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Effects of Ammonium and Non-Ammonium Salt Additions on Methane Oxidation by *Methylosinus trichosporium* OB3b and Maine Forest Soils†

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Additions of ammonium and non-ammonium salts inhibit atmospheric methane consumption by soil at salt concentrations that do not significantly affect the soil water potential. The response of soils to non-ammonium salts has previously raised questions about the mechanism of ammonium inhibition. Results presented here show that inhibition of methane consumption by non-ammonium salts can be explained in part by ion-exchange reactions: cations desorb ammonium, with the level of desorption varying as a function of both the cation and anion added; differential desorption results in differential inhibition levels. Differences in the extent of inhibition among ammonium salts can also be explained in part by the effects of anions on ammonium exchange. In contrast, only minimal effects of cations and anions are observed in liquid cultures of *Methylosinus trichosporium* OB3b. The comparable level of inhibition by equinormal concentrations of NH_4Cl and $(NH_4)_2SO_4$ and the insensitivity of salt inhibition to increasing methane concentrations (from 10 to 100 ppm) are of particular interest, since both of these patterns are in contrast to results for soils. The greater inhibition of methane consumption for NH_4Cl than $(NH_4)_2SO_4$ in soils can be attributed to increased ammonium adsorption by sulfate; increasing inhibition by non-ammonium salts with increasing methane concentrations can be attributed to desorbed ammonium and a physiological mechanism proposed previously for pure cultures.

A number of factors, including gas transport, soil water content, water stress, and temperature, limit atmospheric methane consumption by soils (1, 6, 7, 9, 19, 26, 30, 31). In addition, nitrogen mineralization and ammonium constrain methanotrophic activity (see e.g., references 1, 2, 18, 20–22, 24, 25, 28, and 32). The effects of added ammonium are usually substantial and persistent (see, e.g., references 14, 15, 20–22, and 24). King and Schnell (20, 21) have proposed a model of ammonium inhibition based on the physiological characteristics of known methanotrophic bacteria. This model includes a parabolic inhibition response as a function of methane concentrations and is consistent with observations for forest soils.

Inhibition of methane consumption by non-ammonium salts has also been observed in field and laboratory studies (see, e.g., references 1, 8, 12, 16, and 17), raising questions about the specificity of ammonium and the mechanism of ammonium inhibition. To address these questions, we have compared the responses of a methanotrophic culture (*Methylosinus trichosporium* OB3b) and atmospheric methane consumption by soils to a variety of ammonium and non-ammonium salts. *M. trichosporium* OB3b has been used previously as a model for understanding the physiology of ammonium inhibition. In this study, culture responses have been assayed at low headspace methane concentrations (10 to 100 ppm) and low to modest salt concentrations (0.5 to 8 mM). Salts have been added to soils at levels (e.g., $\leq$1 μmol g [fresh weight]$^{-1}$) that do not significantly affect the total soil water potential and that are comparable to those used in previous studies. We have also examined the effect of various salts on ammonium desorption and adsorption. Our results indicate that many cations desorb ammonium and inhibit methane consumption, as expected from ion-exchange chemistry. In addition, some anions (e.g., nitrate and sulfate) promote ammonium absorption while others (e.g., chloride) promote desorption, further complicating the interpretation of salt effects. The results also support previously proposed mechanisms for ammonium inhibition and suggest that non-ammonium salts cannot be used unequivocally as controls for solute addition.

MATERIALS AND METHODS

Culture assays. *M. trichosporium* OB3b was grown in batch culture with Higgins nitrate mineral salts (NMS) as described previously (see, e.g., references 19 and 21). The cells were harvested by centrifugation (10,000 × *g* at 4°C) after reaching an absorbance at 600 nm of 0.2 to 0.3 (early log phase), washed twice with 10 mM phosphate buffer, and resuspended in a modified NMS medium containing no NaCl. Replicate 100-ml cultures were incubated in sealed 500-ml Erlenmeyer flasks with rotary shaking (200 rpm) at 30°C with headspace methane concentrations of 10 or 100 ppm (about 14.7 and 147 nM, respectively) and various concentrations of either NaCl, KCl, or NH_4Cl (0, 0.5, 2.0, or 8.0 mM); $(NH_4)_2SO_4$ was added to parallel cultures at 0, 0.25, 1.0, or 4 mM. Methane uptake was determined by removing headspace subsamples (0.3 cm^3) with a needle and syringe at intervals for assay by flame ionization gas chromatography (21). Uptake rate constants for duplicates of each treatment were estimated from a regression analysis of the exponential decrease in methane concentration over time.

Soil analyses. The effect of various ammonium and non-ammonium salts on atmospheric methane consumption was determined with sieved soils (2-mm mesh) from the 6- to 10-cm layer of 6.5-cm-inner-diameter cores obtained from a mixed coniferous-hardwood forest at the Darling Marine Center. The 6- to 10-cm layer is the most active for methane uptake; the site has been characterized previously (1, 25, 26). Soil samples (10 g [fresh weight]) were transferred to jars with a headspace of about 110 cm^3. The soil water contents varied between 25 and 30%, a range previously shown to represent a broad optimum for methane consumption (26); the water contents were determined by drying soils for 24 h at 105°C. Salts dissolved in deionized water were added to replicate jars by carefully pipetting 1-ml volumes onto soils and gently mixing them. The final salt concentrations were 1 μmol g (fresh weight)$^{-1}$ for cations unless otherwise indicated. Deionized water with no added salts served as a control. The jars were sealed with butyl rubber stoppers that did not release methane or other organic gases. For most assays, the jars contained atmospheric methane (1.7 to 1.8 ppm; equivalent to 2.5 to 2.6 nM in soil solution), but in some cases methane was

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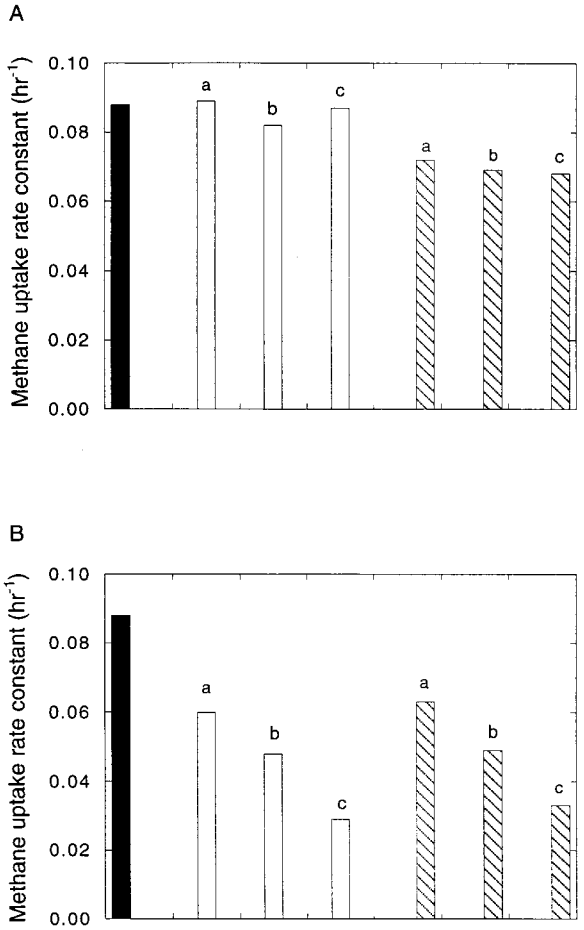

FIG. 1. (A) Methane uptake rate constants for *M. trichosporium* OB3b incubated with 10 ppm of methane and without added salts (solid bar) or with NaCl (open bars; a, 0.5 mM; b, 2 mM; c, 8 mM) or KCl (hatched bars; a, 0.5 mM; b, 2 mM; c, 8 mM). (B) Methane uptake rate constants for *M. trichosporium* OB3b incubated with 10 ppm of methane and without added salts (solid bar) or with NH_4Cl (open bars; a, 0.5 mM; b, 2 mM; c, 8 mM) or $(NH_4)_2SO_4$ (hatched bars; a, 0.25 mM; b, 1 mM; c, 4 mM). Data are means of duplicate determinations; the range for duplicates was less than 10% of the mean.

added to give initial headspace concentrations of 100 ppm. Methane uptake was determined by removing headspace subsamples (0.3 cm^3) with a needle and syringe for processing as above. Uptake rate constants were estimated from triplicate determinations for each treatment by regression analysis of the exponential decrease in methane concentration over time.

The effect of inorganic salts on ammonium desorption was determined by adding salt solutions to 10 g (fresh weight) of soil (final concentrations, 1 μmol of N g [fresh weight] of soil^{-1}). The soils were equilibrated for 1 to 2 h, and then ammonium was extracted by adding deionized water (1 ml g [fresh weight] of soil^{-1}). The soil slurry was vortexed briefly and then centrifuged. Supernatant ammonium concentrations were assayed colorimetrically (3). Similarly, ammonium salts were added to a parallel set of soils that were extracted to determine the extent to which ammonium salt counterions affect adsorption.

Ammonium desorption and adsorption were also examined by suspending 50 g (fresh weight) of sieved soil from the 6- to 10-cm depth in 200 ml of deionized water. The suspension was mixed continuously with a magnetic stirrer while concentrated solutions of LiCl, KCl, or CsCl were added incrementally in fixed volumes to increase the ionic strength. Subsamples of the suspension (2 ml) were obtained after each incremental addition of salt for the ammonium assay as described above.

The soil water potential was measured with a Wescor dew point psychrometer as described by Schnell and King (26). The total water potential was calibrated by using a series of NaCl solutions with known molality. Molality was converted to potential by using the following relationship: $\psi_s = RT \ln (a_w)$, where a_w is the weight-based mole fraction of water in a solution, corrected for nonideal solute behavior.

RESULTS

Cultures. Methane uptake by *M. trichosporium* OB3b did not differ over a range of added NaCl or KCl concentrations from 0.5 to 8 mM in NMS (Fig. 1). However, uptake was consistently higher by about 10% for cultures with NaCl and lower by about 10% for cultures with KCl relative to controls. In contrast, NH_4Cl was inhibitory relative to the alkaline metal salts and unamended controls (Fig. 1). While uptake rate constants for KCl-treated cultures were slightly lower than for those treated with NaCl, no significant differences were observed between the level of inhibition for equinormal concentrations of NH_4Cl and $(NH_4)_2SO_4$. The results for cultures incubated with 100 ppm of methane and either KCl or NaCl were similar to those for cultures incubated with 10 ppm of methane (data not shown). The rate constants at 100 ppm for NaCl and KCl treatments were 93.4 and 114.3% of the values at 10 ppm, respectively. In contrast, inhibition by either NH_4Cl or $(NH_4)_2SO_4$ was greater at 100 ppm than at 10 ppm (NH_4Cl, 48.9 and 38.3% inhibition for 100 and 10 ppm, respectively; $(NH_4)_2SO_4$, 43.2 and 36.1% inhibition at 100 and 10 ppm, respectively).

Soils. Salt additions at ≤1 μmol g (fresh weight) of soil^{-1} had little or no effect on the total soil water potential; all values were approximately ≥−0.05 MPa. Relative to deionized-water treatments, salt additions inhibited atmospheric methane consumption by sieved soils (Table 1). There was a trend for increasing inhibition from LiCl to CsCl (Fig. 2A), although the specific order of inhibition varied somewhat among different batches of soil; $MgCl_2$ was typically more inhibitory than the alkaline metal chlorides. The extent of inhibition by salts increased during the first 24 h after addition but was relatively stable for 4 days thereafter (data not shown). The extent of inhibition by sodium and potassium salts varied as a function of the counteranion added (Table 1; Fig. 2A), with the nitrate, phosphate, and sulfate salts being less inhibitory than the chlorides.

Inhibition by ammonium salts also varied as a function of the added counteranion, with the chloride salt being substantially more potent than the phosphate or sulfate salts (Fig. 2B). As

TABLE 1. Methane consumption by forest soils incubated with methane and various ammonium or non-ammonium salts[a]

Salt added[b]	Uptake (nmol g [dry wt]$^{-1}$ h^{-1}) (% of control) in presence of methane at[c]:	
	1.7 ppm	250 ppm
Expt A		
Control[d]	0.41	2.69
NaCl	0.14 (34.5)	0.62 (23.2)
Na_2SO_4	0.37 (89.5)	2.77 (102.7)
NaH_2PO_4	0.38 (93.3)	2.59 (96.3)
KCl	0.15 (35.5)	0.69 (25.8)
KNO_3	0.23 (55.5)	1.19 (44.2)
$MgCl_2$	0.13 (32.4)	0.74 (27.6)
Expt B		
Control	0.56	4.34
NaCl	0.30 (53.9)	0.50 (11.4)
NH_4Cl	0.29 (52.3)	0.39 (9.1)

[a] The methane was present at atmospheric concentrations (1.7 ppm) or at 250 ppm. The added salts were present at 1 μmol g (fresh weight)$^{-1}$ (0.5 μ g^{-1} for Na_2SO_4). The total soil water potential for all samples was >−0.01 MPa.

[b] Experiments A and B represent independent assays with similar soils.

[c] Results are means of triplicate determinations.

[d] Controls were treated with a volume of deionized water equal to that used for the salt additions.

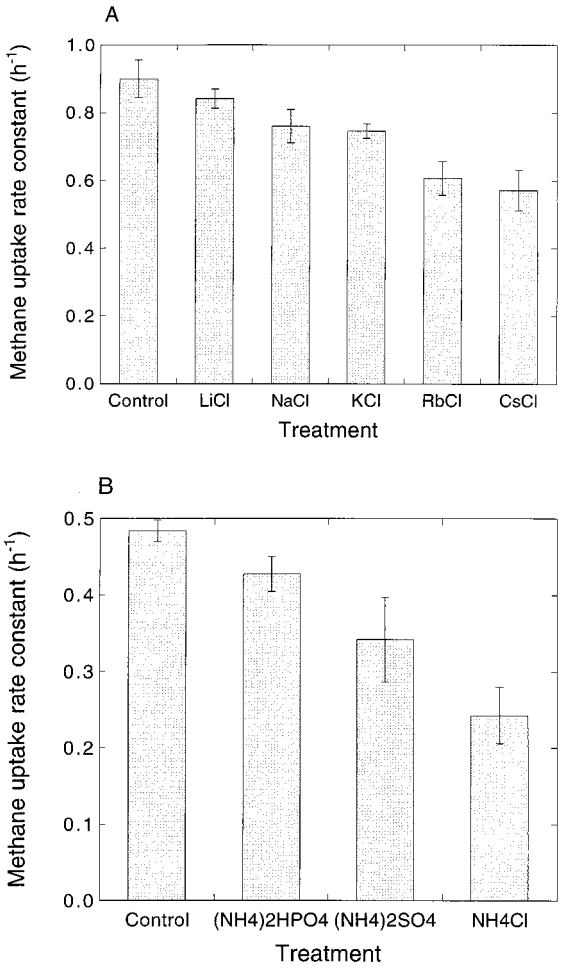

FIG. 2. (A) Atmospheric methane uptake rate constants for 10 g (fresh weight) of soil incubated with 2 μmol of the indicated salts g (fresh weight)$^{-1}$. Data are means of triplicate determinations ± 1 standard error. (B) Atmospheric methane uptake rate constants for 10 g (fresh weight) of soil incubated with 1 μmol of N from the indicated ammonium salts g (fresh weight)$^{-1}$. Data are means of triplicate determinations ± 1 standard error.

with the alkaline metal salts, ammonium inhibition increased to a maximum about 24 h after addition and remained relatively stable subsequently for NH_4Cl (data not shown). Although in some instances inhibition by various chloride salts slightly exceeded that by NH_4Cl, the extent of inhibition was typically similar for salts used at equal concentrations (Fig. 3).

Salt additions altered ammonium concentrations in aqueous extracts of sieved soils (Fig. 4). The concentrations tended to increase with increasing atomic number for the alkaline metal chloride series (Li to Cs). Deionized-water-extractable ammonium levels in soils treated with $MgCl_2$ exceeded those in soils treated with NaCl or $CaCl_2$; the concentrations in extracts from soils treated with sulfate or nitrate salts were consistently lower than those in extracts from soils treated with the analogous chloride salts (Fig. 4). In some instances, extracts from soils treated with sulfate and nitrate salts were not statistically different from extracts from untreated soils. An analysis of ammonium desorption in soil slurries revealed a similar pattern, with desorption increasing in the order Li < K < Cs (Fig. 5). Ammonium concentrations in soils extracts also depended on the counteranion used for ammonium additions. The chloride salt resulted in higher water-extractable concentrations than did either the phosphate or sulfate salt, for which concentrations were similar (Fig. 4).

DISCUSSION

Rapid inhibition of atmospheric methane consumption by ammonium has been well documented (see, e.g., references 1, 2, 4, 5, 10, 15, 24, and 28) and attributed to the combined effect of substrate competition at the level of methane monooxygenase and toxicity of nitrite generated intracellularly as an ammonium oxidation end product (see, e.g., references 20, 21, and 25). However, the fact that non-ammonium salts inhibit methane consumption has led some researchers to speculate that ammonium inhibition may be due all or in part to nonspecific ionic or solute effects (see, e.g., references 8, 16, and 17). Non-ammonium salts have also been proposed as essential controls for partitioning ammonium inhibition between the nonspecific and methane monooxygenase-related mechanisms (17, 23). Results presented here support an enzyme-based model of ammonium inhibition and indicate that non-ammonium salts cannot be used unambiguously as controls to partition inhibition among multiple mechanisms.

Non-ammonium salts are unsuitable as controls for at least two reasons. First, non-ammonium cations desorb ammonium in soils by ion exchange. Ammonium desorption in general and increasing desorption with increasing cation radius in particular (Fig. 4 and 5) are well-known phenomena (see e.g., references 11 and 27) that apparently have not been considered in previous salt inhibition assays. Likewise, the effect of anions on ammonium exchange (Fig. 4) has not been considered previously. Differences in ammonium concentrations in soil solutions as a function of added anions most probably reflect the differential stability of various ion pairs, with increasing stability decreasing the ionic character of a given cation-anion series (29).

The collective observations described both here and previously provide a basis for understanding the effects of ammonium and nonammonium salts on soil methane uptake. Uptake is lowest for cation and anion pairs that promote ammonium desorption (e.g., KCl and $MgCl_2$), and greatest for pairs that limit desorption (e.g., LiCl and Na_2SO_4); (Table 1; Fig. 2). Likewise, uptake is lowest for ammonium salts that are minimally adsorbed (e.g., chloride) and greatest for sulfate and phosphate pairs that are more strongly adsorbed (Fig. 2). In contrast to the situation in soils, ammonium counterions do not affect inhibition in cultures (Fig. 1), since ion exchange is

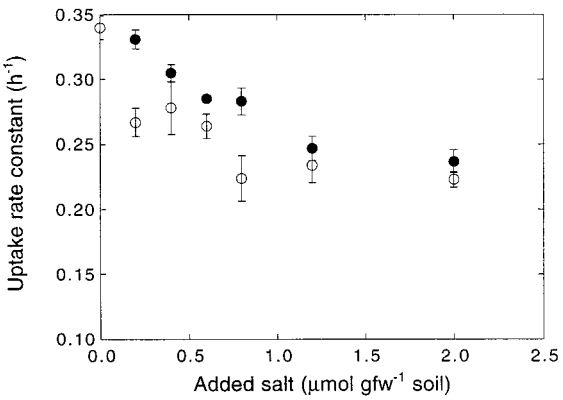

FIG. 3. Atmospheric methane uptake rate constants for 10 g (fresh weight) of soil incubated with various concentrations of NH_4Cl (●) or KCl (○). Data are means of triplicate determinations ± 1 standard error.

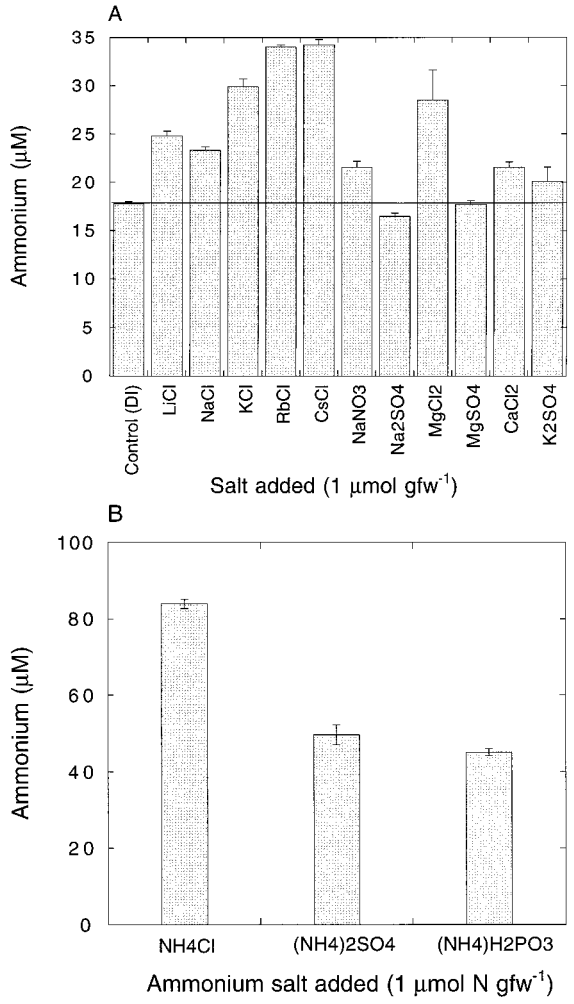

FIG. 4. (A) Ammonium concentrations in aqueous extracts (1 ml of deionized water g [fresh weight] of soil^{-1} for soils treated with the indicated salts. (B) As in panel A but for additions of ammonium salts to soils. Data are means of triplicate determinations ± 1 standard error.

relatively unimportant in the distribution of ions in liquid media. The response of salt-amended soils to elevated methane concentrations (Table 1) can also be understood best in the context of ammonium desorption. Increased inhibition by non-ammonium salts at elevated methane concentrations occurs regardless of the cation-anion pair added to Maine forest soils, although the specific levels of inhibition at elevated methane concentrations vary among salts. This response is consistent with the combined effect of desorption and the mechanism for ammonium inhibition described previously (25).

Non-ammonium salts are also inappropriate controls for ammonium addition because they may have inhibitory effects unrelated to those of ammonium. For example, high concentrations of NaCl or KCl decrease methane oxidation in cultures due to physiological stresses that do not specifically involve methane monooxygenase (25). Furthermore, *M. trichosporium* OB3b consistently oxidizes less methane in media with dilute concentrations (0.5 to 8 mM) of KCl rather than NaCl. This suggests that methanotrophs may be differentially sensitive to potassium and perhaps to other cations. This sensitivity involves a mechanism different from that for ammonium, since ammonium inhibition in cultures increases with increasing methane concentrations from 10 to 100 ppm (Fig. 1) (21) while no such effect is observed with non-ammonium salts.

Since the responses of soils to certain ammonium and non-ammonium salts (e.g., KCl and NH_4Cl [Fig. 3] and NaCl-RbCl [Fig. 2]) are similar while chloride salts are more inhibitory than their sulfate, nitrate, or phosphate analogs (Table 1; Fig. 2), it is tempting to speculate that chloride has an additional, complicating toxicity of its own (13). Although this possibility deserves further attention, the culture data presented here provide no indication that chloride might be inhibitory per se. No differences were observed for *M. trichosporium* OB3b incubated with increasing chloride concentrations (as the sodium or potassium salt) from 0.5 to 8 mM; equinormal NH_4Cl and $(NH_4)_2SO_4$ were similarly inhibitory (Fig. 1). Furthermore, there is no obvious mechanism by which chloride alone could account for increased inhibition in salt-amended soils incubated with increasing methane concentrations from 1.7 to 250 ppm (Table 1); this phenomenon is most reasonably attributed to an effect of added or desorbed ammonium.

Although the data here raise doubts about the efficacy of non-ammonium salts as controls for ammonium addition to soils, the relative response of atmospheric methane consumption to a variety of salts remains interesting in the context of inputs via wet deposition. Sodium and ammonium typically dominate wet deposition, usually dwarfing potassium (33); thus, potassium salts might be ill suited for assays involving nonagricultural soils. With the exception of the situation for coastal sites influenced by marine weather systems, sulfate and nitrate dominate wet deposition, with chloride usually being a minor component (about 10%) and perhaps unrepresentative as a counterion. However, in areas such as coastal Maine, chloride accounts for about 50% of the anions assayed routinely by the National Acid Deposition and Precipitation program (extensive data on the geographical and temporal distribution of acid rain chemistry from the NADP/NTN program are available online from the U.S. Geological Survey at http://btdqs.usgs.gov/acidrain/); in this and similar cases, chloride salts might prove more representative than other choices.

Finally, it should be emphasized that comparisons of the responses of different soils to ammonium and non-ammonium salts must be tempered by recognition of the enormous variation that exists among soils in basic physical-chemical parameters (e.g., pH, water content, ammonium content, mineralol-

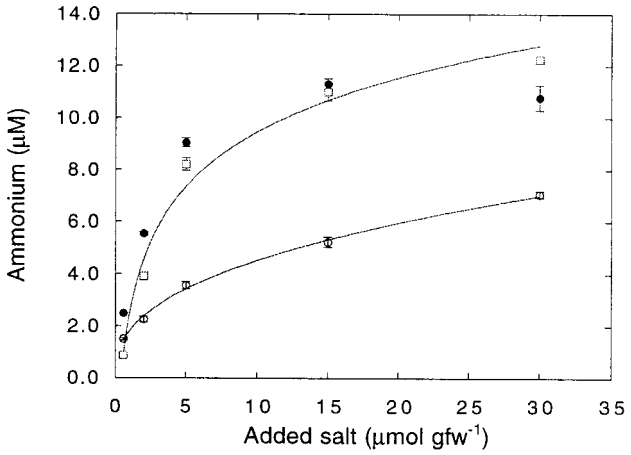

FIG. 5. Ammonium concentrations in a soil slurry (250 ml of deionized water, 50 g [fresh weight] of soil) to which was progressively added increasing concentrations of LiCl (○), KCl (□), or CsCl (●). Data are means of triplicate determinations ± 1 standard error.

ogy, organic content, and dynamics of ammonia oxidation) that determine ion exchange and soil solution ammonium concentrations. Although physical and chemical diversity may complicate comparisons among soils, it is apparent that ammonium is an important determinant of current and future variations in atmospheric methane consumption by soils. Global eutrophication and conversion of forests and grasslands to agricultural use will continue to decrease the relative significance of the soil methane sink, thereby intensifying climate change. Ammonium inputs and dynamics in soils, as shown in the physiology of methanotrophic bacteria, will be a key component of these changes, with or without additional effects of non-ammonium salts.

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