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

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We examined the rates and sustainability of methyl bromide (MeBr) oxidation in moderately low density cell suspensions (∼6 × 10⁷ cells ml⁻¹) of the NH₃-oxidizing bacterium *Nitrosomonas europaea*. In the presence of 10 mM NH₄⁺ 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 NH₄⁺, 18% of the NH₄⁺, and 35% of the NH₄⁺, 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 NH₄⁺-MeBr combinations examined (10 to 20 μmol mg [dry weight] of cells⁻¹). Approximately 90% of the NH₄⁺-dependent O₂ uptake activity and the NO₂⁻-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, NO₂⁻ 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 NO₂⁻ 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 NO₂⁻-producing and MeBr-oxidizing activities could not be attributed directly to NH₄⁺ or NH₃ 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 (NO₂⁻ 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 cultivable, and to determine if cooxidative activity interferes with the regulation of NH₃-oxidizing activity.

*Nitrosomonas europaea*, a chemolithoautotrophic NH₃ 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 NH₃ to hydroxylamine (NH₂OH), the first step in oxidation of NH₃ to NO₂⁻ (30). Preciously described studies of cooxidation of MeBr by halogenated hydrocarbons by NH₃-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 (≤1 h), high-density cell suspensions (10⁹ to 10¹¹ cells ml⁻¹) exhibiting high rates of NO₂⁻ production (∼3 μmol ml⁻¹ h⁻¹), and pH values considered to be optimal for NH₃ oxidation (pH 7.8 to 8.0). Comprehensive studies have not been performed yet with lower-density cell suspensions (<10⁹ cells ml⁻¹) that exhibit NH₃ oxidation rates more typical of environments like nitrifying bioreactors (∼0.1 μmol of NH₄⁺ ml⁻¹ h⁻¹) (1, 2), in which cooxidation may occur (15, 23). Furthermore, the sustainability of cooxidation and the relationship of cooxidation to NO₂⁻ production could not be examined adequately in our previous studies because total ammonium (NH₄⁺ plus NH₃) became limiting very quickly because of high rates of consumption and because a decrease in pH reduced NH₃ 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 NH₃ 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 (∼6 × 10⁷ cells ml⁻¹) that oxidized NH₃ 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 Erlemeyer or Fernbach flasks in the dark at 27°C...
with orbital shaking (150 rpm). The growth medium consisted of 25 mM (NH₄)₂SO₄ and other constituents as described elsewhere (4). Cells were harvested by centrifugation (11,000 × g, 15 min) after the late exponential phase was reached (3 days), washed twice in buffer (50 mM KH₂PO₄·K₂HPO₄, 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 × 10⁷ cells ml⁻¹ when the optical density at 660 nm was 1.0. The average dry weight of the cell suspension was 0.32 ± 0.08 mg ml⁻¹.

Preparation of assay vials. Portions (5 ml) of sterile assay buffer were added to sterile reaction vials (74 ml), which were inverted and incubated in the dark at 27°C. To check for AMO-independent oxidation of MeBr, we included control vials that contained 1% (vol/vol) acetylene, a specific mechanism-based inhibitor of AMO (4). By using these controls we determined that about 10 and 20% of the micromoles of MeBr oxidized or NO₂⁻ production were similar for both exposed and unexposed cells. To determine if either NH₄⁺ limitation or cell inactivation on the sustainability of MeBr oxidation was examined by monitoring the oxidation of 0.22 mM MeBr in 50 mM phosphate buffer (pH 7.0) supplemented with 7.5 mM NH₄Cl. Factorized combinations consisting of 2.5 mM NO₂⁻ and 0.4 mM formamidéhyde were added to the assay vials; these combinations 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 NH₄Cl. The reactions were started by adding 1-ml aliquots of conditioned buffer to the vials that contained 107 cells ml⁻¹ and 0.3 mM NH₄Cl, and NO₂⁻ production was monitored as described above.

(iv) Influence of protein synthesis on NO₂⁻ production during MeBr cooxidation. Either chloramphenicol (final concentration, 200 or 400 µg ml⁻¹ or cycloheximide (10 to 50 µg ml⁻¹) was added to the culture. Control vials containing cells, 10 mM NH₄Cl, and NO₂⁻ production was determined as described above.

Residual NH₄⁺- and NH₄Cl-dependent O₂ uptake activity after oxidation of MeBr. To further examine the effect of MeBr oxidation on the residual activity of N. europaea, cells (6 × 10⁷ cells ml⁻¹) were incubated with 10 mM NH₄Cl 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-µm-pore-size polycarbonate filters and washed by filtering 9 ml of sterile buffer over the cells. Control vials containing cells, 10 mM NH₄Cl, and NO₂⁻ production were treated exactly like the vials that contained MeBr were treated. To determine residual O₂ 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 ml) was filtered through a 0.4-µm-pore-size polycarbonate filter on an O₂ electronic chamber. After 3 to 5 min of stirring, NH₄Cl (final concentration, 10 mM) was added to the chamber, and the NH₄⁺-dependent O₂ uptake rate was measured over a 2- to 5-min interval. NH₄Cl-dependent O₂ uptake was stopped by adding 1-µl 2-M-thiouria (final concentration, 0.1 mM), a reversible inhibitor of AMO (14). Subsequently, NH₂OH (final concentration, 0.6 mM) was added to the chamber to measure NH₄Cl-dependent O₂ uptake.

Recovery of NO₂⁻-producing ability by N. europaea after oxidation of 0.44 mM MeBr for 24 h. N. europaea (6 × 10⁷ cells 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 NO₂⁻ production were similar for both exposed and unexposed cells. At 6-h intervals, samples of cells were recovered, and NO₂⁻ 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 × 10⁷ cells ml⁻¹ (16 µg [dry weight] of cells ml⁻¹). Oxidation of MeBr at concentrations of ≥0.66 and ≤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 ≤10⁷ cells 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 (≤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...
adequate for dealing with the acidity generated during NH\textsubscript{3} oxidation by the concentrations of cells used for at least 24 h. Additional studies of the properties of MeBr oxidation by \textit{N. europaea} were performed by using pH 7.2 and a cell density of 6 \times 10^7 cells ml\textsuperscript{-1}.

By experimenting with moderately low cell densities we were able to examine the initial rates of MeBr oxidation and the accompanying rates of NO\textsubscript{2}\textsuperscript{-} production at different NH\textsubscript{4}\textsuperscript{+} concentrations (Table 1). The highest rates of MeBr oxidation occurred in the presence of 2.5 to 10 mM NH\textsubscript{4}\textsuperscript{+} and 0.22 to 0.44 mM MeBr. The responses of the initial rate of MeBr oxidation to NH\textsubscript{4}\textsuperscript{+} concentration between 1 and 10 mM, despite the fact that the level of NO\textsubscript{2}\textsuperscript{-} production changed fivefold over this concentration range. In the presence of 0.44 mM MeBr, the rate of MeBr oxidation increased twofold, while the level of NO\textsubscript{2}\textsuperscript{-} production decreased threefold as the NH\textsubscript{4}\textsuperscript{+} 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 NH\textsubscript{4}\textsuperscript{+} concentration at concentrations between 1 and 10 mM, despite the fact that the level of NO\textsubscript{2}\textsuperscript{-} 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 NH\textsubscript{4}\textsuperscript{+} concentration at 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 NO\textsubscript{2}\textsuperscript{-} 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 NH\textsubscript{4}\textsuperscript{+} 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 NO\textsubscript{2}\textsuperscript{-} 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 NO\textsubscript{2}\textsuperscript{-} 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 NO\textsubscript{2}\textsuperscript{-} production were very similar (2 to 3 \( \mu \)mol of NO\textsubscript{2}\textsuperscript{-} produced mg [dry weight] of cells\textsuperscript{-1} h\textsuperscript{-1}). Despite relatively large differences in the rate and duration of active MeBr oxidation for the different NH\textsubscript{4}\textsuperscript{+}-MeBr combinations, similar amounts of MeBr were oxidized with a large number of the NH\textsubscript{4}\textsuperscript{+}-MeBr combinations (1 to 2 \( \mu \)mol per vial, 10 to 20 \( \mu \)mol mg [dry weight] of cells\textsuperscript{-1}) (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) NO\textsubscript{2}\textsuperscript{-} and/or formaldehyde accumulation, (ii) a decrease in pH (pH 7.2 to 7.0), (iii) NH\textsubscript{4}\textsuperscript{+} limitation (10 to 7.5 mM), or (iv) inadequate medium composition (buffer versus growth medium) was directly responsible for the decreases in NO\textsubscript{2}\textsuperscript{-} producing ability and MeBr-oxidizing ability. For example, when assays were initiated at pH 7 in the presence of 7.5

### Table 1. NO\textsubscript{2}\textsuperscript{-} production and MeBr oxidation by \textit{N. europaea} in the presence of different combinations of NH\textsubscript{4}\textsuperscript{+} and MeBr\textsuperscript{+}

| NH\textsubscript{4}\textsuperscript{+} concn (mM) | Rate of NO\textsubscript{2}\textsuperscript{-} production in the absence of MeBr | Rate of NO\textsubscript{2}\textsuperscript{-} production | Rate of MeBr oxidation | M/N ratio | Rate of NO\textsubscript{2}\textsuperscript{-} production | Rate of MeBr oxidation | M/N ratio | Rate of NO\textsubscript{2}\textsuperscript{-} production | Rate of MeBr oxidation | M/N ratio |
|----------------|---------------------------------|---------------------------------|----------------------|-----------|---------------------------------|----------------------|-----------|---------------------------------|----------------------|-----------|
| 1              | 5.7                             | 3.7 (0.2)                       | 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 (0.11)          | 0.07      | 6.0 (0.3)                       | 1.23 (0.21)          | 0.21      | 3.1 (0.4)                       | 0.94 (0.27)          | 0.30      |
| 5              | 14.3                            | 10.8 (0.5)                      | 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      |

*Because the durations of the initial rates of NO\textsubscript{2}\textsuperscript{-} production and MeBr oxidation varied considerably for the different combinations of NH\textsubscript{4}\textsuperscript{+} 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.

*The values in parentheses are the standard deviations of the means based on the results obtained for three replicates per treatment.
TABLE 2. MeBr transformation capacities of *N. europaea* when it was incubated in the presence of different combinations of NH$_4^+$ and MeBr

| NH$_4^+$ concn (mM) | MeBr concn (mM) | Amt of MeBr consumed (µmol) | Amt of NO$_2^-$ produced (µmol) |
|---------------------|------------------|-----------------------------|---------------------------------|
| 10                  | 0.11             | 72                          | 1.25 (0.22)$^a$                  | 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)                      |

$^a$ The values in parentheses are the standard deviations of the means based on the results obtained for three replicates per treatment.

mM NH$_4^+$ and different combinations of 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 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 NH$_4^+$ added along with the cells increased the rate of 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 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 NH$_4^+$ concentrations (2.5 to 20 mM NH$_4^+$) (Table 1) (12) over which the rate of 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 NO$_2^-$-producing (data not shown) and NH$_4^+$-dependent O$_2$ uptake (Table 3) activities had been lost. Much less of the whole-cell hydroxylamine (NH$_2$OH)-dependent O$_2$ uptake activity was lost (20 to 30%) after exposure to MeBr. Recovery of 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 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 NO$_2^-$ production, and these cells were used as a control. We found that the NO$_2^-$ concentration increased immediately in a nonlinear manner in uptake (Table 3) activities had been lost.

**FIG. 2.** Effects of NO$_2^-$, formaldehyde, pH, and NH$_4^+$ on the MeBr consumed by *N. europaea*. All assay mixtures contained 6 $\times$ 10$^7$ cells of *N. europaea* ml$^{-1}$, and the initial conditions were as follows: pH 7.0, 7.5 mM NH$_4^+$, and 0.22 mM MeBr. NO$_2^-$ and formaldehyde were added when appropriate to final concentrations of 2.5 and 0.5 mM, respectively. Symbols: □, NO$_2^-$ and formaldehyde both present; ●, NO$_2^-$ present and formaldehyde absent; ◇, NO$_2^-$ absent and formaldehyde present; ▲, 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.

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

| MeBr concn (mM) | O$_2$ uptake rate (µmol mg [dry wt] of cells$^{-1}$ h$^{-1}$) $^b$ | NH$_4^+$ dependent | NH$_2$OH dependent |
|-----------------|---------------------------------------------------------------|-------------------|---------------------|
| 0               | 22.4 (2.6)$^b$                                                 | 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)         |                     |

$^a$ Oxygen uptake rates were determined after incubation for 24 h in the presence of 10 mM 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.

$^b$ The values are means based on three or more replicate experiments. The values in parentheses are the standard deviations of the means.
been reported that formaldehyde (28) and NO₂⁻ formation by the end products of cooxidation. Nonetheless, it has not been possible to assess the magnitude of toxic species generated intracellularly at any time during cooxidation of MeBr 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 NH₄⁺. Symbols: A, growth medium, pH 7.2; B, growth medium, pH 8; C, growth medium, pH 7.2; D, growth medium, pH 8. For clarity error bars are not shown. The standard deviations were ±10% of the mean values regardless of the treatment.

![Diagram](https://via.placeholder.com/150)

**FIG. 4.** Development of NO₂⁻ 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 NH₄⁺. Symbols: A, growth medium, pH 7.2; B, growth medium, pH 8; C, growth medium, pH 7.2; D, growth medium, pH 8. For clarity error bars are not shown. The standard deviations were ±10% of the mean values regardless of the treatment.

both exposed and unexposed cells at both pH values. Although the amount of NO₂⁻ 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 NO₂⁻ 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 NO₂⁻ 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 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 μmol 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 μmol per assay mixture). It is not surprising, therefore, that they did not determine the finite capacity of *N. europaea* to oxidize MeBr and that NO₂⁻ production and NH₄⁺-dependent O₂ uptake activities declined considerably as a consequence of prolonged MeBr oxidation.

At first we were confused by our finding that both NO₂⁻-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 NO₂⁻ in the absence of NH₄⁺ (20) inhibit NH₄ 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 NH₄⁺-dependent O₂ uptake was reduced more severely by exposure to MeBr than NH₂OH-dependent O₂ 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 NH₄⁺-dependent O₂ uptake activity in *N. europaea* was eliminated by specifically inactivating AMO with strong light, NO₂⁻-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 NH₄⁺-dependent O₂ uptake activity was debilitated, required about 48 to 60 h of incubation to exhibit a rate of NO₂⁻ production comparable to the initial rate detected in unexposed cells. Indeed, the effect of long-term oxidation of MeBr on NO₂⁻-producing activity is more similar to what occurred when *N. europaea* lost approximately 90% of its NH₄⁺-dependent O₂ uptake activity during short-term cooxidation of trichloroethylene. In that case, very little NO₂⁻-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 NO₂⁻ 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 NO₂⁻ production probably reflected proliferation of the surviving cells. It is not clear, however, why development of NO₂⁻ production by the cells exposed to MeBr lagged behind development of NO₂⁻ 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 NO₂⁻ 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 NH₄⁺ availability (21, 22), that de novo protein synthesis is extremely limited in cells exposed to 10 mM NH₄⁺ at pH 7 (5), and that production of the mRNA transcript for AMO is limited when rates of NO₂⁻ production are supported by ≤2 mM NH₄⁺ at pH 7.5 (19). The faster development of NO₂⁻ production by the unexposed cells might have been due to upregulation of NH₄⁻-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 NO₂⁻ 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 NH₄ 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 NH₄ 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 NH₄⁻-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 NH\textsubscript{4}\textsuperscript{+} availability.

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