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Differential Inhibition by Allylsulfide of Nitrification and Methane Oxidation in Freshwater Sediment

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Addition of nitrapyrin, allylthiourea, C_2H_2, and CH_3F to freshwater sediment slurries inhibited CH_4 oxidation and nitrification to similar extents. Dicyandiamide and allylsulfide were less inhibitory for CH_4 oxidation than for nitrification. Allylsulfide was the most potent inhibitor of nitrification, and the estimated 50% inhibitory concentrations for this process and CH_4 oxidation were 0.2 and 121 μM, respectively. At a concentration of 2 μM allylsulfide, growth and CH_4 oxidation activity of *Methylosinus trichosporium* OB3b were not inhibited. Allylsulfide at 200 μM inhibited the growth of *M. trichosporium* by approximately 50% but did not inhibit CH_4 oxidation activity. Nitrite production by cells of *M. trichosporium* was not significantly affected by allylsulfide, except at a concentration of 2 mM, when growth and CH_4 oxidation were also inhibited by about 50%. Methane monooxygenase activity present in soluble fractions of *M. trichosporium* was not inhibited significantly by allylsulfide at either 200 μM or 2 mM. These results suggest that the partial inhibition of CH_4 oxidation in sediment slurries by high allylsulfide concentrations may be caused by an inhibition of the growth of methanotrophs rather than an inhibition of methane monooxygenase activity specifically. We conclude that allylsulfide is a promising tool for the study of interactions of methanotrophs and nitrifiers in N cycling and CH_4 turnover in natural systems.

Methanotrophic bacteria, which grow on CH_4 as their sole carbon and energy source, and nitrifying bacteria, which derive their energy from the oxidation of NH_4^+ to NO_2^-, occupy similar niches in natural environments: close to the oxic/anoxic interface, receiving CH_4 and NH_4^+ from the anaerobic mineralization of organic matter. The main oxidative pathways in these bacteria are somewhat similar, involving the oxidation of their substrates by monooxygenases followed by further oxidation of the product by an oxidoreductase or a dehydrogenase (2).

Interactions between nitrifiers and methanotrophs in natural systems such as freshwater sediment are complex and poorly understood so far. It is known that methanotrophs may oxidize NH_4^+ to NH_2OH via their methane monooxygenase (MMO) (reviewed in reference 2) and further oxidize NH_2OH to NO_2^- by a unique hydroxylamine oxidoreductase different from that of nitrifying bacteria (27). Also, nitrifiers can cooxidize CH_4 via their ammonia monooxygenase (AMO). However, in neither case can the bacteria grow on their respective alternative substrate. Competition for O_2 and NH_4^+ between methanotrophs and nitrifiers may also be a significant cause of suppression of nitrifying bacteria by methanotrophs in soil (11) and in sediment (19). The lack of a differential inhibitor that blocks one activity without affecting the other has prevented the assessment of the relative importance of these interactions for N cycling and CH_4 turnover in situ.

Most classical inhibitors of nitrification (nitrapyrin, allylthiourea, and C_2H_2) also inhibit CH_4 oxidation (2). CH_3F was recently reported as an inhibitor of CH_4 oxidation (16, 17) but was also found later to inhibit nitrification (13). Dicyandiamide (DCD) is a nitrification inhibitor which has not yet been tested specifically on CH_4 oxidation except in a field study of CH_4 emission in flooded rice fields (8). Allylsulfide (diallylsulfide) was recently reported to be a mechanism-based inhibitor of the AMO of *Nitrosomonas europaea* (7). It is also a competitive inhibitor of another monooxygenase, cytochrome P-450 (3). It is not known whether allylsulfide affects (i) nitrification or CH_4 oxidation by mixed populations in freshwater sediments or (ii) axenic cultures of methanotrophic bacteria.

Here we report the effect on nitrification and methane oxidation in polluted freshwater sediment of nitrapyrin, allylthiourea, C_2H_2, CH_3F, DCD, and allylsulfide. Allylsulfide inhibited nitrification at concentrations almost 3 orders of magnitude lower than those causing similar inhibition of methane oxidation. The much smaller effect of allylsulfide on methane oxidation in slurries was supported by results of experiments with axenic cultures and soluble fractions of *Methylosinus trichosporium* OB3b. These experiments indicated that allylsulfide may affect the growth of *M. trichosporium* by a mechanism other than a specific inhibition of MMO activity.

MATERIALS AND METHODS

Experiments with sediment. Mixed sediment from a profundal (23-m-deep) site (station 906) was sampled from Hamilton Harbour, Ontario, Canada, by an Ekman dredge (21). Sediment was stored in bags in a cold room (4 to 10°C) before use.

Sediment slurries (500 ml) with a 1:10 sediment-to-water ratio were prepared by diluting sediment with distilled water containing $(NH_4)_2SO_4$ (final concentration, 2 mM) in each of two 2-liter Erlenmeyer flasks. Each flask was capped with a rubber stopper fitted with a small glass tube closed with an 11-mm sleeve rubber septum (Wheaton, Millville, N.J.). In one flask, 21 ml of pure CH_4 (Matheson, Montreal, Canada) was added by syringe to achieve a 1-kPa partial pressure after the withdrawal of an equal volume of air. These slurries were preincubated on a gyratory shaker (250 rpm) at 25°C for 2 days. During the preincubation, headspace CH_4, CO_2, and N_2O were monitored by gas chromatography. Pore water samples (1.5 ml) were withdrawn, microcentrifuged (15,000 × *g* for 10 min), and frozen (−20°C) until determination of NH_4^+, NO_2^-, and NO_3^- concentrations.

After the preincubation, inhibitors were added to aliquots of the slurries (20 ml) in 125-ml Erlenmeyer flasks, which were closed with silicone rubber-filled red rubber serum stoppers (Suba-Seals; William Freeman and Co., Barnsley,

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United Kingdom) in the presence and absence of 1 kPa of CH_4, corresponding to the presence and absence of CH_4 during the preincubation. Because nitrapyrin, allylthiourea, and allylsulfide are not very soluble in water, they were dissolved in dimethyl sulfoxide (DMSO) and 20 μl was added to the slurries. To allow comparison with other inhibitors, DCD was also dissolved in DMSO. DMSO was previously found to be innocuous to methanotrophs (24) and nitrifying bacteria (18), and under the conditions used in our study, it was found to have no significant effect on CH_4 oxidation or nitrification. DMSO was found to interfere with the phenylhypochlorite reaction for the determination of NH_4^+: with the DMSO final concentration used, we obtained a 90% recovery of the same NH_4^+ concentration in distilled water only. Gaseous inhibitors (C_2H_2, CH_3F) and CH_4 were added by syringe, as described for the preincubation. Flasks were then incubated under the same conditions as for the preincubation. Gases (CH_4, CO_2, and N_2O) in the headspace were determined by gas chromatography at the beginning and at desired intervals up to 48 h after addition of inhibitors. After each gas chromatography analysis, headspaces were changed by opening the flasks for 15 min. The CH_4 concentration (1 kPa) was reestablished where needed. Each treatment was done in triplicate. At least 90% of the triplicate flasks at any time during the course of these experiments had a standard error of the mean of no more than 10% of the value of the mean. The concentrations of allylsulfide tested were 0.1, 5, 10, 50, 100, 200, 250, 500, 1,000, and 2,000 μM. The concentrations of DCD were 0.2, 2, 3, 4, and 8 mM. The concentrations of other inhibitors tested were as in Tables 1 and 2.

Experiments with *M. trichosporium* OB3b. Cultures of *M. trichosporium* OB3b (obtained from R. Whittenbury via T. Yoshinari) were grown on 50 ml of nitrate mineral salts (NMS) medium (final copper concentration, 2.4 nM) (6) in 125-ml Erlenmeyer flasks with 10 to 20% CH_4 on a gyratory shaker (250 rpm) at 25°C. For nitrification experiments, *M. trichosporium* was grown on ammonium mineral salts (AMS) medium (6). Phosphate buffer (6) was added to a final concentration of 20 mM in AMS to avoid too great an acidification of the medium following NH_4^+ oxidation. Culture purity was periodically confirmed by plating 0.1 ml of cell suspension on nutrient agar plates (Difco, Detroit, Mich.).

Inhibition assays were carried out in 50-ml sidearm flasks containing sterile NMS or AMS medium (7.5 ml). Each flask was inoculated with 2.5 ml of a late-log-phase to early-stationary-phase bacterial suspension having an A_{430} of 0.2, giving a final A_{430} of 0.05. Allylsulfide was dissolved in DMSO, and 20 μl was added to each flask to obtain the desired final concentrations. DMSO alone was added to control flasks. Flasks were capped with sterile silicone rubber-filled serum stoppers. Pure methane was added through a syringe filter (0.22 μm) to obtain a partial pressure of 1 kPa. Growth was monitored by measuring A_{430} on a spectrophotometer (Spectronic 21; Bausch & Lomb) (12). The headspace was monitored for CH_4 and CO_2 as a measure of CH_4 oxidation activities. Nitrous oxide was measured in headspaces of flasks containing AMS medium. Cell suspensions (1.5 ml) were microcentrifuged (15,000 × *g* for 10 min), and the supernatants were frozen until the determination of nitrogenous ions.

Experiments with soluble fractions of *M. trichosporium*. Cells of *M. trichosporium* OB3b were grown in 2 liters of NMS medium in a 6-liter Erlenmeyer flask and in 500 ml of NMS medium in each of three 2-liter flasks with a CH_4-to-air ratio of 30:70, continuously agitated either by a magnetic bar or on a gyratory shaker (250 rpm), at 25°C. Cells in late log phase were harvested after 5 days of incubation when densities were 0.4 A_{430} unit. Cells were first centrifuged (4,000 × *g* for 30 min at 4°C) and washed twice (23), first in 140 ml and then in 60 ml of 25 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 7.5) (4). Cells were then resuspended in 20 ml of MOPS buffer and sonicated five times for 5 min each in a cup horn sonicator (W-220F; Heat Systems Ultrasonics Inc., Plainview, N.Y.) at power 8 with 5 min of cooling between the cycles. The cell extract was then centrifuged (17,000 × *g* for 10 min at 4°C) to eliminate cell debris and unbroken cells. The supernatant was centrifuged (250,000 × *g* for 90 min at 4°C) to yield a supernatant (soluble fraction) and a pellet (membrane fraction), which was resuspended in 5 ml of MOPS buffer by using a glass homogenizer.

The MMO activity of each fraction was then assayed by a polarographic procedure with furan as substrate (4, 26) and was located in the soluble fraction. MOPS buffer (1.0 ml) was put in a Rank oxygen electrode chamber (3.0 ml). Soluble fraction (2.0 ml) and 100 μl of furan-saturated MOPS buffer (206 μM furan) were added to the chamber. The chamber was closed with a capillary bore plug. A stable baseline was established, and the hydroxylation reaction was initiated by the addition of 10 μl of NADH (500 mM in 10 mM Tris [pH 8.0]). Nonspecific oxidation was determined by adding only NADH to the soluble fraction without furan. For allylsulfide inhibition assays, the soluble fraction was preincubated (5 min) with 10 μl of a stock solution of allylsulfide in DMSO before the addition of substrate and NADH. The significance of the inhibition was assessed by an unpaired and one-tailed *t* test.

Analytical procedures. CO_2, CH_4, and N_2O concentrations were determined by gas chromatography (19, 21). Nitrogenous ion concentrations were determined by automated colorimetric methods on an autoanalyzer (19). Because NO_2^- did not accumulate significantly in sediment slurries of Hamilton Harbour (19), we routinely determined only the sum of NO_2^- + NO_3^- and considered the NO_2^- to be negligible. To further confirm the presence of NO_3^- in AMS medium during methanotrophic nitrification experiments, NO_3^- was also determined by ion capillary electrophoresis (1).

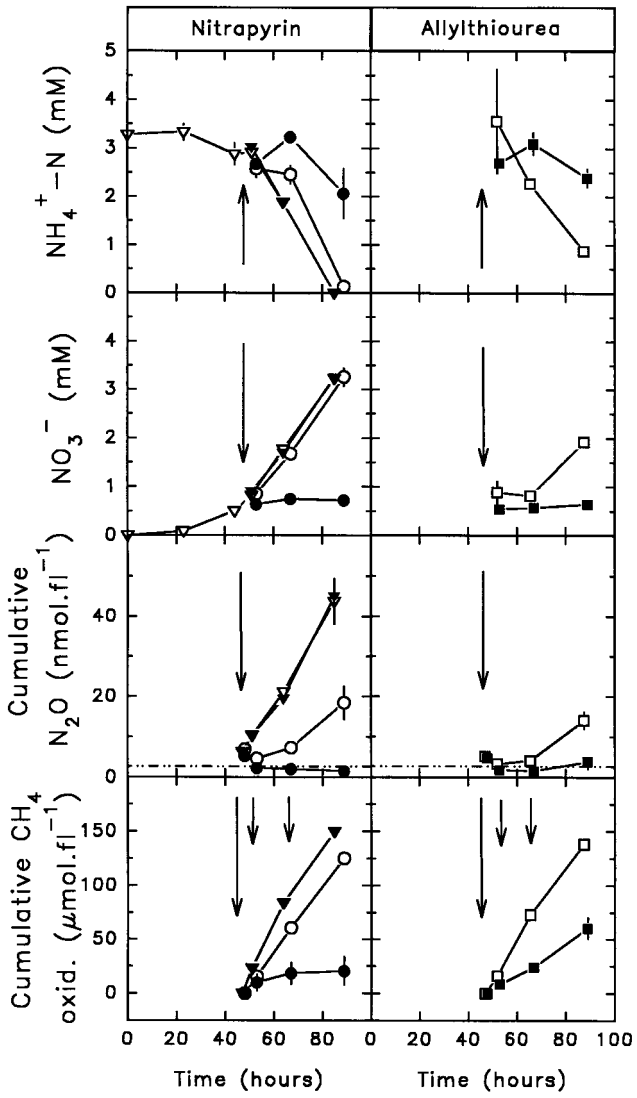

FIG. 1. Inhibition by nitrapyrin and allylthiourea of nitrification and CH_4 oxidation in sediment slurries from Hamilton Harbour incubated at 25°C with shaking at 250 rpm. Symbols: ○, 5 μM nitrapyrin; ●, 50 μM nitrapyrin; □, 5 μM allylthiourea; ■, 50 μM allylthiourea; ▼, +DMSO control; ▽, −DMSO control. Each datum point is the average of results from triplicate flasks ± 1 standard error of the mean. Cumulative N_2O represents the sum of the N_2O amounts produced over the total assay period. Cumulative CH_4 oxidized is the sum of the amounts of CH_4 consumed over the total assay period. The dotted line represents the average N_2O concentration in ambient air. The large arrows indicate the addition of inhibitors, and the small arrows indicate change of headspaces and reestablishment of the CH_4 concentrations.

RESULTS

Sediment slurries. Nitrapyrin suppressed CH_4 oxidation and NO_3^- and N_2O production to about the same extent (Fig. 1; Table 1). Similar results were obtained with allylthiourea (Fig. 1; Table 1). The lowest concentration tested (5 μM) for these two inhibitors gave only partial inhibition of each of the three processes. Acetylene (0.1 kPa or 41 μM) and CH_3F (0.1 kPa or 59 μM) inhibited CH_4 oxidation by 79 and 98% and NO_3^- production by 105 and 59%, respectively. Nitrous oxide production in the sediment slurries was also completely inhibited by both inhibitors (data not shown). In contrast, DCD and allylsulfide were more inhibitory of nitrification than of CH_4 oxidation (Table 2). At 2 mM DCD and 200 μM allylsulfide,

TABLE 1. Effect of nitrapyrin and allylthiourea on CH_4 oxidation and nitrification in slurries of profundal Hamilton Harbour freshwater sediment[a]

Inhibitor	+CH_4 (1 kPa)						−CH_4 (0.001 kPa)			
	CH_4 oxidation		NO_3^- production		N_2O production		NO_3^- production		N_2O production	
	μmol/flask/h	Inhibition (%)	μM/h	Inhibition (%)	nmol/flask/h	Inhibition (%)	μM/h	Inhibition (%)	nmol/flask/h	Inhibition (%)
None (control)	5.82 (0.62)		69.8 (3.0)		0.978 (0.043)		68.7 (2.0)		0.552 (0.049)	
Nitrapyrin (5 μM)	3.11 (0.24)	47	67.0 (3.0)	4	0.280 (0.071)	71	53.6 (8.8)	22	0.065 (0.023)	88
Nitrapyrin (50 μM)	2.00 (1.06)	66	2.1 (1.3)	97	0.000 (0.021)	100	−1.4 (2.6)	102	0.000 (0.005)	100
Allylthiourea (5 μM)	3.22 (0.65)	45	30.7 (6.9)	56	0.208 (0.047)	79	44.8 (3.3)	35	0.075 (0.033)	86
Allylthiourea (50 μM)	1.74 (0.36)	70	2.9 (1.8)	96	0.000 (0.035)	100	0.7 (0.5)	99	0.000 (0.010)	100

[a] Rates were calculated by linear regression of concentrations in triplicate flasks with incubation time. CH_4 oxidation rates were calculated within the initial 6 to 8 h. NO_3^- production rates were calculated over the 48-h period of the inhibition assay. Rates of N_2O production were calculated as cumulative N_2O over the 48 h of the inhibition assay. The standard error of the slope is given in parentheses. Percent inhibition was calculated as [1 − (rate inhibited/rate control)] × 100.

CH_4 oxidation was unaffected or only slightly affected whereas nitrification was completely suppressed.

This differential inhibition of CH_4 oxidation and nitrification by DCD was seen clearly in the DCD concentration range of about 2 to 4 mM (Fig. 2). A much greater differential effect was found with allylsulfide, which gave 50% inhibition of the CH_4 oxidation activity at a concentration (121 μM) which is about 500 times higher than the allylsulfide concentration required for 50% inhibition of nitrification (0.2 μM) (Fig. 3). Nitrous oxide production in aerobic sediment slurries was inhibited by DCD and allylsulfide to the same extent as was nitrification (Table 2).

***M. trichosporium*.** Since the effect of allylsulfide on methanotrophs is unknown, we tested its effect on axenic cultures of *M. trichosporium*, a group II methanotroph. Allylsulfide at a concentration of 2 μM had no effect on CH_4 oxidation activity or growth of *M. trichosporium* in NMS medium (Fig. 4). Growth was reduced by 50% at 200 μM allylsulfide, but, interestingly, no significant effect on CH_4 oxidation was observed at this concentration. We found complete inhibition of growth and CH_4 oxidation only at 2 mM allylsulfide.

To test the effect of allylsulfide on methanotrophic nitrification, we also grew the cells in AMS medium (Fig. 5). Growth and CH_4 oxidation in this medium were slower than in NMS, and allylsulfide was less inhibitory under these conditions. The highest concentration of allylsulfide tested (2 mM) reduced growth and activity by about 50%. No significant effect on growth or CH_4 oxidation was observed for allylsulfide concentrations of 2 or 200 μM. Production of NO_2^- and NO_3^- was observed. Nitrite production was not significantly affected by allylsulfide, except at the highest concentration (about 50% inhibition at 2 mM). Allylsulfide inhibited nitrate production by 36% (2 μM) to 82% (2 mM).

Soluble and membrane fractions of *M. trichosporium* were prepared and tested for MMO activity. Significant MMO activity (when NADH-dependent O_2 consumption is subtracted) in the soluble fraction was detected (Table 3). Allylsulfide did not significantly inhibit the MMO activity, as found by a *t* test.

DISCUSSION

Well-known inhibitors of chemolithotrophic nitrification (nitrapyrin, allylthiourea, and C_2H_2) suppressed both CH_4 oxidation and nitrification in Hamilton Harbour sediment slurries. Our results are consistent with previous reports (2). Methyl fluoride also inhibited CH_4 oxidation and nitrification to the same extent as and at concentrations similar to those already reported (13, 16, 17).

Evaluation of DCD as a nitrification inhibitor in soil indicated 84 to 100% inhibition with addition of 100 μg of DCD·g of soil^{-1} (or 4.0 mM) and up to 80% inhibition with addition of 10 μg·g of soil^{-1} (400 μM) (9). This compares well with the inhibition of nitrification by DCD in freshwater sediment reported here. The minimal inhibition (≤18%) of CH_4 oxidation observed in freshwater sediment by 2 mM DCD clearly indicates the potential of this compound as a differential inhibitor. Further physiological studies are needed to establish the reasons for this differential sensitivity to DCD between methanotrophs and nitrifiers.

Compared with DCD, allylsulfide appears much more interesting as a differential inhibitor because (i) much lower concentrations of allylsulfide (3 to 4 orders of magnitude) are required to completely inhibit nitrification and (ii) the difference in sensitivity to allylsulfide between CH_4 oxidation and nitrification is greater (2 to 3 orders of magnitude) than the difference in sensitivity of these processes to DCD (1 order of

TABLE 2. Effect of DCD and allylsulfide on CH_4 oxidation and nitrification in Hamilton Harbour sediment slurries[a]

Inhibitor	+CH_4 (1 kPa)						−CH_4 (0.001 kPa)			
	CH_4 oxidation		NO_3^- production		N_2O production		NO_3^- production		N_2O production	
	μmol/flask/h	Inhibition (%)	μM/h	Inhibition (%)	nmol/flask/h	Inhibition (%)	μM/h	Inhibition (%)	nmol/flask/h	Inhibition (%)
None (control)	4.07 (0.75)		45.9 (9.0)		0.844 (0.151)		67.4 (7.6)		0.562 (0.094)	
DCD (200 μM)	5.35 (0.43)	0	31.3 (4.6)	32	0.294 (0.194)	65	42.2 (5.1)	37	0.265 (0.135)	53
DCD (2 mM)	4.70 (0.35)	0	−4.8 (1.6)	110	0.042 (0.022)	95	−2.6 (2.1)	104	0.000 (0.009)	100
Allylsulfide (200 μM)	3.20 (0.75)	22	−2.8 (1.2)	106	0.017 (0.007)	98	−2.2 (2.2)	103	0.000 (0.009)	100
Allylsulfide (2 mM)	0.80 (0.48)	80	−3.7 (3.3)	108	0.016 (0.007)	98	−5.5 (1.8)	108	0.000 (0.009)	100

[a] For details, see Table 1, footnote *a*.

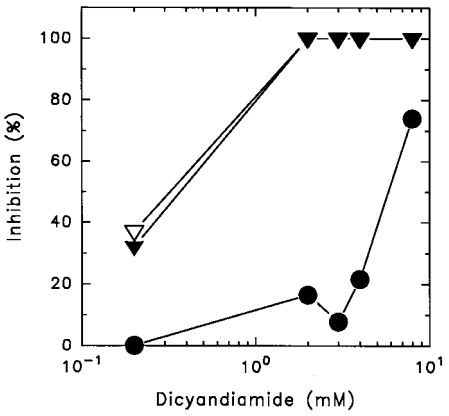

FIG. 2. Inhibition by DCD of nitrification (NO_3^- production in the presence [▼] and absence [▽] of CH_4) and CH_4 oxidation (●) in sediment slurries from Hamilton Harbour incubated at 25°C with shaking at 250 rpm. Datum points are averages of results from triplicate flasks.

magnitude). Although the low water solubility of allylsulfide may be a limitation when compared with DCD, the use of DMSO as a carrier, which appears to have little effect on the processes under study, may easily overcome this limitation. One should also note the greater efficiency of allylsulfide in completely suppressing nitrification and nitrification-dependent N_2O formation at a concentration of 5 μM when compared with classical nitrification inhibitors such as nitrapyrin and allylthiourea, which give only partial inhibition at such concentrations. This is also lower than the 40 μM allylsulfide giving an 80% inhibition of O_2 uptake by *Nitrosomonas europaea* in short-term (5-min) assays (7).

Methane oxidation in sediment slurries was less inhibited by allylsulfide than was nitrification. We calculated that 121 μM inhibited CH_4 oxidation by 50%. This observation was in general agreement with experiments on *M. trichosporium* showing that 200 μM and 2 mM allylsulfide inhibited growth by 50 and 100%, respectively. However, 200 μM allylsulfide, unlike in the slurry experiments, did not inhibit CH_4 oxidation by *M. trichosporium*. This discrepancy between slurry and pure-culture experiments may be explained if the dominant methanotroph in Hamilton Harbour sediment is more sensitive to allylsulfide than is *M. trichosporium*. An alternative explanation may be that CH_4 oxidation in the sediment slurries was growth dependent.

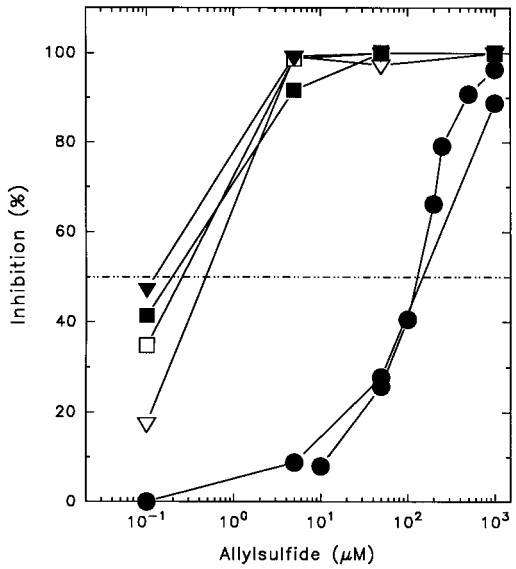

FIG. 3. Inhibition by allylsulfide of nitrification (NO_3^- production [▽, ▼], N_2O production [□, ■]) and CH_4 oxidation (●) in sediment slurries incubated at 25°C with shaking at 250 rpm. Open and solid symbols indicate incubation of flasks in the absence and presence of CH_4 (1 kPa), respectively. Data are averages of results from triplicate flasks. The two plots with the solid circles indicate independent experiments.

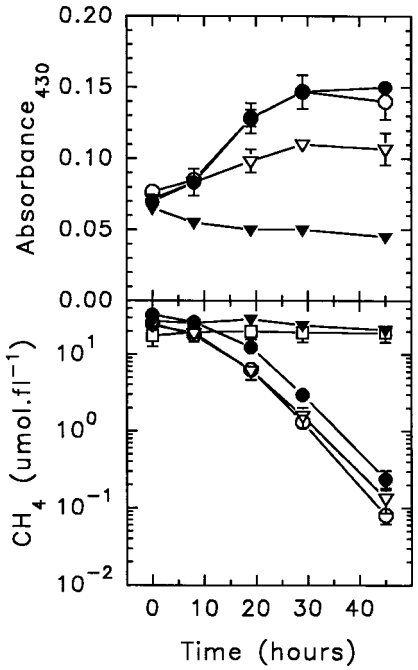

FIG. 4. Effect of allylsulfide on growth (A_{430}) and CH_4 oxidation by *M. trichosporium* OB3b in NMS medium with CH_4 (1 kPa) at 25°C with shaking at 250 rpm. Symbols: ○, no allylsulfide; ●, 2 μM; ▽, 200 μM; ▼, 2 mM. Uninoculated medium (□) was used as a blank. Data are averages of results from triplicate flasks (fl) ± 1 standard error of the mean.

In any case, our data on *M. trichosporium* suggest that allylsulfide does not inhibit CH_4 oxidation at the MMO stage but suppresses growth by inhibiting another process(es) which remains to be identified. Lack of transport of allylsulfide across the cell membrane could be an alternative explanation for this result. However, preliminary experiments with soluble fractions of cells of *M. trichosporium* suggest that allylsulfide at 200 μM and 2 mM does not significantly inhibit the soluble MMO activity. Allylsulfide has not been widely studied as an inhibitor, and it is therefore difficult to speculate about a possible candidate for the inhibition of growth of *M. trichosporium*. One example of an inhibitory effect of allylsulfide is that of the microsomal cytochrome P-450 of rat hepatic cells (3). Cytochromes P-450 form a large family of monooxygenase enzymes, widely distributed among mammals, in which they are involved in steroid hydroxylation (15). Several cytochromes P-450 have been isolated from a number of prokaryotic species (14, 25), but none has been reported yet for a methanotrophic bacterium. Since cytochrome P-450 plays a role in maintaining membrane integrity or in catabolism in prokaryotes (15), such an enzyme, if present in *M. trichosporium*, would be a good candidate to explain the inhibition of growth of these cells by 200 μM and 2 mM allylsulfide. The fact that allylsulfide did not inhibit the growth of *M. trichosporium* in AMS medium as

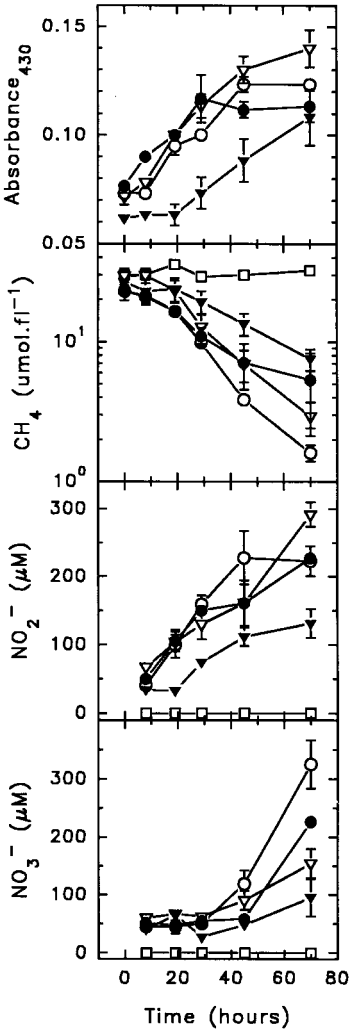

FIG. 5. Effect of allylsulfide on growth (A_{430}), CH_4 oxidation, NO_2^- production, and NO_3^- production by *M. trichosporium* OB3b in AMS medium with CH_4 (1 kPa) at 25°C with shaking at 250 rpm. Other details are described in the legend to Fig. 4.

much as that in NMS medium suggests that allylsulfide may interfere with NO_3^- metabolism in this organism.

The apparent lack of inhibition by allylsulfide (≤200 μM) of NH_4^+ oxidation by *M. trichosporium* should make allylsulfide a useful tool in the study of methanotrophic nitrification in natural systems. Allylsulfide, to our knowledge, is the first nitrification inhibitor with little effect on methanotrophic NO_2^- production. Allylsulfide contrasts with picolinic acid because it affects methanotrophic nitrification less than it affects nitrification by nitrifiers (12a, 22).

TABLE 3. Effect of allylsulfide on MMO activity in soluble fractions of *M. trichosporium* OB3b

Addition	O_2 uptake[a] (μM/min)	Inhibition (%)
NADH	3.82 ± 0.22a	
NADH+furan	5.77 ± 0.82b	
NADH+furan+allylsulfide (200 μM)	6.29 ± 0.35b	0
NADH+furan+allylsulfide (2 mM)	4.86 ± 0.80b	16

[a] Data are means of triplicate measurements ± standard error of the mean. Different letters after the numbers indicate significant difference ($P \leq 0.05$) based on an unpaired and one-tailed *t* test.

Another interesting aspect of our data is the suggestion of a difference in the chemistry of AMO and MMO. *M. trichosporium* harbors two types of MMO: soluble (sMMO) and particulate (pMMO). Expression of sMMO is repressed under conditions of copper sufficiency (≥2 μM) (5). Whole-cell experiments with *M. trichosporium* were performed in medium with a low copper concentration (2.4 nM), which allowed sMMO synthesis. This fact suggests that allylsulfide up to 2 mM does not inhibit the sMMO. The difference in sensitivity to allylsulfide between AMO and MMO may reflect the absence of significant homology between AMO and sMMO (10). It remains to be established what, if any, effect allylsulfide has on the pMMO. Studying the effect of allylsulfide on purified MMO may yield information about the different catalytic mechanisms of AMO and MMO. On the basis of the published literature (7), it is unclear exactly what is inhibiting the AMO: allylsulfide or a product of its oxidation, allylsulfoxide or allylsulfone. It is therefore difficult, at present, to explain the chemical basis of the difference in sensitivity to allylsulfide between AMO and MMO.

In conclusion, allylsulfide appears to be a promising tool for the study of the complex interaction between methanotrophs and nitrifiers in natural systems. The strong mechanism-based inhibition of AMO by allylsulfide and the apparent lack of inhibition of MMO by this compound should, in the future, allow the specific inhibition of nitrifying bacteria with little effect on the population of CH_4-oxidizing bacteria. Applications of low concentrations of allylsulfide may help to elucidate the role of nitrifying bacteria in the oxidation of CH_4 in natural systems and the role of methanotrophic bacteria in the oxidation of NH_4^+. Preliminary experiments indicate that allylsulfide inhibits methanogenesis and N_2O reduction in freshwater sediments only at concentrations higher than those required for the inhibition of CH_4 oxidation (20). However, the specificity of allylsulfide for nitrification remains to be confirmed. The fate of allylsulfide in natural systems should also be investigated to evaluate its suitability for microcosm studies.

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