equivalent dry weight, 0.4 mg ml^{-1}) were transferred to 57-ml serum bottles, which were then stoppered. These transfers were conducted in an anaerobic glove box. Sodium lactate (final concentration, 10 mM) was added to all bottles except for one control, and a reductant (cysteine sulfide; final concentration, 1 mM) and nitrate (final concentration, 10 mM) were added to all bottles by use of a syringe. Bottles were flushed with an 80:20 mixture of N_2-CO_2 for 10 min prior to the addition of CH_3F (20% [vol/vol]) to the headspace of two bottles. Headspace N_2O and aqueous NO_3^-, NO_2^-, and NH_4^+ concentrations were monitored over the course of the incubation (30 h).

Experiments with soils. For examination of the effect of CH_3F and DME on N_2O production in soils, bottle incubations were conducted. Methanotrophic soils were collected from the subsurface (depth, 73 to 100 cm) of an agricultural field (32, 33). Soils (10 g; volume, ~7 ml) were dispensed into serum bottles (57 ml), which were then crimp sealed under air with black butyl rubber stoppers. Controls were heat killed by autoclaving (121°C; 2 atm [ca. 203 kPa]) for 1 h. Inhibitors (CH_3F and DME) were injected at the levels indicated in Results. In some experiments, NH_4Cl (40 μmol) or NH_4NO_3 (50 μmol) was added by use of a pipet prior to sealing or by use of a syringe after sealing. Soils were incubated statically at 20°C in the dark.

Anaerobic soil slurries were prepared by mixing one part soil with two parts deionized water in a blender under flowing nitrogen. Slurries (60 ml) were dispensed into conical flasks (125 ml) and flushed with N_2 for 5 min. $NaNO_3$ (100 μmol) was added by use of a syringe to each flask prior to a final O_2-free N_2 flush (5 min). CH_3F (5% [vol/vol]) or C_2H_2 (30% [vol/vol]) was added to some flasks, while others were incubated without added gases. Controls consisted of heat-killed (121°C for 1 h) slurries with or without the gaseous inhibitors. Slurries were incubated at 20°C with constant shaking (60 rpm) for 14 days.

For determination of the effect of CH_3F and DME on N_2O reductase activity, anaerobic soil incubations with added N_2O were conducted. Soils (10 g) in 57-ml serum bottles were flushed with O_2-free N_2 for 15 min, shaken, flushed again for 15 min, and preincubated at 21°C for 12 days (to remove NO_3^-) before the addition of CH_3F or DME (1.0% [vol/vol]) to the headspace. N_2O (0.1% [vol/vol]) was added to all bottles, including one containing a heat-killed control, and headspace N_2O concentrations were monitored.

Experiments with estuarine sediments. The upper 10 cm of sediment was collected by a diver at a shallow station (2-m depth) in South San Francisco Bay. Sediment with overlying water was transported in sealed glass jars to the laboratory, where experiments were initiated within 1 day. Sediment slurries were prepared by homogenizing one part sediment and one part water. Slurries (40 ml) were dispensed into 250-ml conical flasks under air and incubated at 20°C with rotary shaking (250 rpm) for up to 14 days. Heat-killed controls were prepared by autoclaving (121°C; 2 atm) for 1 h. NH_4Cl (40 μmol) and CH_3F (5% [vol/vol]) were added to some flasks. Headspace N_2O concentrations were monitored during the course of the incubation, while slurries were sacrificed at times corresponding to the beginning, middle, and end of the experiment for the determination of dissolved inorganic nitrogen species. NO_3^- + NO_2^- and NH_4^+ concentrations were determined in water separated from sediment by centrifugation (8,000 × *g*) for 10 min and then filtration (0.4-μm-pore-size Nuclepore filters).

Field experiments with flux chambers. Cylindrical Plexiglas chambers (0.2-m diameter; 4.0-liter volume) were placed over two sites of seasonally exposed lakebed soils along the shoreline of Searsville Lake, Calif. (33). Site A was sandy soil, with a dry surface about 0.5 m above the lake level and 10 m from the lake shoreline, while site B was wet, silty soil nearer the lake shoreline. Site B had been exposed only several weeks earlier by a receding lake level. Some chambers received additions of inhibitors (1% [vol/vol] CH_3F; 5% [vol/vol] C_2H_2) immediately after placement. As an internal standard, sulfur hexafluoride (10^{-5} μmol) was added to all chambers and its level monitored over time to assess the integrity of the seal at the soil surface. The chamber headspace was sampled initially and three additional times over a 24-h period by withdrawing 4 ml into glass syringes and sealing the syringes with small rubber septa contained in modified Luer needle hubs, such that subsamples for gas chromatography (GC) analyses could be withdrawn and atmospheric pressure could be maintained. This storage procedure resulted in a <20% change in N_2O concentrations over several hours, and good agreement was found with duplicate samples collected by use of a syringe and stored by water displacement in sealed glass tubes.

Experiments with intact cores. Five sediment cores (10-cm diameter; 20-cm length) were collected in butyrate tubes between sites A and B at Searsville Lake. One core was immediately sectioned into 1-cm segments from the surface to 8 cm and sampled for dissolved inorganic nitrogen species (NO_3^- + NO_2^- and NH_4^+; see below). The remaining four cores were sealed with rubber stoppers and incubated for 20 to 21 days. Duplicate cores were incubated under a 0.9-liter air headspace with or without added CH_3F (5% [vol/vol]). Sulfur hexafluoride (SF_6) (5.6×10^{-6} μmol) was added to the headspace of all cores as an internal standard. After 20 to 21 days, the four incubated cores were sectioned into 1- or 2-cm segments from the surface to 6 cm. For extraction of gases, sediment (~5 g) was placed in a preweighed 125-ml conical flask containing 35 or 50 ml of 2 M KCl and shaken at 250 rpm for 2 h; headspace analysis by GC was then done (see below). Sediment extracts were centrifuged at 8,000 × *g* for 10 min, filtered through a 0.45-μm-pore-size Nuclepore

TABLE 1. Inhibition of NO_2^- and N_2O production by cell suspensions of *N. europaea* during incubations with various levels of CH_3F and DME[a]

Addition and % (vol/vol)	NO_2^-		N_2O	
	Produced (μmol h^{-1})[b]	Inhibited (%)	Produced (nmol h^{-1})[b]	Inhibited (%)
None	0.61	0	1.05	0
CH_3F				
0.1	0.35	43	0.43	67
1.0	0.11	82	0.17	96
10	0.03	96	0.16	97
DME				
0.1	0.64	−5	1.11	−7
1.0	0.53	14	0.70	38
10	0.15	75	0.26	86
25	0.05	92	0.24	88
No cells	0	100	0.13	100

[a] Assay bottles contained NH_4Cl (4.5 μmol) and (0.7 ± 0.1) × 10^7 cells.
[b] Production was nonlinear; the rate was calculated as the linear slope (0 to 1 h) of the best fit of concentration-time data to a logarithmic equation: $y = (a + b) \ln x$.

filter, and analyzed for dissolved and adsorbed NH_4^+ and $NO_3^- + NO_2^-$ (49).

Analyses. Headspace gases in flasks and chambers were measured by GC. A variable-frequency pulsed (^{63}Ni) electron capture detector (Valco model 140BN) was used for N_2O and SF_6; separation was achieved on a Porapak R column (2.4 m by 0.32 cm [diameter]) with a 20-ml min^{-1} carrier (5% CH_4; balance, N_2) flow rate and at a 45°C oven temperature. An eight-port injection valve fitted with matched columns was used to prevent C_2H_2 from reaching the detector (36). CH_3F and DME levels were determined by use of a flame ionization detector (HNU model 301) following separation on a Porapak S column (2.4 m by 0.32 cm [diameter]) at 50°C and with a 30-ml min^{-1} carrier (He) flow rate. Nitrite levels were measured in aqueous samples from incubations of *N. europaea* and the *Nitrobacter* sp. by spectrophotometry (45). NO_3^- and NO_2^- levels were measured in centrifuged aqueous samples from incubations of SES-3 by ion chromatography (35). Ammonium levels were determined by the phenol-hypochlorite method (43), and $NO_3^- + NO_2^-$ levels were determined by flow injection analyses following cadmium reduction of NO_3^- to NO_2^- (22).

RESULTS

Experiments with bacterial cultures. CH_3F and DME inhibited NO_2^- and N_2O production from NH_4^+ during nitrification by washed-cell suspensions of *N. europaea* (Table 1). Uninhibited cells produced 1.1 μmol of NO_2^- and 1.8 nmol of N_2O from 4.5 μmol of NH_4^+ in 4.9 h, but production was nonlinear. To distinguish the initial production rates, we fit the concentration-time data to a logarithmic equation, which was linearized for the period from 0 to 1 h. A cell-free control did not produce NO_2^- and formed <0.2 nmol of N_2O. Increasing headspace concentrations of an inhibitor (0.1 to 10% [vol/vol] CH_3F and 0.1 to 25% [vol/vol] DME) resulted in increasing degrees of inhibition, with the highest inhibitor concentrations resulting in >88% inhibition of both NO_2^- and N_2O production.

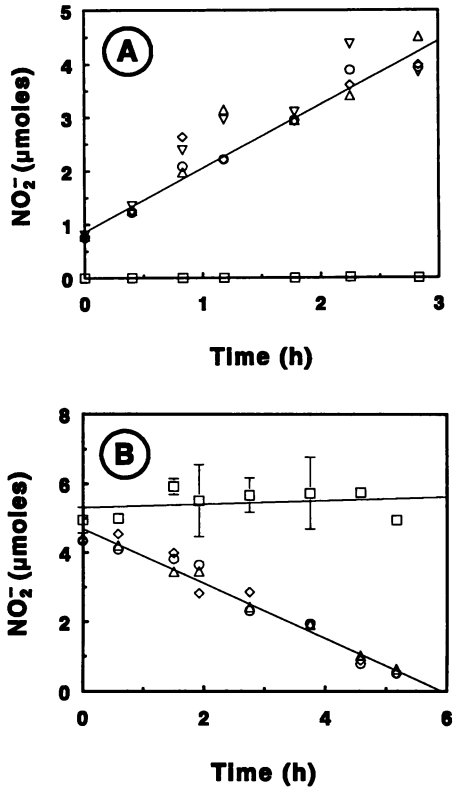

FIG. 1. Nitrite production from hydroxylamine by *N. europaea* (A) and nitrite consumption by *Nitrobacter* sp. (B) washed-cell suspensions (final cell density, ~10^7 cells ml^{-1}). Symbols: ○, no additions; ◇, 10% (vol/vol) CH_3F; △, 10% (vol/vol) DME; ▽, 25% (vol/vol) DME; □, no cells. Lines represent linear regressions of control data (no additions, A and B; no cells, B).

CH_3F and DME did not affect the production of NO_2^- from NH_2OH by *N. europaea* (Fig. 1A). Uninhibited cells, as well as those incubated with CH_3F or DME, all produced 4.08 ± 0.30 μmol of NO_2^- ($n = 4$) from 4.5 μmol of NH_2OH in 2.8 h. CH_3F and DME had no effect on the production of N_2O from NH_2OH in aerobic incubations (12 ± 1 nmol of N_2O produced in 4.8 h; $n = 3$).

CH_3F and DME had no effect on the oxidation of NO_2^- by a washed-cell suspension of a *Nitrobacter* sp. (Fig. 1B). Uninhibited cells and those incubated with CH_3F and DME all consumed 4.5 μmol of NO_2^- at the same rate over 5 h. N_2O was not detected in any of the experimental bottles.

In experiments with the respiratory NO_3^- reducer (SES-3), CH_3F had no effect on the transformation of inorganic nitrogen species during the dissimilatory reduction of 300 μmol of NO_3^- (data not shown). Cells incubated without an inhibitor as well as duplicate samples containing 20% (vol/vol) CH_3F consumed all of the NO_3^- and produced up to 140 μmol of NO_2^- (transient), 227 ± 5 μmol of NH_4^+ ($n = 3$), and 0.60 ± 0.15 μmol of N_2O ($n = 3$) during the course of the incubation (30 h). A control without an electron donor (lactate) did not consume NO_3^- and did not produce NO_2^-, NH_4^+, or N_2O.

Experiments with soils and sediments. Soils amended with 40 μmol of NH_4^+ produced up to 1.4 μmol of N_2O in 14 days (Fig. 2A). Production was completely inhibited by autoclaving and by CH_3F, while samples incubated with C_2H_2 produced a small quantity of N_2O (0.1 μmol). Headspace

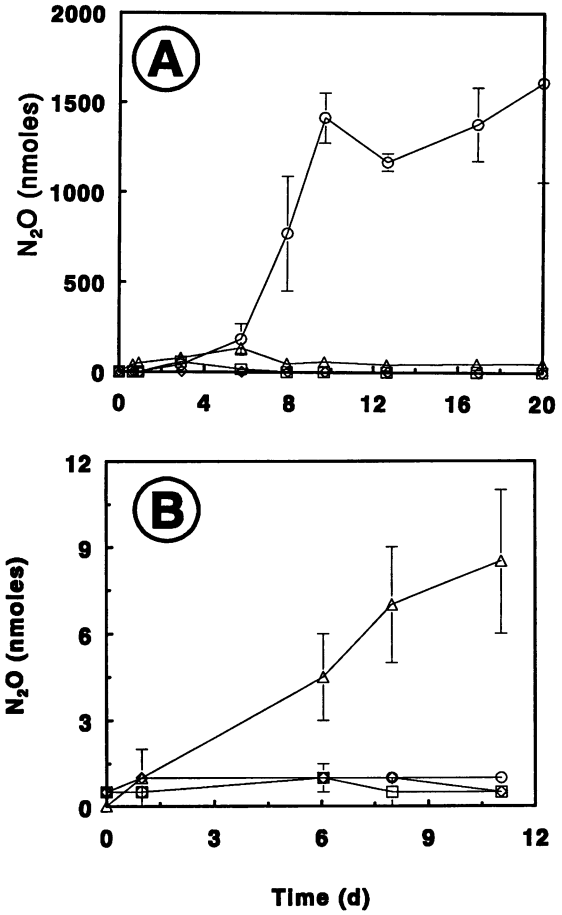

FIG. 2. Nitrous oxide production in aerobic soils with 40 μmol of added NH_4Cl (A) or 50 μmol of added NH_4NO_3 (B). Symbols: ○, no additions; ◇, 5% (vol/vol) CH_3F; △, 15% (vol/vol) C_2H_2; □, heat-killed control.

C_2H_2 concentrations remained constant over the 20-day incubation, while the CH_3F concentration decreased by 30% (data not shown). Soils with added NH_4NO_3 (50 μmol) did not produce N_2O either in controls or in the presence of CH_3F, but C_2H_2 stimulated the formation of a small but significant ($P < 0.025$) amount of N_2O, presumably from denitrification in anaerobic microsites (Fig. 2B).

The efficacies of CH_3F and DME as inhibitors of nitrification-linked N_2O production in soils were examined at several inhibitor concentrations. N_2O production during soil nitrification could be fully inhibited by CH_3F and DME, but CH_3F was more effective than DME at the lowest concentrations applied (0.1% [vol/vol]) (Table 2). Uninhibited soils produced 0.39 μmol of N_2O from 40 μmol of added NH_4^+ in 14 days, while heat-killed controls produced none. Headspace concentrations of inhibitors remained constant over the first week of the incubations, except for that in 0.1% (vol/vol) DME-amended bottles, which decreased by 30% (data not shown).

For determination of whether CH_3F could block nitrification in sediments as well as soils, experiments were conducted with San Francisco Bay sediment slurries. N_2O production in these sediments was inhibited by CH_3F (Table 3). The addition of NH_4^+ to slurries stimulated N_2O production ~27-fold (from 0.3 to 8 μmol), while CH_3F blocked N_2O production in both NH_4^+-amended and unamended slurries. Elevated levels of $NO_3^- + NO_2^-$ were observed in unamended and NH_4^+-amended flasks (1.5 and 13 μmol of NO_3^- and NO_2^-, respectively) after 1 week of incubation. After 2 weeks, $NO_3^- + NO_2^-$ concentrations were lower and similar in all flasks, presumably because of microbial consumption. NH_4^+ was consumed in the uninhibited slurries with and without added NH_4^+; in contrast, a net accumulation of NH_4^+ occurred in all flasks amended with CH_3F.

TABLE 2. N_2O production and percent inhibition during incubations of soil with various levels of CH_3F and DME[a]

Addition and % (vol/vol)	N_2O	
	Produced (nmol)[b]	Inhibited (%)
None	390 ± 150	0
CH_3F		
0.1	7 ± 4	98
1.0	4 ± 1	99
DME		
0.1	270 ± 120	30
1.0	3 ± 2	99
10	21 ± 30	95
Heat-killed control	1 ± 0	100

[a] Assay bottles contained NH_4Cl (40 μmol) and 10 g of soil.
[b] Mean N_2O produced in 14 days ± 1 SD ($n = 3$).

The effect of CH_3F and DME on N_2O metabolism associated with denitrification was examined in experiments with anaerobic soil slurries and dry soils. N_2O production during denitrification was unaffected by the addition of CH_3F. Uninhibited soil slurries produced levels of N_2O comparable to those produced in the presence of CH_3F (Fig. 3A). However, C_2H_2 caused a 10-fold enhancement of N_2O production, indicating that N_2O reductase was inhibited (1). Heat-killed controls (with or without added CH_3F and C_2H_2) did not produce N_2O. The consumption of added N_2O during denitrification was slightly affected by the addition of CH_3F or DME (Fig. 3B). Uninhibited dry soils consumed N_2O linearly over 5 h at a rate of 7.8 nmol g^{-1} h^{-1} ($n = 18$; $r^2 = 0.93$), while those incubated with CH_3F and DME consumed N_2O at rates of 6.8 ($r^2 = 0.92$) and 12 ($r^2 = 0.84$) nmol g^{-1} h^{-1}, respectively. No N_2O consumption was observed in heat-killed controls.

TABLE 3. Effect of CH_3F on nitrification-linked production (or consumption) of N_2O, $NO_3^- + NO_2^-$, and NH_4^+ in estuarine sediment slurries

Addition	μmol[a] of:		
	N_2O	$NO_3^- + NO_2^-$	NH_4^+
None	0.30 ± 0.46	0.24 ± 0.83	−0.92 ± 0.33
NH_4^+[b]	8.00 ± 1.31	1.96 ± 1.98	−25.4 ± 3.07
5% (vol/vol) CH_3F	0	0.37 ± 0.10	25.7 ± 2.36
5% (vol/vol) CH_3F + NH_4^+[b]	0	0.33 ± 0.05	28.7 ± 13.6

[a] Mean produced in 14 days ± 1 SD ($n = 3$ slurries). The initial NH_4^+ concentration was 7.44 μmol; the initial $NO_3^- + NO_2^-$ concentration was 0.68 μmol. A minus sign denotes consumption.
[b] 40.0 μmol of NH_4Cl added; slurry volume, 40 ml.

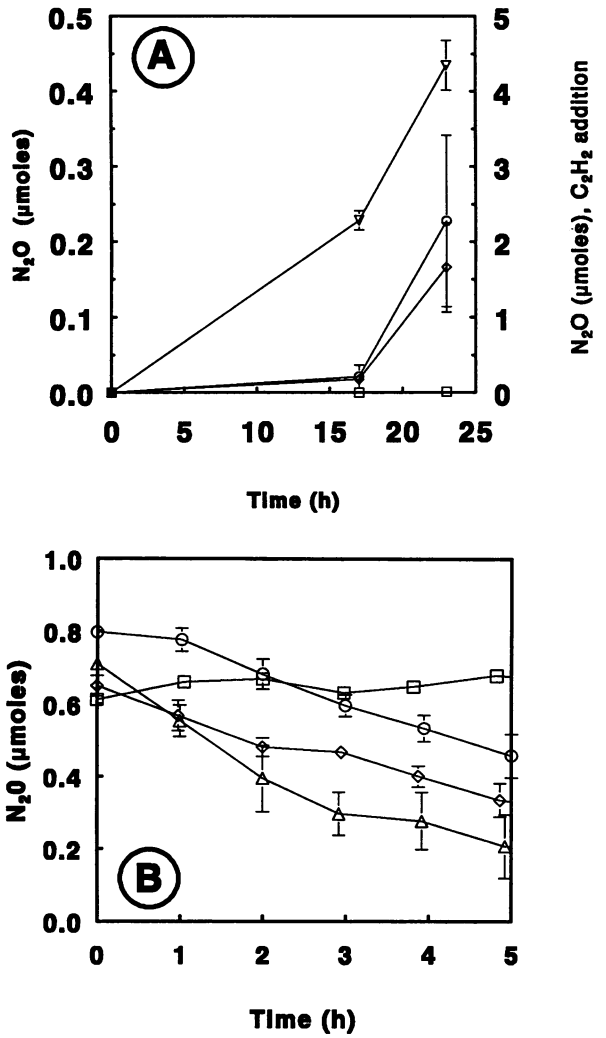

FIG. 3. Nitrous oxide production in anaerobic soils with 100 μmol of added NO_3^- (A) and N_2O consumption in anaerobic soils with ~0.7 μmol of added N_2O (B). Symbols: ○, no additions; ◇, 1% (vol/vol) CH_3F; △, 1% (vol/vol) DME; ▽, 30% C_2H_2, right-hand vertical axis in panel A; □, heat-killed control.

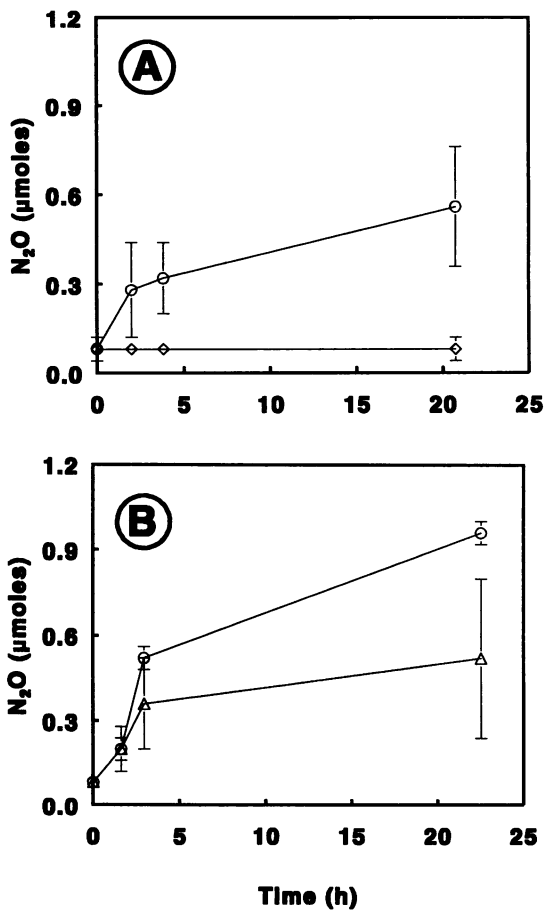

FIG. 4. Nitrous oxide accumulation in flux chambers at two sites (A = high and dry; B = low and wet) on the Searsville Lake shoreline. Symbols: ○, no additions; ◇, 1% (vol/vol) CH_3F.

Field experiments with flux chambers. The accumulation of N_2O in chamber headspaces was inhibited by the addition of CH_3F. At site A, chambers without additions accumulated 0.51 ± 0.36 μmol of N_2O in 21 h, while those with CH_3F added did not accumulate N_2O (Fig. 4A). At site B, chambers without additions accumulated 0.96 ± 0.04 μmol of N_2O in 23 h, while chambers with CH_3F added accumulated half as much N_2O (Fig. 4B). Acetylene markedly stimulated N_2O accumulation at site B (data not shown). Chambers without additions accumulated 0.76 ± 0.37 μmol of N_2O in 23 h, while those with C_2H_2 added accumulated 21 ± 8.0 μmol.

Intact-core incubations. N_2O production during intact-core incubations was initially inhibited by CH_3F (data not shown). Net N_2O production (0.19 ± 0.08 μmol in 20 days) occurred in cores without additions, while net N_2O consumption (−0.11 ± 0.03 μmol in 21 days) occurred in cores with added CH_3F. Constant SF_6 headspace concentrations indicated that no leaks existed between the cores and outside air. The initial soil content of NO_3^- + NO_2^- ranged from 0.39 μmol g^{-1} at the surface to below the detection limit (<0.02 μmol g^{-1}) at depths below 4 cm (Fig. 5A). Final NO_3^- + NO_2^- contents in cores without additions were similar at the surface (0.38 ± 0.04 μmol g^{-1}) but decreased to 0.09 ± 0.02 μmol g^{-1} at depths of 4 to 6 cm. Cores with added CH_3F were strongly depleted of NO_3^- + NO_2^- at the surface and had no detectable NO_3^- + NO_2^- below 1.8 cm after 21 days. The initial soil NH_4^+ concentration was below the detection limit (<0.3 μmol g^{-1}) at all depths (Fig. 5B). Final NH_4^+ concentrations in cores without additions were only slightly elevated at the surface (0.9 ± 0.5 μmol g^{-1}) and were below the detection limit at deeper depths. Cores with added CH_3F had markedly elevated NH_4^+ concentrations, ranging from 40 ± 0.5 μmol g^{-1} at the surface to 1.5 ± 0.7 μmol g^{-1} at depths of 4 to 6 cm. Final CH_3F contents in the soil column ranged from 0.03 ± 0.02 μmol g^{-1} at the surface to 0.06 ± 0.02 μmol g^{-1} at depths of 4 to 6 cm and averaged 0.05 ± 0.02 μmol g^{-1} (n = 10).

DISCUSSION

CH_3F and DME blocked NO_2^- and N_2O production (nitrification) from NH_4^+ in experiments with *N. europaea* (Table 1). Inhibition occurred immediately. The inhibitors appeared to act at the level of the AMO enzyme because nitrite production from NH_2OH was unaffected by inhibitor

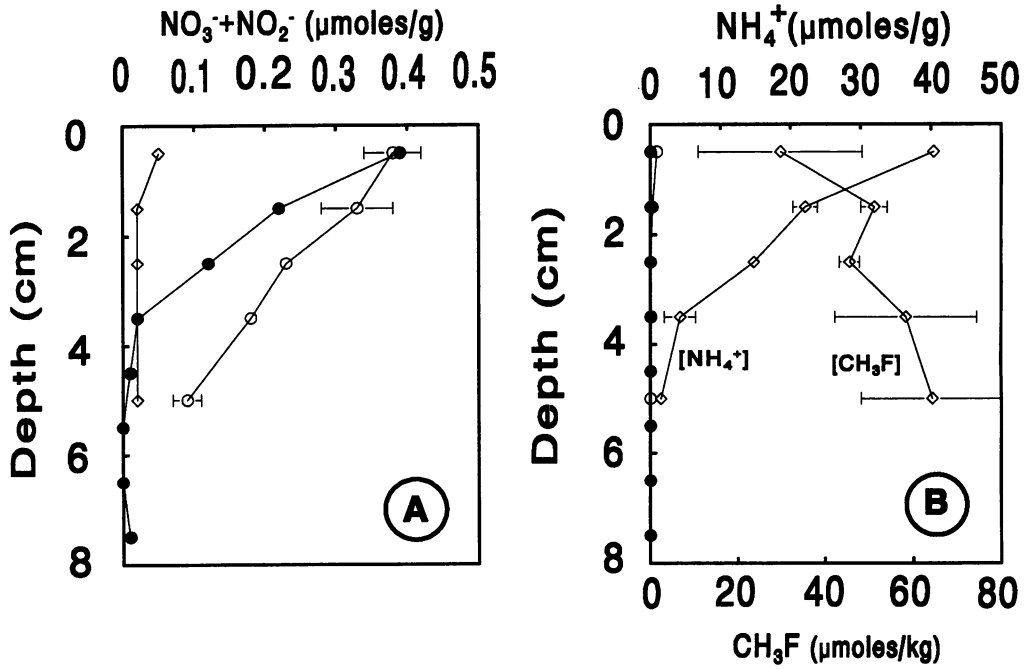

FIG. 5. Nitrate plus nitrite (A) and ammonia and CH_3F (B) profiles in the soil column of Searsville Lake. Solid symbols represent concentrations observed at the time at which the cores were collected. Open symbols represent concentrations observed after 3 weeks of incubation. Symbols: ○, uninhibited; ◇, 5% added CH_3F.

concentrations that completely blocked NH_4^+ oxidation (Fig. 1A). Likewise, both inhibitors failed to block NO_2^- uptake during the final oxidative step of nitrification, NO_3^- production by a *Nitrobacter* sp. (Fig. 1B). These results lead us to identify both CH_3F and DME as specific inhibitors of AMO enzyme activity.

CH_3F and DME blocked N_2O production in aerobic soil incubations (Table 2). Soils amended with NH_4Cl or NH_4NO_3 produced N_2O in the presence of C_2H_2 but not CH_3F (Fig. 2), suggesting that denitrification within anaerobic microsites occurred and was unaffected by CH_3F. Preformed NO_3^- within the soil may have provided a substrate for denitrification in the NH_4Cl-amended incubations (soil water below 100 cm in these experiments contained 0.3 mM NO_3^-, data not shown).

CH_3F blocked N_2O production (with or without added NH_4Cl) in estuarine sediments (Table 3). Ammonium losses and NO_3^- + NO_2^- increases were inhibited in flasks amended with CH_3F relative to flasks without the inhibitor. However, only the results from the NH_4^+ addition experiments were unequivocal, because denitrification of up to 13.0 μmol of NO_3^- + NO_2^- formed by 1 week of the experiment occurred in flasks without the inhibitor.

Neither CH_3F nor DME affected the production or consumption of N_2O during reductive nitrogen transformations. Dissimilatory nitrate reduction by SES-3 resulted in the consumption of added NO_3^- commensurate with the production of NO_2^-, NH_4^+, and N_2O. CH_3F had no effect on the rate of these transformations. By contrast, Kaspar and Tiedje (24) reported that C_2H_2 inhibited both NO_2^- reduction to NH_4^+ and N_2O production during dissimilatory reduction in the bovine rumen. Denitrification in soil slurry experiments produced N_2O from added NO_3^-, and this production also was not influenced by the presence of CH_3F. Because N_2O production was stimulated by the addition of C_2H_2, we suggest that CH_3F does not block N_2O reductase as C_2H_2 does (1, 51).

To determine the effect of CH_3F and DME on N_2O reductase activity in intact soils, we applied a procedure previously developed for sediments containing low NO_3^- levels whereby the consumption of a small amount of added N_2O is monitored over time (30). However, in situ soil NO_3^- concentrations were sufficiently high (>0.3 mM) to require substantial preincubation under anaerobic conditions so that N_2O production from NO_3^- could be eliminated. N_2O consumption rates were similar in amended and unamended flasks following preincubation (Fig. 3B), further suggesting that neither CH_3F nor DME blocks N_2O reductase activity.

The conclusion that CH_3F and DME are specific inhibitors of AMO and are "transparent" to the reductive transformations of nitrogenous compounds renders them extremely useful in studies of the source and fate of N_2O in complex systems in which both nitrification and denitrification are occurring. We tested this utility with chamber experiments, the results of which, from Searsville Lake, provided sufficient information to separate the sources of N_2O from two sites that differed primarily in soil moisture content. Nitrification only occurred at site A, the drier of the two soils. We conclude that all of the N_2O flux (15 ± 7 μmol m^{-2} day^{-1}) originated from the nitrification of ammonia because the flux was completely inhibited by the addition of CH_3F (Fig. 4). The ammonia concentration in the soil was low (Fig. 5), so the level of N_2O flux due to nitrification in the soil was also low. Nitrification and denitrification both occurred at site B. The level of N_2O flux (28 ± 2 μmol m^{-2} day^{-1}) was higher than that at site A; however, only half of that flux was inhibited by CH_3F. The residual N_2O flux (13 ± 7 μmol m^{-2} day^{-1}) was derived from the denitrification of preexisting NO_3^- in the soil. The soil NO_3^- concentration was high (Fig. 5), so the low level of N_2O flux resulted from high N_2O

reductase activity (6). High N_2O reductase activity was corroborated by high N_2O flux (770 ± 280 μmol m^{-2} day^{-1}) in chambers amended with C_2H_2 at site B.

Nitrous oxide fluxes in Searsville Lake soils were similar in magnitude to fluxes reported for dry tropical soils (16 μmol m^{-2} day^{-1}) (47), lake sediments (17 μmol m^{-2} day^{-1}) (40), and coastal marine sediments (44 μmol m^{-2} day^{-1}) (41). However, these previous studies could only rely on a general characterization of the redox state of the environment to distinguish the process (nitrification or denitrification) responsible for the N_2O flux. We infer, using a specific inhibitor of nitrification, that denitrification contributes about half of the N_2O flux at a wet soil site and is unimportant at a dry soil site. We observed similar effects of CH_3F on N_2O flux during intact-core incubations of Searsville Lake soils collected between sites A and B. N_2O was produced by nitrification during the first 6 days of the incubation (0.32 ± 0.04 μmol), and >80% of this N_2O flux could be inhibited by CH_3F (data not shown).

Nitrite and nitrate are products of nitrification in soils and sediments and are substrates for dissimilatory processes, including denitrification (28, 31). Nitrate consumed during denitrification may be derived from outside the zone of denitrification, or its production and consumption may be coupled, as in the suboxic water columns of oceans and lakes (11, 14) and, more commonly, in surface sediments (13, 19, 21, 27, 31, 36, 40, 46) and soils (18, 26). If NO_2^- and NO_3^- production from nitrification can be eliminated without affecting dissimilatory processes, then assessment of these reductive processes can be made independently of ongoing NO_2^- and NO_3^- production. In addition, the degree of coupling (i.e., the extent to which nitrification contributes NO_2^- and NO_3^- for denitrification) may be determined by inhibiting nitrification and monitoring the effects of continued denitrification.

We assessed coupling by inhibiting nitrification during intact-core incubations of Searsville Lake soils (Fig. 5). Uncoupling of nitrification and denitrification resulted in a net loss of NO_3^- + NO_2^- over 3 weeks because of a diminished nitrate supply from nitrification and continued denitrification. Nitrogen was lost as N_2O and presumably as N_2. This loss was accompanied by a much larger increase in the level of ammonia resulting from the inhibition of NH_4^+ oxidation and a continued ammonia supply. The mass deficit (ammonia increase minus NO_3^- + NO_2^- decrease) represents the potential nitrification-coupled denitrification if the uninhibited cores were at the steady state. This estimation scheme would overestimate potential coupling if N_2O loss during nitrification were significant.

Coupling of nitrification and denitrification has been invoked to explain the high rates of denitrification in sediments in which the supply of nitrate is limited (4) and also anomolously high pore water concentrations of dissolved N_2 (2) and NO_3^- + NO_2^- (19). Uncoupling of nitrification and denitrification has been invoked to explain the net fluxes of nitrate out of sediments in which denitrification is occurring (5, 20, 40). We conclude that the use of specific inhibitors of nitrification to determine the degree of coupling under these and similar circumstances shows promising results. Additionally, both CH_3F and DME can be used as alternatives to nitrapyrin and C_2H_2 in studies of nitrification by use of the difference between inhibited and uninhibited NH_4^+ fluxes in aquatic sediments (42).

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