

# New Insights into Methyl Bromide Cooxidation by *Nitrosomonas europaea* Obtained by Experimenting with Moderately Low Density Cell Suspensions†

KHRYSTYNE N. DUDDLESTON,<sup>1,‡</sup> PETER J. BOTTOMLEY,<sup>1,2,\*</sup> ANGELA J. PORTER,<sup>1</sup>  
AND DANIEL J. ARP<sup>3</sup>

Department of Microbiology,<sup>1</sup> Department of Crop and Soil Science,<sup>2</sup> and The Laboratory for N<sub>2</sub> Fixation Research,  
Department of Botany and Plant Pathology,<sup>3</sup> Oregon State University, Corvallis, Oregon 97331

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We examined the rates and sustainability of methyl bromide (MeBr) oxidation in moderately low density cell suspensions ( $\sim 6 \times 10^7$  cells ml<sup>-1</sup>) of the NH<sub>3</sub>-oxidizing bacterium *Nitrosomonas europaea*. In the presence of 10 mM NH<sub>4</sub><sup>+</sup> and 0.44, 0.22, and 0.11 mM MeBr, the initial rates of MeBr oxidation were sustained for 12, 12, and 24 h, respectively, despite the fact that only 10% of the NH<sub>4</sub><sup>+</sup>, 18% of the NH<sub>4</sub><sup>+</sup>, and 35% of the NH<sub>4</sub><sup>+</sup>, respectively, were consumed. Although the duration of active MeBr oxidation generally decreased as the MeBr concentration increased, similar amounts of MeBr were oxidized with a large number of the NH<sub>4</sub><sup>+</sup>-MeBr combinations examined (10 to 20  $\mu$ mol mg [dry weight] of cells<sup>-1</sup>). Approximately 90% of the NH<sub>3</sub>-dependent O<sub>2</sub> uptake activity and the NO<sub>2</sub><sup>-</sup>-producing activity were lost after *N. europaea* was exposed to 0.44 mM MeBr for 24 h. After MeBr was removed and the cells were resuspended in fresh growth medium, NO<sub>2</sub><sup>-</sup> production increased exponentially, and 48 to 60 h was required to reach the level of activity observed initially in control cells that were not exposed to MeBr. It is not clear what percentage of the cells were capable of cell division after MeBr oxidation because NO<sub>2</sub><sup>-</sup> accumulated more slowly in the exposed cells than in the unexposed cells despite the fact that the latter were diluted 10-fold to create inocula which exhibited equal initial activities. The decreases in NO<sub>2</sub><sup>-</sup>-producing and MeBr-oxidizing activities could not be attributed directly to NH<sub>4</sub><sup>+</sup> or NH<sub>3</sub> limitation, to a decrease in the pH, to the composition of the incubation medium, or to toxic effects caused by accumulation of the end products of oxidation (NO<sub>2</sub><sup>-</sup> and formaldehyde) in the medium. Additional cooxidation-related studies of *N. europaea* are needed to identify the mechanism(s) responsible for the MeBr-induced loss of cell activity and/or viability, to determine what percentages of cells damaged by cooxidative activities are culturable, and to determine if cooxidative activity interferes with the regulation of NH<sub>3</sub>-oxidizing activity.

*Nitrosomonas europaea*, a chemolithoautotrophic NH<sub>3</sub> oxidizer, oxidizes a variety of compounds, including alkanes, alkenes, alkynes (6, 10), halogenated hydrocarbons (12, 18, 27), and aromatic compounds (9, 13), with ammonia monooxygenase (AMO). AMO is the broad-substrate-range oxygenase that is responsible for oxidation of NH<sub>3</sub> to hydroxylamine (NH<sub>2</sub>OH), the first step in oxidation of NH<sub>3</sub> to NO<sub>2</sub><sup>-</sup> (30). Previously described studies of cooxidation of halogenated hydrocarbons by NH<sub>3</sub>-oxidizing bacteria have focused primarily on determining the range of compounds cooxidized by *N. europaea* (6, 7, 13, 16–18) and, to a lesser degree, on kinetic parameters (12). The majority of these studies were conducted by using short incubation periods ( $\leq 1$  h), high-density cell suspensions ( $10^9$  to  $10^{11}$  cells ml<sup>-1</sup>) exhibiting high rates of NO<sub>2</sub><sup>-</sup> production ( $\sim 3 \mu$ mol ml<sup>-1</sup> h<sup>-1</sup>), and pH values considered to be optimal for NH<sub>3</sub> oxidation (pH 7.8 to 8.0). Comprehensive studies have not been performed yet with lower-density cell suspensions ( $< 10^8$  cells ml<sup>-1</sup>) that exhibit NH<sub>3</sub> oxidation rates more typical of environments like nitrifying bioreactors ( $\sim 0.1 \mu$ mol of NH<sub>4</sub><sup>+</sup> ml<sup>-1</sup> h<sup>-1</sup>) (1, 2), in which

cooxidation may occur (15, 23). Furthermore, the sustainability of cooxidation and the relationship of cooxidation to NO<sub>2</sub><sup>-</sup> production could not be examined adequately in our previous studies because total ammonium (NH<sub>4</sub><sup>+</sup> plus NH<sub>3</sub>) became limiting very quickly because of high rates of consumption and because a decrease in pH reduced NH<sub>3</sub> availability. The issue of sustainability and the factors that affect it need to be studied in order to understand the long-term effects of cooxidation of halogenated hydrocarbons on NH<sub>3</sub> oxidizers and to better assess the potential use of these organisms in bioremediation of contaminants.

Methyl bromide (MeBr) is a soil fumigant that is used to control weeds, soilborne plant pathogens, and nematodes (25, 29, 31). MeBr has been categorized as a class 1 ozone-depleting chemical by the U.S. Environmental Protection Agency and is scheduled for complete phase-out within a few years (25). Thus, the fate of MeBr has some applied significance, and this compound also is an excellent model compound for examining cooxidation by *N. europaea* because the end products of MeBr cooxidation (formaldehyde and HBr) have been identified (11, 12, 17). The objective of this study was to examine cooxidation of MeBr by a moderately low-density suspension of *N. europaea* cells ( $\sim 6 \times 10^7$  cells ml<sup>-1</sup>) that oxidized NH<sub>3</sub> at a rate similar to the rates measured in nitrifying bioreactors (1, 2).

## MATERIALS AND METHODS

**Cell growth and preparation.** Batch cultures (750 or 1,500 ml) of *N. europaea* ATCC 19718 were grown in Erlenmeyer or Fernbach flasks in the dark at 27°C

\* Corresponding author. Mailing address: Department of Microbiology, 220 Nash Hall, Oregon State University, Corvallis, OR 97331. Phone: (541) 737-1844. Fax: (541) 737-0496. E-mail: bottompl@ucs.orst.edu.

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‡ Present address: Department of Biological Sciences, University of Alaska Anchorage, Anchorage, AK 99508.

with orbital shaking (150 rpm). The growth medium consisted of 25 mM  $(\text{NH}_4)_2\text{SO}_4$  and other constituents as described elsewhere (4). Cells were harvested by centrifugation ( $11,000 \times g$ , 15 min) after the late exponential phase was reached (3 days), washed twice in buffer (50 mM potassium phosphate, pH 7.2), and resuspended in buffer to an optical density at 660 nm of approximately 1.0. All assays were initiated with aliquots of this cell suspension within 1 h of preparation. Epifluorescence microscopic counting of 4',6'-diamidino-2-phenylindole (DAPI)-stained cells confirmed that the cell density was  $1.2 \times 10^9 \pm 0.3 \times 10^9$  cells  $\text{ml}^{-1}$  when the optical density at 660 nm was 1.0. The average dry weight of the cell suspension was  $0.32 \pm 0.08$  mg  $\text{ml}^{-1}$ .

**Preparation of assay vials.** Portions (5 ml) of sterile assay buffer were added to sterile glass vials (capacity, 74 ml), which were sealed with gray butyl stoppers (Kimble, Owens, Ill.) and aluminum crimp top seals (Wheaton, Millville, N.J.). The assay buffer contained 5 mM  $(\text{NH}_4)_2\text{SO}_4$  and 50 mM  $\text{KH}_2\text{PO}_4$ - $\text{K}_2\text{HPO}_4$  (pH 7.2) unless otherwise noted. An MeBr stock vial was prepared by flushing a sealed vial containing 5 to 10 glass beads for 1 min (with periodic shaking to disrupt air pockets) with MeBr (99.5% pure; Matheson Gas Products, Inc., Newark, Calif.) from a lecture bottle. After an overpressure of gas was established in the stock vial, appropriate amounts of MeBr were added to assay vials by using gas-tight Hamilton microsyringes equipped with sidebore needles. Experiments were initiated by adding an aliquot of the cell suspension to each vial so that the final cell density was  $6 \times 10^7 \pm 1.5 \times 10^7$  cells  $\text{ml}^{-1}$  ( $16 \pm 4$   $\mu\text{g}$  [dry weight] of cells  $\text{ml}^{-1}$ ,  $80 \pm 20$   $\mu\text{g}$   $\text{vial}^{-1}$ ) unless otherwise noted. The vials were inverted and incubated in the dark at 27°C.

**Analytical procedures.** MeBr oxidation was measured by monitoring the disappearance of MeBr from the gas phase in the assay vials by using a Shimadzu model GC-14 gas chromatograph. The gas chromatograph was equipped with a stainless steel column (outside diameter, 0.32 cm; length, 91 cm) packed with Porapak-Q (80–100 mesh; Waters Associates Inc., Framingham, Mass.) (column temperature, 120°C) and a flame ionization detector (detector and injector temperature, 200°C) interfaced with a Shimadzu model CR501 integrator. At time intervals, 60- to 200- $\mu\text{l}$  aliquots of headspace gas were removed from the assay vials with a gas-tight Hamilton microsyringe equipped with a sidebore needle. To check for AMO-independent oxidation of MeBr, we included control vials that contained 1% (vol/vol) acetylene, a specific mechanism-based inactivator of AMO (4). By using these controls we determined that about 10 and 20% of the MeBr that disappeared from vials in 24-h assays and 48- to 72-h assays, respectively, could be considered AMO independent. These quantities were routinely subtracted from the MeBr depletion values that were obtained with vials that did not contain acetylene. The amounts of MeBr in the vials were determined by comparison with standards containing known amounts of MeBr in 74-ml vials containing 5 ml of sterile assay buffer. A dimensionless Henry's Law constant for MeBr of 0.25 was utilized (24), and approximately 22% of the total amount of MeBr added to the vials partitioned into the liquid phase. For all assays the amount of MeBr added was expressed as the concentration in the liquid phase, while the amount transformed was expressed in micromoles. For purposes of comparison, when 10  $\mu\text{mol}$  of MeBr was added to a vial, the aqueous phase concentration of MeBr was 0.44 mM.  $\text{NO}_2^-$  production was determined by removing aliquots (20 to 100  $\mu\text{l}$ ) of cell suspensions from the sealed vials with a gas-tight syringe and determining the  $\text{NO}_2^-$  contents colorimetrically (3).

**Response of the initial rate of MeBr oxidation to cell density, pH, and  $\text{NH}_4^+$  and MeBr concentrations.** (i) **Cell density.** Aliquots of diluted cell suspensions of *N. europaea* were added to triplicate assay vials containing buffer, 10 mM  $\text{NH}_4^+$ , and 0.5 to 3  $\mu\text{mol}$  of MeBr  $\text{vial}^{-1}$  (aqueous concentration, 0.02 to 0.13 mM) in order to obtain cell densities of  $6 \times 10^6$ ,  $6 \times 10^7$ , and  $6 \times 10^8$  cells  $\text{ml}^{-1}$ . Samples of headspace gas were recovered at 3- to 12-h intervals for up to 3 days and examined to determine if MeBr depletion could be measured.

(ii) **pH.** *N. europaea* cell suspensions were prepared in phosphate buffer pre-adjusted to pH 6.2, 7.2, or 8.2. One-milliliter aliquots ( $6 \times 10^7$  cells  $\text{ml}^{-1}$ ) were injected into vials that already contained either 0.11, 0.22, or 0.44 mM MeBr and 10 mM  $\text{NH}_4^+$  in the appropriate buffer. Samples of the headspace gas and liquid contents of the vials were obtained and used to determine the MeBr and  $\text{NO}_2^-$  contents at 3- to 6-h intervals over a 24-h period.

(iii)  **$\text{NH}_4^+$  and MeBr concentrations.** *N. europaea* cells ( $6 \times 10^7$  cells  $\text{ml}^{-1}$ ) were incubated in a factorized design experiment with combinations consisting of 0.11, 0.22, or 0.44 mM MeBr and 2.5, 5, or 10 mM  $\text{NH}_4^+$ . MeBr oxidation and  $\text{NO}_2^-$  production were monitored at 2- to 6-h intervals. The rates of MeBr oxidation and  $\text{NO}_2^-$  production were calculated by using the linear regression feature in SigmaPlot 3.0 (Jandel Scientific, San Rafael, Calif.). The correlation coefficients for all regressions were  $\geq 0.96$ . The initial rates were expressed in micromoles of MeBr oxidized or  $\text{NO}_2^-$  produced per milligram (dry weight) per hour.

**Examination of the factors that might influence the sustainability of MeBr oxidation.** (i) **Influence of buffer, growth medium, and  $\text{Na}_2\text{CO}_3$  on the sustainability of MeBr oxidation.** *N. europaea* cells ( $6 \times 10^7$  cells  $\text{ml}^{-1}$ ) were incubated in either phosphate buffer or complete growth medium (pH 7.2) that was supplemented or not supplemented with  $\text{Na}_2\text{CO}_3$  (4 mM). The pH of each solution was adjusted to 7.2, and 4 mM  $\text{Na}_2\text{SO}_4$  was added to vials that did not receive  $\text{Na}_2\text{CO}_3$ . Both the cell suspensions and the  $\text{NH}_4^+$  stock solution were prepared by using the appropriate buffer or growth medium. MeBr disappearance was monitored over a 36-h period.

(ii) **Influence of  $\text{NH}_4^+$  limitation or cell inactivation on the sustainability of MeBr oxidation.** To determine if either  $\text{NH}_4^+$  limitation or cell inactivation was the primary reason for the loss of MeBr-oxidizing activity, a preparation containing  $6 \times 10^7$  cells  $\text{ml}^{-1}$  was incubated with 10 mM  $\text{NH}_4^+$  and 0.22 mM MeBr until the initial rate of MeBr oxidation declined (after approximately 9 h of incubation). Then replicate vials received either (i) additional cells ( $3 \times 10^8$  cells), (ii) additional cells plus  $\text{NH}_4^+$  (equivalent to an additional 10 mM), or (iii)  $\text{NH}_4^+$  alone. MeBr oxidation and  $\text{NO}_2^-$  production were monitored for another 15 h.

(iii) **Effects of the end products of  $\text{NH}_3$  and MeBr oxidation,  $\text{NH}_4^+$  depletion, and pH decline on sustainability of MeBr oxidation.** The effects of accumulation of the end products of MeBr and  $\text{NH}_3$  oxidation on the sustainability of MeBr oxidation were examined by monitoring the oxidation of 0.22 mM MeBr in 50 mM phosphate buffer (pH 7.0) supplemented with 7.5 mM  $\text{NH}_4^+$ . Factorialized combinations consisting of 2.5 mM  $\text{NO}_2^-$  and 0.4 mM formaldehyde were added to the assay vials; these concentrations were chosen because they represented the approximate conditions in the assay vials after 24 h of oxidation of 0.22 mM MeBr and 10 mM  $\text{NH}_4^+$ . The reactions were started by adding 1-ml aliquots of cells suspended in phosphate buffer (pH 7.0), and MeBr disappearance and  $\text{NO}_2^-$  production were monitored as described above.

(iv) **Influence of inhibition of protein synthesis on  $\text{NO}_2^-$  production during MeBr cooxidation.** Either chloramphenicol (final concentration, 200 or 400  $\mu\text{g}/\text{ml}$ ) or kanamycin (10 to 50  $\mu\text{g}/\text{ml}$ ) was dissolved in 50 mM phosphate buffer (pH, 7.2). Aliquots of the buffer were then supplemented with 10 mM  $\text{NH}_4^+$  and injected into sealed vials containing enough MeBr so that the MeBr concentration in the aqueous phase was 0.22 or 0.44 mM. The reactions were started by adding *N. europaea* ( $6 \times 10^7$  cells  $\text{ml}^{-1}$ ), and  $\text{NO}_2^-$  production was monitored during 7 h of incubation.

**Residual  $\text{NH}_4^+$  and  $\text{NH}_2\text{OH}$ -dependent  $\text{O}_2$  uptake activity after oxidation of MeBr.** To further examine the effect of MeBr oxidation on the residual activity of *N. europaea*, cells ( $6 \times 10^7$  cells  $\text{ml}^{-1}$ ) were incubated with 10 mM  $\text{NH}_4^+$  and 0.11, 0.22, or 0.44 mM MeBr for 24 h. Following incubation, the vials were opened and vented for 5 min. The cell suspensions were filtered through 25-mm diameter 0.4- $\mu\text{m}$ -pore-size polycarbonate filters and washed by filtering 9 ml of sterile buffer over the cells. Control vials containing cells, 10 mM  $\text{NH}_4^+$ , and no MeBr were treated exactly like the vials that contained MeBr were treated. To determine residual  $\text{O}_2$  uptake activity, the filters were placed in 2-ml portions of buffer, and the cells were washed off with gentle shaking. An aliquot of the cell suspension (1.6 ml, 64  $\mu\text{g}$  [dry weight] of cells) was added to an  $\text{O}_2$  electrode chamber. After 3 to 5 min of stirring,  $\text{NH}_4^+$  (final concentration, 10 mM) was added to the chamber, and the  $\text{NH}_4^+$ -dependent  $\text{O}_2$  uptake rate was measured over a 2- to 5-min interval.  $\text{NH}_4^+$ -dependent  $\text{O}_2$  uptake was stopped by adding 1-allyl-2-thiourea (final concentration, 0.1 mM), a reversible inhibitor of AMO (14). Subsequently,  $\text{NH}_2\text{OH}$  (final concentration, 0.6 mM) was added to the chamber to measure  $\text{NH}_2\text{OH}$ -dependent  $\text{O}_2$  uptake.

**Recovery of  $\text{NO}_2^-$ -producing ability by *N. europaea* after oxidation of 0.44 mM MeBr for 24 h.** *N. europaea* ( $6 \times 10^7$  cells  $\text{ml}^{-1}$ ) was exposed to 0.44 mM MeBr in phosphate buffer (pH 7.2) for 24 h as described above. Vials containing cells that were not exposed to MeBr were included as controls. Cells were harvested from the buffer, washed, and resuspended in complete growth medium at either pH 7.2 or pH 8. Unexposed cells were diluted another 10-fold in growth medium so that the initial rates of  $\text{NO}_2^-$  production were similar for both exposed and unexposed cells. At 6-h intervals, samples of cells were recovered, and  $\text{NO}_2^-$  production was determined as described above.

## RESULTS

In preliminary experiments (data not shown) we established that oxidation of MeBr at concentrations up to 0.44 mM could be measured accurately with a cell density of  $6 \times 10^7$  cells  $\text{ml}^{-1}$  (16  $\mu\text{g}$  [dry weight] of cells  $\text{ml}^{-1}$ ). Oxidation of MeBr at concentrations of  $>0.66$  and  $\leq 0.88$  mM could be measured, but the rates decreased rapidly after only 1 to 4 h of incubation and thus were not studied in detail. At cell densities of  $<10^7$  cells  $\text{ml}^{-1}$ , the incubation time required to accurately measure MeBr disappearance with a gas chromatograph equipped with a flame ionization detector was 24 h or more, and such determinations could be made only at low MeBr concentrations ( $\leq 0.04$  mM). The MeBr-oxidizing ability of *N. europaea* was examined at three pH values (pH 6.2, 7.2, and 8.2) representing the range of pH values likely to be encountered in many natural environments. At each of the three MeBr concentrations evaluated (0.11, 0.22, and 0.44 mM) MeBr was oxidized significantly faster at pH 7.2 than at either pH 6.2 or pH 8.2 (data not shown). After 24 h of incubation the pH had changed very little in the pH 7.2 preparation (final pH, 7.0 to 7.1), which implied that the buffering capacity of 50 mM phosphate was

TABLE 1.  $\text{NO}_2^-$  production and MeBr oxidation by *N. europaea* in the presence of different combinations of  $\text{NH}_4^+$  and MeBr<sup>a</sup>

$\text{NH}_4^+$ concn (mM)	Rate of $\text{NO}_2^-$ production in the absence of MeBr	0.11 mM MeBr			0.22 mM MeBr			0.44 mM MeBr		
		Rate of $\text{NO}_2^-$ production	Rate of MeBr oxidation	M/N ratio	Rate of $\text{NO}_2^-$ production	Rate of MeBr oxidation	M/N ratio	Rate of $\text{NO}_2^-$ production	Rate of MeBr oxidation	M/N ratio
1	5.7	3.7 (0.2) <sup>b</sup>	0.77 (0.1)	0.21	2.2 (0.2)	0.92 (0.17)	0.42	0.7 (0.1)	0.21 (0.31)	0.30
2.5	10.2	7.8 (0.4)	0.54 (0.1)	0.07	6.0 (0.3)	1.23 (0.21)	0.21	3.1 (0.1)	0.94 (0.27)	0.30
5	14.3	10.8 (0.3)	0.56 (0.15)	0.05	9.3 (0.1)	1.17 (0.15)	0.13	6.4 (0.4)	1.52 (0.31)	0.24
10	17.5	12.5 (0.3)	0.38 (0.19)	0.03	11.0 (0.3)	1.17 (0.58)	0.11	8.8 (0.4)	1.38 (0.40)	0.16

<sup>a</sup> Because the durations of the initial rates of  $\text{NO}_2^-$  production and MeBr oxidation varied considerably for the different combinations of  $\text{NH}_4^+$  and MeBr, the linear regression feature of SigmaPlot 3.0 was used to determine the time interval over which rates were constant. The rates are expressed in micromoles per milligram (dry weight) per hour.

<sup>b</sup> The values in parentheses are the standard deviations of the means based on the results obtained for three replicates per treatment.

adequate for dealing with the acidity generated during  $\text{NH}_3$  oxidation by the concentrations of cells used for at least 24 h. Additional studies of the properties of MeBr oxidation by *N. europaea* were performed by using pH 7.2 and a cell density of  $6 \times 10^7$  cells  $\text{ml}^{-1}$ .

By experimenting with moderately low cell densities we were able to examine the initial rates of MeBr oxidation and the accompanying rates of  $\text{NO}_2^-$  production at different  $\text{NH}_4^+$  concentrations (Table 1). The highest rates of MeBr oxidation occurred in the presence of 2.5 to 10 mM  $\text{NH}_4^+$  and 0.22 to 0.44 mM MeBr. The responses of the initial rate of MeBr oxidation to  $\text{NH}_4^+$  concentration were different at different MeBr concentrations. In the presence of 0.11 mM MeBr, the rate of MeBr oxidation increased twofold, while the level of  $\text{NO}_2^-$  production decreased threefold as the  $\text{NH}_4^+$  concentration decreased from 10 to 1 mM. In the presence of 0.22 mM MeBr, the initial rates of MeBr oxidation were relatively insensitive to changes in the  $\text{NH}_4^+$  concentration at concentrations between 1 and 10 mM, despite the fact that the level of  $\text{NO}_2^-$  production changed fivefold over this concentration range. In the presence of 0.44 mM MeBr, the initial rate of MeBr oxidation responded to most incremental changes in the  $\text{NH}_4^+$  concentration and decreased at  $\text{NH}_4^+$  concentrations between 5 and 2.5 mM and between 2.5 and 1 mM. When we examined the ratio of amount of MeBr oxidized to amount of  $\text{NO}_2^-$  produced (M/N ratio), we found that the maximum initial rates of MeBr oxidation occurred at almost identical M/N ratios (the M/N ratios were 0.24, 0.21, and 0.21 for MeBr concentrations of 0.44, 0.22, and 0.11 mM, respectively) regardless of the MeBr and  $\text{NH}_4^+$  concentrations. We also observed another trend: M/N ratios of  $\geq 0.30$  and  $< 0.1$  were associated with suboptimal initial rates of MeBr oxidation.

The rates of MeBr oxidation invariably declined when the cells were incubated for more than 12 h, and they declined to zero within 12 to 24, 36 to 48, and 48 to 72 h in the presence of 0.44, 0.22, and 0.11 mM MeBr, respectively (Fig. 1a). Although the rate of  $\text{NO}_2^-$  production was constant for at least 12 h in the control lacking MeBr, in the presence of 0.11, 0.22, and 0.44 mM MeBr the rates of  $\text{NO}_2^-$  production were constant for approximately 12, 6, and 3 h, respectively, and then gradually declined to zero over ranges of time similar to the ranges of time as described above for MeBr oxidation (Fig. 1b). Whereas the rates of oxidation of 0.11, 0.22, and 0.44 mM MeBr definitely decreased at different times (36 to 48, 24 to 36, and 12 to 24 h, respectively), the corresponding rates of  $\text{NO}_2^-$  production were very similar (2 to 3  $\mu\text{mol}$  of  $\text{NO}_2^-$  produced mg [dry weight] of cells<sup>-1</sup> h<sup>-1</sup>). Despite relatively large differences in the rate and duration of active MeBr oxidation for the different  $\text{NH}_4^+$ -MeBr combinations, similar amounts of MeBr were oxidized with a large number of the  $\text{NH}_4^+$ -MeBr combinations (1 to 2  $\mu\text{mol}$  per vial, 10 to 20  $\mu\text{mol}$  mg [dry weight] of

cells<sup>-1</sup>) (Table 2). In general, this was attributed to the fact that while the rates of MeBr oxidation were about two- to threefold lower when 0.11 MeBr was used than when 0.22 and 0.44 mM MeBr were used, the length of the period of active oxidation was inversely proportional to the MeBr concentration (e.g., 72, 48, and 24 h) (Table 2).

We obtained no evidence which supported the possibility that either (i)  $\text{NO}_2^-$  and/or formaldehyde accumulation, (ii) a decrease in pH (pH 7.2 to 7.0), (iii)  $\text{NH}_4^+$  limitation (10 to 7.5 mM), or (iv) inadequate medium composition (buffer versus growth medium) was directly responsible for the decreases in  $\text{NO}_2^-$ -producing ability and MeBr-oxidizing ability. For example, when assays were initiated at pH 7 in the presence of 7.5

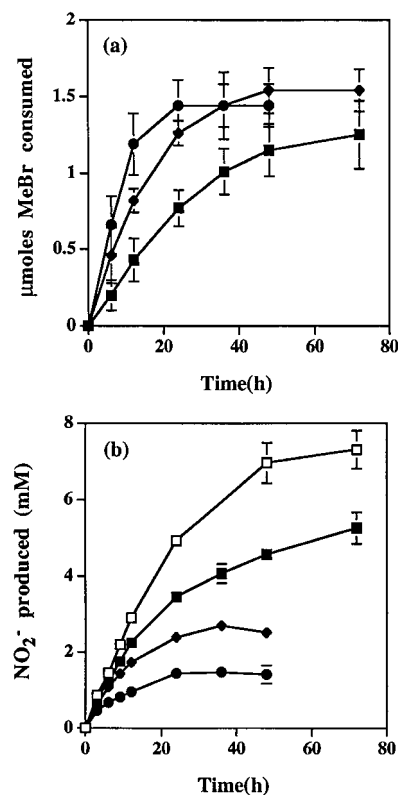


FIG. 1. Time courses of MeBr consumption (a) and  $\text{NO}_2^-$  production (b) by *N. europaea* over a 48- to 72-h period. All vials contained 10 mM  $\text{NH}_4^+$ , phosphate buffer (pH 7.2),  $6 \times 10^7$  cells of *N. europaea*  $\text{ml}^{-1}$ , and either no MeBr ( $\square$ ), 0.11 mM MeBr ( $\blacksquare$ ), 0.22 mM MeBr ( $\blacklozenge$ ), or 0.44 mM MeBr ( $\bullet$ ). The error bars indicate the standard deviations of the means based on the results obtained for three replicate vials per treatment.

TABLE 2. MeBr transformation capacities of *N. europaea* when it was incubated in the presence of different combinations of  $\text{NH}_4^+$  and MeBr

$\text{NH}_4^+$ concn (mM)	MeBr concn (mM)	Amt of time (h)	Amt of MeBr consumed ( $\mu\text{mol}$ )	Amt of $\text{NO}_2^-$ produced ( $\mu\text{mol}$ )
10	0.11	72	1.25 (0.22) <sup>a</sup>	26.3 (2.0)
	0.22	48	1.54 (0.16)	12.6 (0.5)
	0.44	24	1.44 (0.14)	6.4 (0.1)
5	0.11	72	1.37 (0.19)	17.7 (1.2)
	0.22	48	1.50 (0.18)	7.5 (1.4)
	0.44	24	1.30 (0.17)	4.1 (0.1)
2.5	0.11	72	1.27 (0.12)	9.4 (0.4)
	0.22	48	1.35 (0.02)	4.9 (0.2)
	0.44	24	0.74 (0.22)	1.9 (0.1)
1	0.11	72	1.17 (0.04)	3.9 (0.1)
	0.22	24	0.98 (0.19)	1.6 (0.1)
	0.44	24	0.20 (0.12)	0.5 (0.1)

<sup>a</sup> The values in parentheses are the standard deviations of the means based on the results obtained for three replicates per treatment.

mM  $\text{NH}_4^+$  and different combinations of  $\text{NO}_2^-$  (2.5 mM) and formaldehyde (0.3 mM), the characteristics and amounts of MeBr oxidized were similar to the characteristics and amounts obtained under typical assay conditions (Fig. 2). Furthermore, the initial rates of MeBr oxidation and  $\text{NO}_2^-$  production could be sustained in the same assay vials for several additional hours if a second aliquot of cells ( $30 \times 10^7$  cells) was added to the assay mixture after 9 h of incubation (Fig. 3). Although 10 mM  $\text{NH}_4^+$  added along with the cells increased the rate of  $\text{NO}_2^-$  production, it did not increase the rate of MeBr oxidation to a value greater than the value obtained when only cells were added. The increase in  $\text{NO}_2^-$  production without a concomitant increase in MeBr oxidation is consistent with other data which showed that the same rate of oxidation of 0.22 mM MeBr could be supported by a range of  $\text{NH}_4^+$  concentrations (2.5 to 20 mM  $\text{NH}_4^+$ ) (Table 1) (12) over which the rate of  $\text{NO}_2^-$  production doubled.

When cells were recovered from the incubation vials after 24 h of exposure to MeBr, approximately 80 to 90% of their  $\text{NO}_2^-$ -producing (data not shown) and  $\text{NH}_4^+$ -dependent  $\text{O}_2$

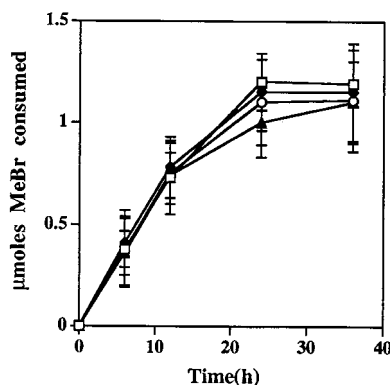


FIG. 2. Effects of  $\text{NO}_2^-$ , formaldehyde, pH, and  $\text{NH}_4^+$  on the MeBr consumed by *N. europaea*. All assay mixtures contained  $6 \times 10^7$  cells of *N. europaea*  $\text{ml}^{-1}$ , and the initial conditions were as follows: pH 7.0, 7.5 mM  $\text{NH}_4^+$ , and 0.22 mM MeBr.  $\text{NO}_2^-$  and formaldehyde were added when appropriate to final concentrations of 2.5 and 0.5 mM, respectively. Symbols:  $\square$ ,  $\text{NO}_2^-$  and formaldehyde both present;  $\blacklozenge$ ,  $\text{NO}_2^-$  present and formaldehyde absent;  $\circ$ ,  $\text{NO}_2^-$  absent and formaldehyde present;  $\blacktriangle$ ,  $\text{NO}_2^-$  and formaldehyde both absent. The error bars indicate the standard deviations of the means based on the results obtained for three replicate vials per treatment.

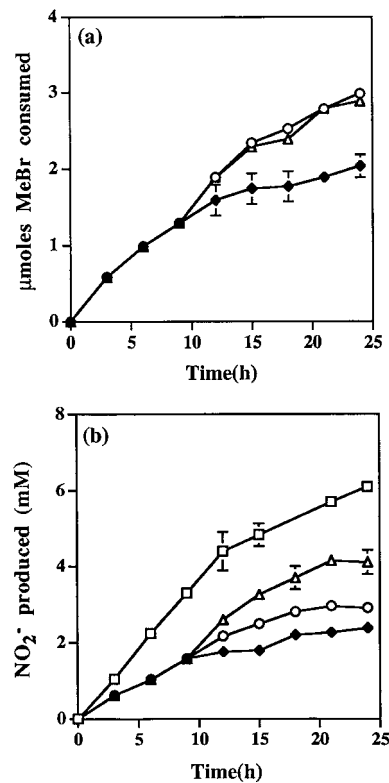


FIG. 3. Effects of  $\text{NH}_4^+$  and fresh cell supplements MeBr consumption (a) and  $\text{NO}_2^-$  production (b) by *N. europaea*. At time zero, all vials (except the control) contained 0.22 mM MeBr, 10 mM  $\text{NH}_4^+$ , and  $3 \times 10^8$  cells of *N. europaea*. After 9 h of incubation, triplicate vials received  $3 \times 10^8$  cells ( $\circ$ ),  $3 \times 10^8$  cells plus 10 mM  $\text{NH}_4^+$  ( $\triangle$ ), or no additional supplement ( $\blacklozenge$ ). The control vials ( $\square$ ) were incubated without MeBr and did not receive an additional supplement. The error bars indicate the standard deviations of the means based on the results obtained for three replicate vials per treatment.

uptake (Table 3) activities had been lost. Much less of the whole-cell hydroxylamine ( $\text{NH}_2\text{OH}$ )-dependent  $\text{O}_2$  uptake activity was lost (20 to 30%) after exposure to MeBr. Recovery of  $\text{NO}_2^-$  production by MeBr-exposed cells was monitored after the cells were resuspended in fresh growth medium (pH 7.2 or 8) containing 20 mM  $\text{NH}_4^+$  (Fig. 4). Cells that were not exposed to MeBr but otherwise treated identically were diluted 10-fold to obtain a similar initial rate of  $\text{NO}_2^-$  production, and these cells were used as a control. We found that the  $\text{NO}_2^-$  concentration increased immediately in a nonlinear manner in

TABLE 3. Effect of incubation of *N. europaea* with MeBr for 24 h on residual  $\text{NH}_4^+$ - and  $\text{NH}_2\text{OH}$ -dependent  $\text{O}_2$  uptake

MeBr concn (mM)	$\text{O}_2$ uptake rate ( $\mu\text{mol mg} [\text{dry wt}]$ of cells $^{-1}$ h $^{-1}$ ) <sup>a</sup>	
	$\text{NH}_4^+$ dependent	$\text{NH}_2\text{OH}$ dependent
0	22.4 (2.6) <sup>b</sup>	7.1 (0.8)
0.11	7.0 (0.8)	5.6 (0.5)
0.22	3.8 (0.8)	5.0 (0.2)
0.44	2.7 (0.7)	5.1 (0.3)

<sup>a</sup> Oxygen uptake rates were determined after incubation for 24 h in the presence of 10 mM  $\text{NH}_4^+$  and MeBr as described in Materials and Methods. The rates obtained for the preparation containing no MeBr at zero time and after 24 h of incubation were not significantly different.

<sup>b</sup> The values are means based on three or more replicate experiments. The values in parentheses are the standard deviations of the means.

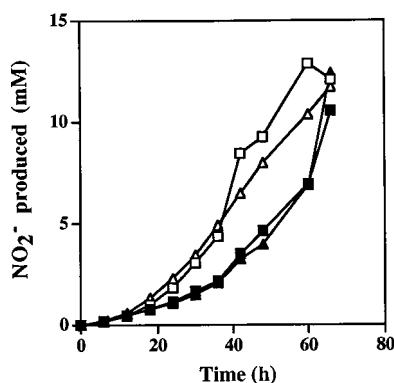


FIG. 4. Development of  $\text{NO}_2^-$  production by *N. europaea* cells resuspended in fresh growth medium after they were exposed (solid symbols) or not exposed (open symbols) to 0.44 mM MeBr in phosphate buffer (pH 7.2). The growth medium contained 20 mM  $\text{NH}_4^+$ . Symbols:  $\blacktriangle$ , growth medium, pH 7.2;  $\blacksquare$ , growth medium, pH 8;  $\triangle$ , growth medium, pH 7.2;  $\square$ , growth medium, pH 8. For clarity error bars are not shown. The standard deviations were  $\leq 10\%$  of the mean values regardless of the treatment.

both exposed and unexposed cells at both pH values. Although the amount of  $\text{NO}_2^-$  produced during the first 6 h of incubation by the cells exposed to MeBr was about the same as the amount produced by the unexposed cells, the rate of  $\text{NO}_2^-$  accumulation was lower in the former preparation. A 48- to 60-h recovery period was required before the cells exposed to MeBr exhibited the same rate of  $\text{NO}_2^-$  production that they exhibited before they oxidized MeBr.

## DISCUSSION

By experimenting with moderately low-density cell suspensions we gained insight into characteristics of MeBr oxidation by *N. europaea* that were not detected in previous studies performed in our laboratory. For example, Rasche et al. (17) and Keener and Arp (12) used 0.5 to 4 mg (dry weight) of cells  $\text{ml}^{-1}$  in their analyses of MeBr oxidation by *N. europaea*. Because the capacity of *N. europaea* to transform MeBr is between 10 to 20  $\mu\text{mol}$  of MeBr mg (dry weight) of cells $^{-1}$ , we know that the quantities of cells used by our colleagues could transform approximately 10-fold more MeBr than the amounts used routinely in these types of studies (2 to 10  $\mu\text{mol}$  per assay mixture). It is not surprising, therefore, that they did not determine the finite capacity of *N. europaea* to oxidize MeBr and that  $\text{NO}_2^-$  production and  $\text{NH}_4^+$ -dependent  $\text{O}_2$  uptake activities declined considerably as a consequence of prolonged MeBr oxidation.

At first we were confused by our finding that both  $\text{NO}_2^-$ -producing and MeBr-oxidizing activities were lost during transformation of MeBr because Rasche et al. (16) had concluded from short-term studies that monohalogenated aliphatic compounds could be degraded by *N. europaea* without AMO inactivation by the end products of cooxidation. Nonetheless, it has been reported that formaldehyde (28) and  $\text{NO}_2^-$  in the absence of  $\text{NH}_4^+$  (20) inhibit  $\text{NH}_3$  oxidation in *N. europaea*, yet we obtained no evidence that these end products were inhibitory to MeBr oxidation at the concentrations generated in our assays and under our experimental conditions. At this time, however, we cannot rule out the possibility that formaldehyde generated intracellularly might be more toxic to *N. europaea* than externally applied material is or that some oxidatively generated brominated chemical species might be the cause of toxicity. Although  $\text{NH}_4^+$ -dependent  $\text{O}_2$  uptake was reduced

more severely by exposure to MeBr than  $\text{NH}_2\text{OH}$ -dependent  $\text{O}_2$  uptake was reduced, our data indicate that prolonged MeBr oxidation resulted in a more general toxic effect on the cells than inactivation of AMO per se. For example, previous studies in our laboratory showed that when approximately 90% of the  $\text{NH}_4^+$ -dependent  $\text{O}_2$  uptake activity in *N. europaea* was eliminated by specifically inactivating AMO with strong light,  $\text{NO}_2^-$ -producing activity could be restored completely within 4 h of the time when cells were resuspended in fresh growth medium (8). In contrast, our studies showed that cultures exposed to MeBr for 24 h, in which approximately 90% of the  $\text{NH}_4^+$ -dependent  $\text{O}_2$  uptake activity was debilitated, required about 48 to 60 h of incubation to exhibit a rate of  $\text{NO}_2^-$  production comparable to the initial rate detected in unexposed cells. Indeed, the effect of long-term oxidation of MeBr on  $\text{NO}_2^-$ -producing activity is more similar to what occurred when *N. europaea* lost approximately 90% of its  $\text{NH}_4^+$ -dependent  $\text{O}_2$  uptake activity during short-term cooxidation of trichloroethylene. In that case, very little  $\text{NO}_2^-$ -producing activity was observed after 8 h of incubation, presumably because the cells had suffered too much nonspecific damage during trichloroethylene oxidation (8).

Because cells exposed to MeBr exhibit about one-tenth the rate of  $\text{NO}_2^-$  production that unexposed cells exhibit, it seems reasonable to conclude that approximately 10% of the cells survived the 24-h MeBr oxidation period and that the exponential recovery of  $\text{NO}_2^-$  production probably reflected proliferation of the surviving cells. It is not clear, however, why development of  $\text{NO}_2^-$  production by the cells exposed to MeBr lagged behind development of  $\text{NO}_2^-$  production by the diluted, unexposed control cells when the two inocula were adjusted so that the initial activities were similar. It is possible that some of the residual  $\text{NO}_2^-$  production by the cells exposed to MeBr originated from cells that were no longer capable of cell division. A recent study has shown that when methane-grown *Methylocystis trichosporium* OB3b oxidizes some chlorinated ethylenes, cell viability decreases more rapidly than the activity of methane monooxygenase decreases (26). Other studies performed in our laboratory have shown that AMO activity can be either upregulated or downregulated in response to  $\text{NH}_4^+$  availability (21, 22), that de novo protein synthesis is extremely limited in cells exposed to 10 mM  $\text{NH}_4^+$  at pH 7 (5), and that production of the mRNA transcript for AMO is limited when rates of  $\text{NO}_2^-$  production are supported by  $\leq 2$  mM  $\text{NH}_4^+$  at pH 7.5 (19). The faster development of  $\text{NO}_2^-$  production by the unexposed cells might have been due to upregulation of  $\text{NH}_3$ -oxidizing activity (21), and the cells exposed to MeBr might have lacked this ability.

Finally, during the initial optimum phase of cooxidation of 0.44 mM MeBr, the rate of  $\text{NO}_2^-$  production declined to approximately 30% of the initial rate before any effect on MeBr oxidation was observed (Fig. 1), and the M/N ratio increased from 0.13 to 0.47. Although it is not known how MeBr oxidation could cause  $\text{NH}_3$  oxidation to decrease while it allows MeBr oxidation to continue unabated, cell viability might decrease if reductant generation became insufficient to meet the combined needs of  $\text{NH}_3$  oxidation, MeBr cooxidation, and the essential maintenance requirements of the cell.

By carrying out cooxidation experiments with moderately low cell densities before we conducted ecologically based studies, we identified a number of additional physiological and molecular biological questions worth pursuing with *N. europaea*. Additional studies will be required (i) to determine the mechanism responsible for the MeBr-induced decreases in  $\text{NH}_3$ -oxidizing activity and cell viability in *N. europaea*; (ii) to examine in more detail the sequence of events that occur

during recovery of cells that have reached their cooxidative transformation capacity; and (iii) to determine if cooxidative activity interferes with regulation of AMO activity and gene regulation in response to  $\text{NH}_4^+$  availability.

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