

# Kinetics of Inhibition of Methane Oxidation by Nitrate, Nitrite, and Ammonium in a Humisol

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Received 27 February 1995/Accepted 1 June 1995

**The kinetics of inhibition of CH<sub>4</sub> oxidation by NH<sub>4</sub><sup>+</sup>, NO<sub>2</sub><sup>-</sup>, and NO<sub>3</sub><sup>-</sup> in a humisol was investigated. Soil slurries exhibited nearly standard Michaelis-Menten kinetics, with half-saturation constant [ $K_{m(app)}$ ] values for CH<sub>4</sub> of 50 to 200 parts per million of volume (ppmv) and  $V_{max}$  values of 1.1 to 2.5 nmol of CH<sub>4</sub> g of dry soil<sup>-1</sup> h<sup>-1</sup>. With one soil sample, NH<sub>4</sub><sup>+</sup> acted as a simple competitive inhibitor, with an estimated  $K_i$  of 8 μM NH<sub>4</sub><sup>+</sup> (18 nM NH<sub>3</sub>). With another soil sample, the response to NH<sub>4</sub><sup>+</sup> addition was more complex and the inhibitory effect of NH<sub>4</sub><sup>+</sup> was greater than predicted by a simple competitive model at low CH<sub>4</sub> concentrations (<50 ppmv). This was probably due to NO<sub>2</sub><sup>-</sup> produced through NH<sub>4</sub><sup>+</sup> oxidation. Added NO<sub>2</sub><sup>-</sup> was inherently more inhibitory of CH<sub>4</sub> oxidation at low CH<sub>4</sub> concentrations, and more NO<sub>2</sub><sup>-</sup> was produced as the CH<sub>4</sub>-to-NH<sub>4</sub><sup>+</sup> ratio decreased and the competitive balance shifted. NaNO<sub>3</sub> was a noncompetitive inhibitor of CH<sub>4</sub> oxidation, but inhibition was evident only at >10 mM concentrations, which also altered soil pHs. Similar concentrations of NaCl were also inhibitory of CH<sub>4</sub> oxidation, so there may be no special inhibitory mechanism of nitrate per se.**

Methane (CH<sub>4</sub>) is an important greenhouse gas that is increasing in atmospheric concentration (13). Much interest has focused on the role of aerobic soils as a sink for CH<sub>4</sub> and on the ecological and land use practices that affect its magnitude (26). Field studies have shown that fertilization with nitrogen, especially in the form of ammonium (NH<sub>4</sub><sup>+</sup>) or urea, can reduce CH<sub>4</sub> oxidation rates in soils (5, 10, 15, 19, 24, 28, 30) and sediments (4). In some cases, this is a long-term effect of repeated fertilizer applications rather than an immediate inhibition of methanotrophic bacteria or the methane monooxygenase (MMO) enzyme (17, 30). However, fertilization can also have an immediate effect on CH<sub>4</sub> oxidation in the field (5, 10, 15, 19). In laboratory incubations of soils and sediments, inhibition is caused by NH<sub>4</sub><sup>+</sup> (1, 6, 9, 19, 25, 28), nitrite (NO<sub>2</sub><sup>-</sup>) (19, 28), and high (>10 mM) concentrations of nitrate (NO<sub>3</sub><sup>-</sup>) (1, 25).

MMO can oxidize a variety of substrates besides CH<sub>4</sub>; these should therefore compete with CH<sub>4</sub> for the active site of this enzyme. One such cosubstrate is ammonia (NH<sub>3</sub>), which is oxidized to NO<sub>2</sub><sup>-</sup> via hydroxylamine (11). Pure culture studies with *Methylococcus capsulatus* (7), *Methylomonas methanica* (14), and *Methylosinus trichosporium* (27) have shown that NH<sub>3</sub> acts as a competitive inhibitor of CH<sub>4</sub> oxidation. Two of these studies (7, 27) noted a significant pH effect on the  $K_i$  measured as the NH<sub>4</sub><sup>+</sup> concentration, but the  $K_i$  was more constant if NH<sub>3</sub> rather than NH<sub>4</sub><sup>+</sup> was considered to be the inhibitor. The competition between NH<sub>3</sub> and CH<sub>4</sub> for the active sites of MMO in methanotrophs and of ammonia monooxygenase in nitrifiers has led to speculation regarding the contribution of nitrifiers to CH<sub>4</sub> oxidation and of methanotrophs to nitrification in natural environments (2, 31, 32).

Inhibition patterns that are more complex than simple enzymatic competition between CH<sub>4</sub> and NH<sub>3</sub> are occasionally evident in methanotrophs, for example, in low-copper *Meth-*

*ylococcus capsulatus* cells (7). The requirement of MMO for cosubstrates oxygen and NADH can complicate the interpretation of NH<sub>3</sub> inhibition (2). The addition of CH<sub>4</sub> may stimulate rather than inhibit NH<sub>3</sub> oxidation by methanotrophs (20, 21, 23, 27, 28), presumably because of alleviation of NADH limitation. Hydroxylamine (16) and NO<sub>2</sub><sup>-</sup> (18, 20, 27) produced through methanotroph oxidation of NH<sub>3</sub> are themselves inhibitors of methanotrophic activity. In a forest soil, the inhibitory effect of NO<sub>2</sub><sup>-</sup> was shown to be greater and more enduring than the direct effect of NH<sub>3</sub> (19, 28).

The half-saturation constant [ $K_{m(app)}$ ] for soil CH<sub>4</sub> oxidation of 30 to 50 nM (3) is several orders of magnitude lower than the values (1 to 66 μM) for pure cultures of methanotrophs (2). Known methanotrophs should not be capable of surviving solely on atmospheric CH<sub>4</sub> (8). Those methanotrophs active in aerobic soils might therefore employ a CH<sub>4</sub>-oxidizing system unlike that of known methanotrophs. Two forms of MMO, one particulate and one soluble, are known. Although these exhibit different substrate affinities, neither approaches the  $K_{m(app)}$  measured in soils (2, 3). This paper presents some experiments on the kinetics of CH<sub>4</sub> oxidation in a humisol designed to examine the competitive strength of NH<sub>3</sub> and to determine the amounts of NH<sub>4</sub><sup>+</sup>, NO<sub>2</sub><sup>-</sup>, and NO<sub>3</sub><sup>-</sup> needed to cause inhibition of CH<sub>4</sub> oxidation.

## MATERIALS AND METHODS

The study site, a humisol on the Central Experimental Farm of Agriculture Canada in Ottawa, Canada, has been described previously (12). Soil samples were taken from a depth of 0 to 20 cm on 17 August 1993 and 30 June 1994 and stored at 12°C. The 1993 soil sample was stored for up to 10 months before experiments were performed. The 1994 soil sample was stored for no more than 40 days. Although NH<sub>3</sub> rather than NH<sub>4</sub><sup>+</sup> is thought to be an inhibitor of MMO, we shall refer primarily to measured NH<sub>4</sub><sup>+</sup> concentrations. When given, NH<sub>3</sub> concentrations have been calculated on the basis of average pH values of 6.6 and 6.9 for 1993 and 1994 soil samples, respectively.

To investigate NH<sub>4</sub><sup>+</sup> inhibition of CH<sub>4</sub> oxidation, aggregates were broken by hand and a 1:3 ratio of field-moist soil (81% H<sub>2</sub>O, 1993 sample; 136% H<sub>2</sub>O, 1994 sample) to distilled deionized H<sub>2</sub>O was homogenized 30 min on a magnetic stirrer. Fifteen-milliliter portions (2.5 to 3.1 g of dry soil) were distributed into 60-ml serum vials, and diluted NH<sub>4</sub>Cl solutions were added to give final volumes of 20 ml. Of the NH<sub>4</sub><sup>+</sup> added, 60 to 70% was not recoverable from the slurry liquid phase shortly after addition and was assumed to be held on exchange sites. CH<sub>4</sub> concentrations of approximately 2, 30, 75, 100, 150, 200, 300, 400, 500, and

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TABLE 1. Effects of added  $\text{NH}_4\text{Cl}$  on the kinetic coefficients of  $\text{CH}_4$  oxidation in humisol slurries

Soil sample and experiment no.	$\text{NH}_4^+$ ( $\mu\text{M}$ ) <sup>a</sup>	$\text{NO}_2^-$ ( $\mu\text{M}$ ) <sup>a</sup>	Hyperbolic model <sup>b</sup>				Hill model <sup>b</sup>		
			$\text{CH}_4$ range (ppmv)	No. of $\text{CH}_4$ levels	$K_m(\text{app})$ (ppmv)	$V_{\text{max}}$ (nmol $\text{g}^{-1} \text{h}^{-1}$ )	$n_{\text{app}}$	$K_s^*$ (ppmv)	$V_{\text{max}}$ (nmol $\text{g}^{-1} \text{h}^{-1}$ )
1993 soil									
1	12.7	NT <sup>c</sup>	15–730	9	189	1.65	1.06	150	1.47
	20.8 <sup>d</sup>	NT	35–730	8	349	2.06	1.19	174	1.42
2	34.3	NT	40–730	8	413	1.81	1.22	340	1.21
	94.2	NT	70–730	7	1,016	2.01	1.20	464	1.25
	16.5	NT	15–730	9	184	2.79	1.05	144	2.22
	35.2 <sup>d</sup>	NT	15–730	9	377	2.99	1.12	178	1.89
	47.9	NT	65–730	7	579	3.36	1.14	328	2.47
	121	NT	65–730	7	982	2.89	1.37	409	1.83
3	26.6	29.8	45–750	9	111	1.38	0.91	101	1.31
	57.5	51.6	45–750	9	275	1.35	1.07	256	1.29
	136	65.4	45–750	9	991	2.03	1.23	623	1.17
1994 soil									
4 <sup>e</sup>	20.4	20.9	30–450	8	88.7	1.51			
	54.6	54.2	30–450	8	316	1.29			
	138	78.1	50–450	7	370	0.71			
5 <sup>e</sup>	27.7	28.0	40–550	9	60.2	1.31			
	52.2	52.7	40–550	9	173	1.27			
	96.2	53.2	70–550	8	153	0.79			
6	24.1	30.8	15–550	16	86.3	1.61	1.16	60.2	1.37
	70.3	48.4	40–550	13	172	1.13	1.39	97.6	0.85
7	19.5	15.8	15–550	14	107	1.64	1.27	56.1	1.22
	48.8	42.3	40–550	11	144	1.16	1.62	68.7	0.80
8	3.4	4.1	15–350	13	86.5	1.48	1.12	63.5	1.28
	94.4	42.2	60–350	10	370	1.52	1.53	119	0.77

<sup>a</sup>  $\text{NH}_4^+$  and  $\text{NO}_2^-$  concentrations are averages of concentrations at the start and end of each incubation.

<sup>b</sup> Kinetic constants were estimated by using the linear portion of Lineweaver-Burk plots for the Michaelis-Menten hyperbolic model or by fitting the data to the Hill cooperative model (see reference 29).

<sup>c</sup> NT, not tested.

<sup>d</sup> Added  $\text{NH}_4^+$  was completely consumed by the end of the experiment and was not used in  $K_f$  calculations or statistics.

<sup>e</sup> The Hill model could not be applied to experiments 4 and 5 because of high data variability.

700 parts per million of volume (ppmv) were added to duplicate vials and incubated on a gyratory shaker at 250 rpm and 25°C. For each experiment, a complete set of  $\text{CH}_4$  concentrations was run for control soil samples (no added  $\text{NH}_4^+$ ) and up to three levels of added  $\text{NH}_4^+$ . Specific details of each experiment are given in Table 1.

Three more rigorous experiments (no. 6, 7, and 8) with the 1994 soil sample used initial  $\text{CH}_4$  concentrations of approximately 2, 5, 10, 15, 20, 30, 40, 50, 60, 80, 100, 125, 150, 175, 200, 250, 300, 350, 400, and 500 ppmv (the two highest levels were omitted in experiment 8). Slurries were shaken for 20 to 24 h before  $\text{NH}_4\text{Cl}$  and  $\text{CH}_4$  addition in an attempt to alleviate two problems noted in earlier trials. First, slurring caused transiently elevated  $\text{NO}_2^-$  levels which could be depleted through nitrification in preincubation. Second, initial net  $\text{CH}_4$  production rather than consumption was occasionally observed at 2-ppmv  $\text{CH}_4$ . This soil has considerable methanogenic capacity (12), and although no methanogenesis should occur in diluted aerobic slurries, a short period of net  $\text{CH}_4$  production may result from equilibration of  $\text{CH}_4$  already in the soil, perhaps hydrophobically bound to organic matter, with the gas phase of vials.

$\text{CH}_4$  levels were measured at 1 to 1.5 and 5.5 to 6 h after the addition of  $\text{NH}_4\text{Cl}$  and  $\text{CH}_4$  by injections of 2-ml headspace volumes into a Shimadzu gas chromatograph and flame ionization detector equipped with a 1.8-m Porapak Q column and 0.5-ml sample loop. At the same times, 1-ml slurry samples were taken into microcentrifuge tubes and frozen immediately. In calculating  $\text{CH}_4$  oxidation rates, compensation was made for the removal of gas during sampling. Vials containing 20 ml of  $\text{H}_2\text{O}$  and several  $\text{CH}_4$  concentrations were included as checks on standardization and leakage. In the event of nonzero  $\text{CH}_4$  oxidation rates calculated for these vials, linear regression of the calculated rate versus  $\text{CH}_4$  concentration was used to correct soil  $\text{CH}_4$  oxidation rates.

Inhibition by  $\text{NaNO}_3$  or  $\text{NaNO}_2$  was tested by essentially the same procedure. Incubations for experiments with  $\text{NO}_2^-$  and  $\text{NH}_4^+$  supplements were limited to 5 to 6 h because nitrification rapidly depleted these ions, but  $\text{NO}_3^-$  concentrations remained constant and thus incubation times could be longer. The possibility of phase transfer limitation was examined with triplicate 20-ml slurries containing 1, 2, 3, or 4 g of 1993 soil at 100-ppmv  $\text{CH}_4$ . To test the reversibility of  $\text{NO}_2^-$  and  $\text{NH}_4^+$  inhibition, vials from representative  $\text{NH}_4^+$  and  $\text{NO}_2^-$  experiments were incubated until all added  $\text{NO}_2^-$  or  $\text{NH}_4^+$  was oxidized and then  $\text{CH}_4$  oxidation was retested.

For nitrogen analyses, slurry samples in microcentrifuge tubes were thawed by centrifugation for 5 min at  $13,800 \times g$ , resuspended by manual shaking, and centrifuged again for 15 min.  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ , and  $\text{NH}_4^+$  levels in the supernatant were measured colorimetrically by using an automated analysis system (Chem-Lab Instruments, Hornchurch, Essex, England) (23). Initial concentrations were estimated from 2-ppmv  $\text{CH}_4$  vials only (this was valid since the  $\text{CH}_4$  concentration did not affect the nitrification rate [see Results]). Final concentrations were the averages of at least six vials. pH values were not affected by experimental  $\text{NH}_4^+$  additions.

The  $\text{CH}_4$ ,  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , and  $\text{NO}_2^-$  concentrations presented and used in kinetic calculations are the arithmetic means of measurements at the beginning and end of each incubation. The arithmetic mean is an overestimate of the actual average  $\text{CH}_4$  concentration over this time, but since maximum  $\text{CH}_4$  depletion is 17%, the error incurred should be minor (29). All kinetic and inhibition coefficients were estimated as described by Segel (29). Statistical procedures were performed with SYSTAT (SYSTAT, Inc., Evanston, Ill.). Except when noted, statistical analyses were multivariate analyses of variance (MANOVAs) with soil sample and inhibitor ( $\text{NH}_4^+$ ,  $\text{NO}_2^-$ , and  $\text{NO}_3^-$ ) concentrations as independent variables and kinetic coefficients as dependent variables. Variance assumptions were checked by using residual plots, and data were occasionally log transformed to satisfy these. Multiple comparisons are Bonferroni contrasts.

## RESULTS

**Phase transfer limitation and initial rates.**  $\text{CH}_4$  oxidation rates increased nearly linearly with increasing soil contents in slurries (Fig. 1), indicating that slurries were not limited by phase transfer and diffusion of  $\text{CH}_4$ . The slight curvature of the plot may result from dilution alterations of slurry pH values and  $\text{NH}_3$  concentrations.

Slurries used in inhibition experiments were never incubated

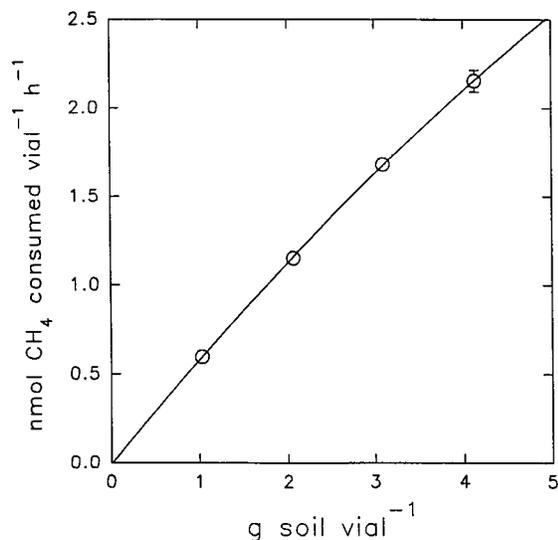


FIG. 1. Oxidation rates of 100-ppmv CH<sub>4</sub> in 20-ml slurries containing different amounts of 1993 soil. Each point is the mean of triplicate vials ± 1 standard error of the mean. When error bars are not visible, they are contained within the symbol.

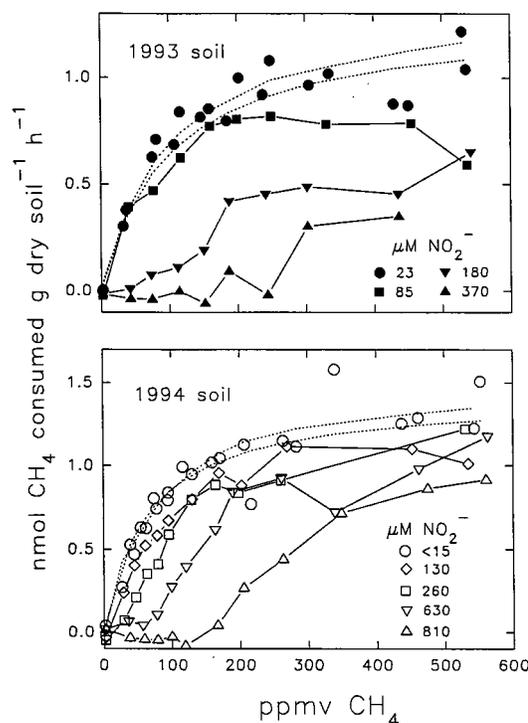


FIG. 2. Effects of NaNO<sub>2</sub> additions on the kinetic curves of CH<sub>4</sub> oxidation in two soil samples. Each graph includes curves for two trials; circles represent control rates. The average NO<sub>2</sub><sup>-</sup> concentrations during incubations are indicated.

for more than 24 h (usually <7 h) with added CH<sub>4</sub>. During these periods, kinetic coefficients were nearly constant for slurries without added NH<sub>4</sub><sup>+</sup> (Table 2). Methanotrophic activity was stimulated by several orders of magnitude by exposure to 10% CH<sub>4</sub> (data not shown), suggesting that the growth of methanotrophs is not nitrogen limited and should not be affected by NH<sub>4</sub><sup>+</sup> additions. The rates presented in this paper are therefore based on initial enzyme concentrations and are true initial rates, with one potential exception. When significant accumulations of NO<sub>2</sub><sup>-</sup> occurred during incubation through oxidation of added NH<sub>4</sub><sup>+</sup>, measured rates may have gradually decreased over time. We have attempted to compensate for this in our calculations (see below).

**NO<sub>2</sub><sup>-</sup> inhibition.** The addition of NaNO<sub>2</sub> inhibited CH<sub>4</sub> oxidation in both soil samples (Fig. 2). Although each graph contains curves from two separate experiments, in each case the two control curves are similar. Progressively higher NaNO<sub>2</sub> additions resulted in increasingly sigmoidal kinetics, with the highest relative inhibition of CH<sub>4</sub> oxidation occurring at the lowest CH<sub>4</sub> concentrations.

**NH<sub>4</sub><sup>+</sup> inhibition.** The experiments comparing CH<sub>4</sub> oxidation kinetics with and without added NH<sub>4</sub><sup>+</sup> are summarized in Table 1. The CH<sub>4</sub> oxidation rates in soil slurries without added NH<sub>4</sub><sup>+</sup> agreed well with a Michaelis-Menten hyperbolic model

TABLE 2. Kinetic constants calculated at various times after the addition of CH<sub>4</sub> to soil slurries

Soil sample	Incubation time (h)	V <sub>max</sub> (nmol g <sup>-1</sup> h <sup>-1</sup> )	K <sub>m(app)</sub> (ppmv)
1994 soil <sup>a</sup>	3.5-9.0	1.50	68
	9.0-22.5	1.54	92
	22.5-28.5	1.73	92
1994 soil + 74 mM NO <sub>3</sub> <sup>a</sup>	3.5-9.0	0.86	43
	9.0-22.5	1.00	77
	22.5-28.5	1.11	60
1993 soil <sup>b</sup>	1.5-7.5	1.12	
	7.5-27.0	1.09	
	27.0-32.0	1.49	

<sup>a</sup> For more details, see results for experiment 11 in Table 3.

<sup>b</sup> Based on duplicate flasks at >400-ppmv CH<sub>4</sub>.

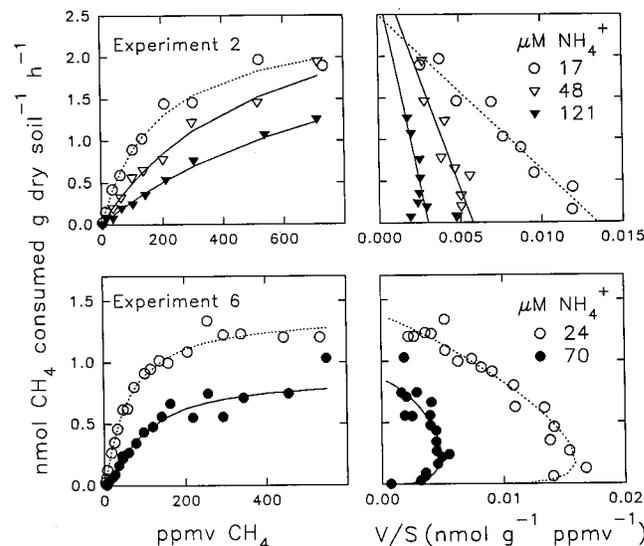


FIG. 3. Representative kinetic curves of CH<sub>4</sub> oxidation in 1993 (experiment 2) and 1994 (experiment 6) soil samples with and without NH<sub>4</sub>Cl additions as well as Eadie-Hofstee replots to show deviations from true hyperbolic curves at low CH<sub>4</sub> oxidation rates for 1994 soil. Lines were fit to the curves by using a Michaelis-Menten hyperbolic model for 1993 soil and a Hill cooperative model for 1994 soil. Points are means of duplicate vials. The average liquid-phase NH<sub>4</sub><sup>+</sup> contents are indicated.

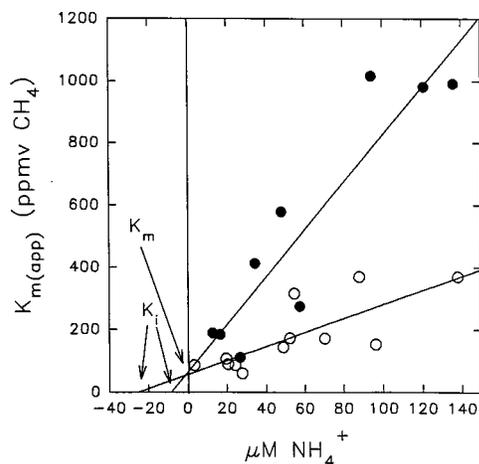


FIG. 4.  $K_{m(app)}$  values of  $\text{CH}_4$  oxidation, estimated by using Lineweaver-Burk replots, plotted against average liquid-phase  $\text{NH}_4^+$  concentrations in 1993 (●) and 1994 (○) humisol slurries. The  $K_m$  of  $\text{CH}_4$  oxidation in an  $\text{NH}_4^+$ -free system and the  $K_i$  of  $\text{NH}_4^+$  for  $\text{CH}_4$  oxidation are estimated from axis intercepts.

(Fig. 3). However, when  $\text{NH}_4^+$  was added, kinetic curves often became sigmoidal and curvature of Lineweaver-Burk and Eadie-Hofstee plots was evident at low velocities, especially for the 1994 soil sample (Fig. 3). Replots used to estimate the Michaelis-Menten kinetic coefficients in Table 1 were linearized by deleting the lowest substrate concentrations. This procedure gives valid estimates when curves of  $v$  plotted against  $s$  are only slightly sigmoidal, as was the case with the 1993 soil sample. However, it is not an ideal treatment of sigmoidal kinetics (29) and gives biased estimates for the 1994 soil sample. Therefore,  $1/v$ -versus- $1/s$  plots were also fit to the Hill cooperative model (29) by using the curve-fit function of SigmaPlot 5.1 (Jandel Scientific, San Raphael Calif.). Because of weighting toward low-velocity points, the lowest points were deleted until an unbiased agreement with the equation was obtained (in practice, this usually meant deleting any velocity that was  $<0.1$ ). Representative experiments showing good agreement with the hyperbolic model for the 1993 soil sample and agreement with the Hill model for the 1994 soil sample are given in Fig. 3.

Ammonium acted as a simple competitive inhibitor in the 1993 soil sample. The  $K_{m(app)}$  values for  $\text{CH}_4$  increased with increases in the  $\text{NH}_4^+$  concentration ( $P < 0.001$ ; log-transformed data), but the  $V_{max}$  values were unaffected ( $P = 0.003$ ; log-transformed data). Plot curvature was minor, as shown by low  $n_{app}$  values (Table 1). Replots of  $K_{m(app)}$  versus  $\text{NH}_4^+$  concentration were used to estimate the  $K_i$  of  $\text{NH}_4^+$  and the true  $K_m$  of  $\text{CH}_4$  oxidation in the absence of  $\text{NH}_4^+$  (Fig. 4). These estimates were a  $K_i$  of  $8.3 \mu\text{M NH}_4^+$  ( $18.5 \text{ nM NH}_3$ ) and a  $K_m$  of  $63.5 \text{ ppmv}$  for  $\text{CH}_4$  ( $88.2 \text{ nM}$  dissolved).

The 1994 soil sample shows a more complex response to  $\text{NH}_4^+$  addition. The  $V_{max}$  decreased, whether estimated by the hyperbolic or Hill model ( $P = 0.004$ ), while the  $K_{m(app)}$  (and the  $K_s^*$ ) again increased ( $P < 0.001$ ) with increasing  $\text{NH}_4^+$  concentrations (Table 1). Unlike those for the 1993 soil sample, the  $K_{m(app)}$  estimates for the 1994 soil sample are not particularly reliable because of sigmoidal  $v$ -versus- $s$  plots. These estimates would give a  $K_i$  of  $25.2 \mu\text{M NH}_4^+$  ( $112 \text{ nM NH}_3$ ) and a  $K_m$  for  $\text{CH}_4$  of  $56.7 \text{ ppmv}$  ( $78.7 \text{ nM}$  dissolved). Methanotrophs in the 1994 soil sample seemed to have a lower affinity for  $\text{NH}_4^+$  than those in the 1993 soil sample.

At levels below about  $50\text{-ppmv CH}_4$ , measured  $\text{CH}_4$  oxidation rates were less than predicted by a strictly Michaelis-

Menten hyperbolic model, resulting in sigmoidal kinetics.  $n_{app}$  values increased with increasing  $\text{NH}_4^+$  concentrations (Table 1), especially in the 1994 soil sample, whose kinetic plots were often strikingly sigmoidal (Fig. 3). The competitive model of  $\text{NH}_4^+$  inhibition is therefore conservative, underestimating inhibition at low  $\text{CH}_4$  concentrations. The  $n_{app}$  parameter should probably be considered simply a measure of plot curvature rather than in its correct sense as a measure of cooperativity, since we hypothesize that the sigmoidal curves result from  $\text{NO}_2^-$  toxicity. While the data could result from an allosteric enzyme, the Hill model might fit because (i)  $\text{NO}_2^-$  is more inhibitory of  $\text{CH}_4$  oxidation at low  $\text{CH}_4$  concentrations and (ii) if  $\text{NH}_4^+$  is competitively oxidized by MMO,  $\text{NO}_2^-$  production decreases with increasing  $\text{CH}_4$  concentrations. This system may thereby mimic a cooperative system whereby increasing  $\text{CH}_4$  concentrations result in optimal oxidation.

The possibility of a false competitive oxidation curve simulated by increased  $\text{NO}_2^-$  production with increasing  $\text{CH}_4$  concentrations, a trend noted elsewhere (20), exists. However, this was not evident from our nitrogen measurements and would in any case require increased enzyme activity at high  $\text{CH}_4$  concentrations. Enzyme activity did not increase during our incubations.

**Nitrification.** The maximum nitrification rate, measured as the production of  $\text{NO}_3^-$  and  $\text{NO}_2^-$  in slurries with  $\text{NH}_4^+$  added ( $>30 \mu\text{M}$ ), was  $0.18$  to  $0.25 \mu\text{mol g}^{-1} \text{ h}^{-1}$  for the 1994 soil sample (experiments 5 through 8) and  $0.11$  to  $0.25 \mu\text{mol g}^{-1} \text{ h}^{-1}$  for the 1993 soil sample (experiments 1 and 2 and data from reference 12). The control 1993 soil sample also accumulated  $\text{NO}_3^-$  and  $\text{NO}_2^-$  at  $0.03$  to  $0.15 \mu\text{mol g}^{-1} \text{ h}^{-1}$ , while nitrification was undetectable in the control 1994 soil sample ( $<0.03 \mu\text{mol g}^{-1} \text{ h}^{-1}$ ), indicating a lower rate of  $\text{NH}_4^+$  mineralization in the latter. The net  $\text{NH}_4^+$  depletion rate in the 1993 soil sample with added  $\text{NH}_4^+$  was only  $0.08$  to  $0.10 \mu\text{mol g}^{-1} \text{ h}^{-1}$ . Nitrification was rapid enough to consume a large proportion of the added  $\text{NH}_4^+$  during incubation, introducing error into the determination of its  $K_i$  for  $\text{CH}_4$  oxidation. However, the arithmetic mean should be a valid estimate of the slurry  $\text{NH}_4^+$  concentration since measured nitrification rates were nearly constant regardless of the amount of  $\text{NH}_4^+$  added [i.e., the  $K_{m(app)}$  of soil nitrification was probably lower than  $30 \mu\text{M}$  (data not shown)].

The effects of  $\text{CH}_4$  concentrations on nitrification were examined by measuring postincubation  $\text{NO}_3^-$  and  $\text{NO}_2^-$  concentrations in all vials from experiment 3 and the  $94.4 \mu\text{M NH}_4^+$  treatment of experiment 8. A two-way MANOVA with the  $\text{CH}_4$  concentration as a regression factor and  $\text{NH}_4^+$  addition as a categorical factor revealed no significant effects of  $\text{CH}_4$  concentrations on total nitrification (final concentration of  $\text{NO}_2^-$  and  $\text{NO}_3^-$ ,  $P = 0.16$ ), but the effects on net  $\text{NO}_2^-$  production were significant ( $P = 0.014$ ; overall Pillai trace,  $P = 0.025$ ). This difference probably simply reflects the more precise measurement of  $\text{NO}_2^-$  than of  $\text{NO}_3^-$  ( $\text{NO}_2^-$  was typically  $<50 \mu\text{M}$ , while the pool of  $\text{NO}_3^-$  was  $>800 \mu\text{M}$ ). While statistically more  $\text{NO}_2^-$  was produced in slurries with low  $\text{CH}_4$  concentrations, the difference was small compared with absolute  $\text{NO}_2^-$  concentrations. The average regression slope was only  $-0.015 \mu\text{M NO}_2^- \text{ ppmv CH}_4^{-1}$ , and the effects of  $\text{CH}_4$  concentrations on total nitrification were therefore minute.

**$\text{NaNO}_3$  inhibition.** Three experiments investigated  $\text{NaNO}_3$  inhibition (Table 3); one of them is shown in Fig. 5. A non-competitive mechanism of inhibition [the  $K_{m(app)}$  was unaffected, and the  $V_{max}$  decreased] is indicated. Statistical tests were not performed because of the paucity of curves, but the 1993 soil sample appeared to be more sensitive to  $\text{NaNO}_3$  inhibition than the 1994 soil sample was. An increase in plot

TABLE 3. Effects of added  $\text{NaNO}_3$  on kinetic coefficients of  $\text{CH}_4$  oxidation in humisol slurries

Soil sample and experiment no.	Incubation time (h)	$\text{NO}_3^-$ (mM)	$\text{NO}_2^-$ ( $\mu\text{M}$ )	pH	$\text{CH}_4$ range (ppmv)	Hyperbolic model			Hill model		
						No. of $\text{CH}_4$ levels	$K_m(\text{app})$ (ppmv)	$V_{\text{max}}$ ( $\text{nmol g}^{-1} \text{h}^{-1}$ )	$n_{\text{app}}$	$K_i^*$ (ppmv)	$V_{\text{max}}$ ( $\text{nmol g}^{-1} \text{h}^{-1}$ )
1993 soil, 9	5.5	1.17	26.0	6.70	30–500	8	158	1.57	1.46	61.0	0.99
		37.8	72.5	6.47	70–550	7	141	0.80	1.38	86.7	0.64
		58.7	105	6.46	70–550	7	195	0.53	2.27	86.7	0.34
1994 soil 10	7.0	0.31	<3.0	NT <sup>a</sup>	30–350	9	65.3	1.21	1.25	52.3	1.07
		18.2	11.9	NT	30–350	9	77.2	1.24	1.49	48.4	0.98
		33.9	29.6	NT	30–350	9	71.4	1.07	1.57	44.8	0.85
11	13.5	0.44	<3.0	7.14	25–500	9	91.7	1.54	1.11	74.5	1.41
		74.2	37.4	6.74	25–550	9	82.0	1.07	1.18	61.6	0.96
		102	57.8	6.70	45–550	8	73.1	0.64	1.73	52.0	0.53

<sup>a</sup> NT, not tested.

curvature ( $n_{\text{app}}$ ) also accompanied  $\text{NaNO}_3$  addition, probably because of  $\text{NO}_2^-$  contained as a contaminant or produced through  $\text{NO}_3^-$  reduction (Table 3).

This noncompetitive inhibition is not necessarily due to  $\text{NO}_3^-$  per se. Slurry pH values were altered by  $\text{NaNO}_3$  additions, although greater than 40 mM increases continued to decrease the  $V_{\text{max}}$  values without further affecting the pH values (Table 3). Similar concentrations of  $\text{NaCl}$  also inhibited  $\text{CH}_4$  oxidation. In experiment 9, the  $\text{CH}_4$  oxidation rates (at 160-ppmv  $\text{CH}_4$ ) in slurries supplemented with 35 or 55 mM  $\text{NaCl}$  were not significantly different from those in slurries with 38 or 59 mM  $\text{NaNO}_3$ , respectively (two-way analysis of variance [ANOVA] with four Bonferroni contrasts,  $P = 0.009$ ). The pH of slurries with added  $\text{NaCl}$  (6.49) was nearly the same as that of slurries with added  $\text{NaNO}_3$ . Smaller  $\text{NaCl}$  additions (up to 10 mM) did not significantly affect  $\text{CH}_4$  oxidation rates at 2-, 8-, 32-, and 120-ppmv  $\text{CH}_4$  in the 1994 soil sample (two-way ANOVA of log-transformed data,  $P = 0.61$ ); therefore, dissolved salts should not have affected the results of  $\text{NH}_4\text{Cl}$  or  $\text{NaNO}_2$  inhibition experiments.

**Reversibility of inhibition.** After 70 h, 0.7 or 1 mM of added  $\text{NO}_2^-$  (1994 soil) reached background, control levels. In 7-h incubations at 2-, 50-, 85-, 200-, and 380-ppmv  $\text{CH}_4$ , these slurries then had the same  $\text{CH}_4$  oxidation rate as slurries that had never received  $\text{NO}_2^-$  additions (two-way ANOVA,  $P = 0.70$ ). In experiment 5, the effects of  $\text{NH}_4^+$  additions on the  $\text{CH}_4$  oxidation rate were also not significant after the  $\text{NH}_4^+$

was nitrified in 5-h incubations at 2-, 120-, 275-, and 450-ppmv  $\text{CH}_4$  (two-way ANOVA of log-transformed data,  $P = 0.30$ ). Inhibition by both  $\text{NH}_4^+$  and  $\text{NO}_2^-$  at these concentrations was fully reversible.

## DISCUSSION

It should be stressed that the statistically significant effects of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  on kinetic coefficients of  $\text{CH}_4$  oxidation do not justify the basic assumption that the kinetic response of MMO is being measured. Measuring the kinetic coefficients of a preparation as crude and ecologically complex as soil is problematic. However, the  $\text{CH}_4$  oxidation rates measured in control soil slurries were true initial rates, there was no diffusion limitation, and  $v$ -versus- $s$  curves usually fit a hyperbolic model well.  $\text{CH}_4$  oxidation is the initial step in an oxidative pathway, and  $\text{CH}_4$  is freely diffusible across the cell membrane. This system may therefore be relatively simple and allow for a kinetic interpretation. If this is accepted, then we can make some conclusions on the mechanisms of inhibition and the strengths of various inhibitors.

Ammonium acted as a competitive inhibitor of  $\text{CH}_4$  oxidation in the 1993 soil sample. Our kinetic plots cannot distinguish between simple competitive inhibition and partial competitive inhibition (29). However, since the  $\text{CH}_4$  oxidation rate was driven to zero with very high levels of  $\text{NH}_4^+$  additions (>1 mM [data not shown]), the mechanism is probably simple competition. The two samples produced similar  $K_m$  estimates (about 60-ppmv  $\text{CH}_4$ ). Nevertheless, while the results of the 1993 soil sample were easily interpretable as simple competition between  $\text{CH}_4$  and  $\text{NH}_4^+$ , the results of the 1994 soil sample were more complex, with decreased  $V_{\text{max}}$  values and strongly sigmoidal kinetics at high  $\text{NH}_4^+$  concentrations.  $\text{NH}_4^+$  also probably acted as a competitive inhibitor in this sample, and although accurate estimation of the  $K_i$  was impossible, this soil seemed less sensitive to  $\text{NH}_4^+$ .

Explaining the differences between the two samples is difficult. There were differences in storage time and experimental protocol (experiments 6 to 8 included 24 h of preshaking and more intense sampling at lower  $\text{CH}_4$  values). Sampling variations may have influenced the microbial flora present, and if measured  $K_m(\text{app})$  values resulted from the activities of several enzymes rather than the activity of one, their ratios could have varied between samples. The more rapid  $\text{NH}_4^+$  depletion rate of the 1994 soil sample might also have protected methanotrophs from the inhibitory effects of  $\text{NH}_4^+$ . Considering  $\text{NH}_3$  to be the inhibitor instead of  $\text{NH}_4^+$  increases the dispar-

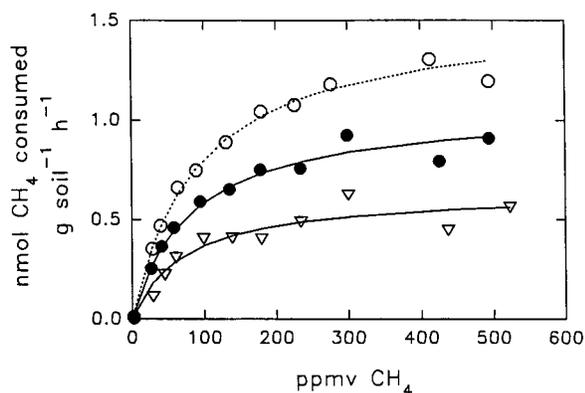


FIG. 5. Effects of  $\text{NaNO}_3$  addition on the kinetics of  $\text{CH}_4$  oxidation in the 1994 soil sample (experiment 11 [Table 3]). The average  $\text{NO}_3^-$  concentrations are 0.4 ( $\circ$ ), 74 ( $\bullet$ ), and 102 ( $\nabla$ ) mM.

ities in  $K_i$  values, but the pH could certainly affect the enzyme system in ways that are distinct from its effects on the  $\text{NH}_4^+$ -to- $\text{NH}_3$  ratio.

Errors in determining  $K_{m(\text{app})}$  values mean that the  $K_i$  of 8 to 25  $\mu\text{M}$   $\text{NH}_4^+$  (18 to 112 nM  $\text{NH}_3$ ) should be considered a rough estimate only, but it does suggest that in situ soil  $\text{NH}_4^+$  concentrations inhibit  $\text{CH}_4$  oxidation. Similar to the finding that the  $K_{m(\text{app})}$  values for soil  $\text{CH}_4$  oxidation are much lower than those for pure cultures of methanotrophs (2, 3), our soil  $K_i$  is lower than the  $K_i$  values of 1.7 to 18 mM  $\text{NH}_4^+$  at pH 6 to 7 (8 to 56  $\mu\text{M}$   $\text{NH}_3$ ) determined for pure cultures (2, 7, 14, 27). However, our results also agree with those of King and Schnell (19, 28) that the inhibition caused by the addition of  $\text{NH}_4^+$  may not be simply competitive dilution but may also be the result of production of toxic  $\text{NO}_2^-$ .

Kinetic curves became increasingly sigmoidal with increased  $\text{NH}_4^+$  or  $\text{NO}_2^-$  additions. This pattern would result if MMO exhibited cooperative behavior which was influenced by  $\text{NO}_2^-$  or  $\text{NH}_4^+$ . It could also result from  $\text{NO}_2^-$  toxicity. The relative inhibition caused by  $\text{NO}_2^-$  was highest at low  $\text{CH}_4$  concentrations, a pattern also noted for another soil (28). This pattern, combined with the shift in competitive balance of MMO as the ratio of  $\text{NH}_4^+$  to  $\text{CH}_4$  increases, could contribute to the sigmoidal  $\text{CH}_4$  oxidation curves of soils with  $\text{NH}_4^+$  added. In other words, more  $\text{NO}_2^-$  is produced in soil at low  $\text{CH}_4$  concentrations, at which it is also inherently more inhibitory of  $\text{CH}_4$  oxidation. Nitrite is an inhibitor of formate dehydrogenase and can contribute to NADH limitation (18), explaining its greater influence on  $\text{CH}_4$ -limited cells. A high metabolic rate might also be necessary to export toxic, cellular  $\text{NO}_2^-$ . The  $\text{NO}_2^-$  concentrations measured in  $\text{NH}_4^+$  inhibition experiments were slightly lower (<100  $\mu\text{M}$ ) than the levels of exogenously added  $\text{NO}_2^-$  required to cause inhibition of  $\text{CH}_4$  oxidation, but if methanotrophs themselves produce  $\text{NO}_2^-$ , intracellular concentrations would be higher.

In this soil, as in many other soils (22), nitrification was not affected or was only slightly affected by up to 800-ppmv  $\text{CH}_4$ . Methanotrophs can occasionally contribute to soil nitrification (2), and a methane-dependent nitrifying consortium was isolated from a similar humisol (23), but methanotrophs probably contributed little to nitrification in our humisol. Slightly elevated soil  $\text{NO}_2^-$  concentrations were noted as  $\text{CH}_4$  concentrations decreased. This indicates a competitive effect of  $\text{CH}_4$  on  $\text{NH}_4^+$  oxidation by nitrifiers or methanotrophs, but the effect was too small to greatly affect the overall nitrification rate.

The competitive nature of  $\text{NH}_4^+$  inhibition in our soil, whereby inhibition decreased at high  $\text{CH}_4$  levels because of competitive dilution, is the complete opposite of a trend found in a forest soil (28). The same authors also showed that  $\text{NO}_2^-$  production from  $\text{NH}_4^+$  in methanotroph cultures increased with increasing  $\text{CH}_4$  concentrations (20) and advanced this as an explanation of the ammonium inhibition pattern observed in soil (28). Other reports (21, 23, 27) also note that while large amounts of added  $\text{CH}_4$  may inhibit  $\text{NH}_4^+$  oxidation by methanotrophs, small amounts of added  $\text{CH}_4$  may actually stimulate it. The former effect is presumably competitive, with the latter stemming from energetic limitation of methanotrophs at low  $\text{CH}_4$  concentrations. Increased  $\text{NH}_4^+$  oxidation and  $\text{NO}_2^-$  production can result from increased MMO activity, through the alleviation of NADH limitation or induction of enzyme production and population growth. In the study of forest soil noted above (28), the authors make no mention of the linearity of their rates over time at high  $\text{CH}_4$  levels; it seems likely that the methanotrophic population was stimulated. Our short incubations provide a look at initial rates based on initial enzyme

concentrations, a "snapshot" indicating that  $\text{NH}_4^+$  or  $\text{NH}_3$  probably acts as a competitive inhibitor. It is perhaps also noteworthy that the initial  $\text{CH}_4$  concentrations in our soil were greater than ambient-air  $\text{CH}_4$  concentrations, presumably because of methanogenesis in anaerobic soil microsites, so methanotrophs may not have suffered from NADH limitation. The slurring of soil, as well as other disturbances (such as sonication), results in transient  $\text{CH}_4$  efflux.

There is a discrepancy between the complete reversibility of  $\text{NO}_2^-$  and  $\text{NH}_4^+$  inhibition in our humisol and the persistence noted in other studies (19, 25). Perhaps the absolute concentration and time of exposure affect the ability of methanotrophs to recover from  $\text{NH}_4^+$  and  $\text{NO}_2^-$  inhibition, and perhaps the extremely high natural nitrification rate of this humisol shields methanotrophs from  $\text{NO}_2^-$  and  $\text{NH}_4^+$ . The levels of our  $\text{NH}_4^+$  additions were much lower than those used in other studies (19, 25), although in a parallel field study, we also found no effects of the addition of 100 kg of urea  $\text{ha}^{-1}$  on  $\text{CH}_4$  oxidation (12).

These studies demonstrate the competitive nature of  $\text{NH}_4^+$  inhibition of soil  $\text{CH}_4$  oxidation and further implicate  $\text{NO}_2^-$  as a significant inhibitor, as previously noted (20, 28). Methanotrophic activity in soil has a higher affinity for  $\text{NH}_4^+$  than that noted for pure methanotroph cultures. As found in other studies (1, 25), inhibition by  $\text{NaNO}_3$  was evident only at concentrations at which salts had a similar inhibitory effect. This inhibition was not purely attributable to pH, and only the maximum catalytic rate of the enzyme was affected.

#### ACKNOWLEDGMENTS

This work was supported by an Agriculture Canada Green Plan contract to R.K. P.D. was supported by an Eco-Research (Canada) fellowship.

Many thanks to Chris Tauchner for endless routine analyses.

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