APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1995, p. 1480–1487 Vol. 61, No. 4
0099-2240/95/$04.00+0

Inhibition, Inactivation, and Recovery of Ammonia-Oxidizing Activity in Cometabolism of Trichloroethylene by *Nitrosomonas europaea*

MICHAEL R. HYMAN,[1]* STERLING A. RUSSELL,[1] ROGER L. ELY,[2] KENNETH J. WILLIAMSON,[2] AND DANIEL J. ARP[1]

Laboratory for Nitrogen Fixation Research, Department of Botany and Plant Pathology,[1] and Department of Civil Engineering,[2] Oregon State University, Corvallis, Oregon 97331-2902

Received 4 November 1994/Accepted 1 February 1995

The kinetics of the cometabolism of trichloroethylene (TCE) by the ammonia-oxidizing soil bacterium *Nitrosomonas europaea* in short-term (<10-min) incubations were investigated. Three individual effects of TCE cometabolism on this bacterium were characterized. First, we observed that TCE is a potent competitive inhibitor of ammonia oxidation by *N. europaea*. The K_i value for TCE (30 μM) is similar to the K_m for ammonia (40 μM). Second, we examined the toxicity associated with TCE cometabolism by *N. europaea*. Stationary-phase cells of *N. europaea* oxidized approximately 60 nmol of TCE per mg of protein before ammonia-oxidizing activity was completely inactivated by reactive intermediates generated during TCE oxidation. At the TCE concentrations used in these experiments, ammonia did not provide significant protection against inactivation. Third, we have determined the ability of cells to recover ammonia-oxidizing activity after exposure to TCE. Cells recovering from TCE inactivation were compared with cells recovering from the specific inactivation of ammonia-oxidizing activity by light. The recovery kinetics were indistinguishable when 40% or less of the activity was inactivated. However, at increased levels of inactivation, TCE-inactivated cells did not recover as rapidly as light-inactivated cells. The kinetics of recovery appear to be dependent on both the extent of inactivation of ammonia-oxidizing activity and the degree of specificity of the inactivating treatment.

Over the past 40 years, trichloroethylene (TCE) has been used extensively as a solvent and degreasing agent. A legacy of this large-scale use is that TCE is currently the most widely distributed organic groundwater pollutant in the United States (27, 38). In view of the widespread environmental distribution and the suspected carcinogenicity of TCE (25), considerable effort has been focused on methods to remediate this contamination. Biological processes have received much attention, principally because the costs of bioremediation are potentially lower than physical methods of removing TCE from contaminated soils. Although no microorganism which can grow on TCE as a sole carbon or energy source has been isolated, alternative approaches using cometabolism have been more successful and several physiological types of bacteria which can cometabolize and partially or fully degrade TCE have been identified (1, 11, 14, 28, 32). Cometabolism is the fortuitous biotransformation of a non-growth-supporting compound by a microorganism; the biotransformation is catalyzed by a nonspecific enzyme or cofactor. Cometabolism also requires that these transformations be concurrent with the metabolism of a growth-supporting substrate or another transformable compound (8).

In anaerobic organisms such as methanogens (35), TCE can be cometabolically transformed as a non-growth-supporting electron acceptor. Reduction of TCE results in dechlorination reactions which generate products with progressively decreasing levels of chlorination (35). In contrast, TCE cometabolism under aerobic conditions can be catalyzed by various nonspecific monooxygenase enzymes which are normally produced to initiate the oxidation of growth-supporting substrates (12). To date, TCE has been shown to be oxidized by bacteria that oxidize toluene (37), phenol (14), methane (29), propane (28, 36), propylene (11), cumene (7), isoprene (13), and ammonia (4, 32).

In the present study, we considered the effects of TCE cometabolism on the ammonia-oxidizing bacterium *Nitrosomonas europaea*, which obtains all of its energy for growth from the oxidation of ammonia to nitrite. Ammonia is initially oxidized to hydroxylamine by the enzyme ammonia monooxygenase (AMO) (39) as follows: $NH_3 + O_2 + 2[H] \rightarrow NH_2OH + H_2O$.

The further four-electron oxidation of hydroxylamine to nitrite is catalyzed by hydroxylamine oxidoreductase (HAO) as follows: $NH_2OH + O_2 \rightarrow NO_2^- + H_2O + H^+$.

In vivo, this second reaction is the only source of the two electrons required to maintain steady-state AMO activity. The remaining two electrons are utilized for ATP synthesis through a conventional electron transport chain (39). Previous studies have shown that whole cells of *N. europaea* can oxidize a wide variety of alternative hydrocarbon substrates through the action of AMO (20–23). These substrates include alkanes, alkenes, and aromatic compounds. More recently, it has been shown that *N. europaea* can also oxidize a wide variety of chlorinated aliphatic compounds, including TCE (30, 31, 34). In general, the oxidation products obtained from alternative substrates are not assimilated or further metabolized by *N. europaea* and accumulate extracellularly.

Much of the interest surrounding cometabolism of TCE is focused on practical issues such as the relative merits of aerobic and anaerobic systems. The principal advantage of aerobic processes over anaerobic cometabolic processes is that TCE can be mineralized to CO_2, H_2O, and chloride ions without the accumulation of stable and carcinogenic intermediates such as vinyl chloride (35). Another important practical issue is the rate of TCE degradation. Previous studies of TCE degradation have often considered the maximal short-term rate. However,

* Corresponding author. Phone: (503) 737-4214. Fax: (503) 737-3573. Electronic mail address: hymanm@bcc.orst.edu.

because of the large amounts of TCE present at many contaminated sites and the protracted periods often required for biological cleanup, a more appropriate rate to consider is the maximal sustainable rate of TCE oxidation.

In the present study, three factors which influence the maximal sustainable rate of TCE degradation are considered. The first factor is the competition which often exists between the growth-supporting substrate and TCE for oxidation by the nonspecific monooxygenases (12). In simple enzymatic terms, competition occurs because of the simultaneous presence of two substrates for the same enzyme. For an enzyme considered in isolation, this competition results in an inhibition of the oxidation of the primary growth-supporting substrate. However, at the whole-cell level this effect is more complex and competition can inhibit the overall cometabolism process through a combination of direct and indirect effects. For example, ammonia oxidation by *N. europaea* generates hydroxylamine and hydroxylamine oxidation supplies the reductant required to support continued AMO activity. The direct inhibition of ammonia oxidation by TCE determines the relative amounts of ammonia oxidation and TCE oxidation. The indirect effects of TCE occur through the effects of decreased ammonia oxidation on hydroxylamine formation and the overall rate of electron flux through both HAO and AMO. A further complication to the question of competition is that several aerobic TCE-degrading bacteria require specific inducer compounds to allow these bacteria to simultaneously grow and produce the correct TCE-degrading enzymes. These inducing compounds are typically also substrates for the TCE-degrading nonspecific monooxygenase (12).

The second factor which influences the sustainability of TCE oxidation is the toxicity associated with TCE oxidation. This toxicity is usually observed as an irreversible inactivation of monooxygenase activity and is distinct from the reversible inhibition of monooxygenase activity discussed above. It has been suggested that this toxicity is caused by a reaction of cell components with short-lived reactive intermediates generated during the oxidation of TCE (12, 15, 29, 32). For *N. europaea*, [^{14}C]TCE oxidation by whole cells results in covalent attachment of ^{14}C label to the 27-kDa active-site-containing polypeptide of AMO, in addition to other proteins (32).

A third factor affecting the maximal sustainable rate of TCE oxidation is the ability of cells to recover from the damage caused by the toxicity associated with TCE oxidation. We have previously observed that cells of *N. europaea* exposed to TCE can recover from the inactivating effects of TCE oxidation in a process which requires de novo protein synthesis (19, 32). While this effect is not likely to be significant in short-term reactions (e.g., a few minutes), it will be an important factor in sustainable reactions extended over many hours and days. The maximal sustainable rate of TCE oxidation is likely to be obtained at the point where the ability of the cells to oxidize TCE is balanced against the ability to repair and recover from the concurrent cellular damage caused by TCE oxidation products.

The aim of the experiments described in this study has been to examine each of these three effects of TCE cometabolism in as much isolation from the other effects as possible. The results indicate that inhibition, inactivation, and recovery of ammonia-oxidizing activity are all important factors that contribute to determining the maximal sustainable rate of TCE degradation by *N. europaea.*

MATERIALS AND METHODS

Materials. Cells of *N. europaea* (ATCC 19178) were grown and harvested as described previously (19), except that cells were washed and stored in chloride-free 50 mM sodium phosphate buffer, pH 7.8. TCE (spectrophotometric grade, >99.9%) was obtained from Aldrich Chemical Co. (Milwaukee, Wis.). Allylthiourea was obtained from Eastman Kodak Co. (Rochester, N.Y.). All other chemicals were of reagent grade.

O_2 uptake measurements. Ammonia- and hydrazine-dependent O_2 uptake measurements were determined with a Clark (Yellow Springs, Ohio) style O_2 electrode mounted in a glass water-jacketed reaction vessel (1.8 ml), as described previously (19). The effects of TCE on ammonia-dependent O_2 uptake activity were determined by incubating cells with NH_4Cl (1, 2.5, or 10 mM) and various concentrations of TCE. Once a steady rate of ammonia-dependent O_2 uptake had been established, TCE from a TCE-saturated solution in buffer was added to the reaction mixture. The subsequent time-dependent loss of ammonia-dependent O_2 uptake activity was used to estimate both an initial inhibitory effect of TCE on ammonia oxidation and the rate of inactivation of ammonia-oxidizing activity resulting from the oxidation of TCE.

The inactivating effects of TCE on ammonia-oxidizing activity in fixed-time assays were also determined by measuring O_2 uptake rates. Cells were exposed to TCE for 10 min and then were washed to remove residual TCE (see the paragraph below). The rate of ammonia-dependent O_2 uptake was then determined for cells incubated in the presence of 10 mM NH_4Cl. Allylthiourea (100 μM) was then added to inhibit further ammonia-dependent O_2 uptake. Hydrazine hydrochloride was then added to a final concentration of 750 μM. The hydrazine-dependent O_2 uptake was measured and was used as an estimate of residual HAO activity. All substrates and inhibitors were added from aqueous stock solutions by means of gas-tight microsyringes. The solubility of O_2 in air-saturated buffer was assigned a value of 230 μM (33). Estimates of the quantities of TCE used in each incubation were based on the aqueous solubility of TCE of 8.4 mM at 20°C (18).

Incubations with TCE. The effects of TCE-dependent inactivation on ammonia- and hydrazine-oxidizing activities were determined by incubating cells with TCE in glass serum vials (10 ml). The vials were prepared by adding 50 mM sodium phosphate buffer, pH 7.8 (up to 900 μl), and $(NH_4)_2SO_4$ (up to 25 mM NH_4^+). The vials were sealed with Teflon-lined silicone stoppers (Alltech Associates Inc., Deerfield, Ill.), and various volumes of TCE-saturated buffer (up to 900 μl) were added. The vials were placed inverted in a shaking water bath (30°C, 150 rpm) for 5 min to allow an equilibrium distribution of TCE between the gas and liquid phases to be established. The reactions were initiated by the addition of cells (100 μl; approximately 2 mg of protein) to yield a final reaction volume of 1 ml. The vials were then returned to the shaking water bath. After 10 min, the vials were removed and a sample of the reaction medium (900 μl) was added to a microcentrifuge tube (1.5 ml) and centrifuged for 30 s (14,000 × *g*). A sample (750 μl) of the resulting supernatant was removed and stored at 4°C for subsequent analysis for nitrite and chloride ions. The remaining supernatant was decanted, and the cell pellet was quickly resuspended in fresh buffer (1 ml). The cells were sedimented again by centrifugation and were then resuspended in fresh buffer (200 μl). The washed cells were stored at 4°C in the dark until analysis for residual ammonia and hydrazine-dependent O_2 uptake activity, as described above. The ammonia-dependent activities of cells stored in this way remained constant for at least 3 h.

Inactivation of ammonia-oxidizing activity by light. The ammonia-oxidizing activity in cells was selectively inactivated by light, as described previously (19). In summary, a fresh cell suspension (50 ml at approximately 0.2 mg of protein per ml) in sodium phosphate buffer was added to a 160-ml glass serum vial mounted on an orbital shaker (200 rpm). The cells were illuminated by a 500-W tungsten-halogen projector bulb at a distance of 30 cm. After various periods of illumination, samples (5 ml) were removed from the vial. The residual ammonia-dependent O_2 uptake activity was determined by using an aliquot (200 μl) of this sample added directly to the oxygen electrode chamber. The remainder of the sample was stored at 4°C in the dark. The residual ammonia- and hydrazine-oxidizing activities of cells stored in this way remained constant for at least 3 h (data not shown). Once all the samples had been prepared, the cells were sedimented by centrifugation (10,000 × *g* for 10 min). The sedimented cells were then resuspended in 1 ml of sodium phosphate buffer and were used for further experiments within 1 h.

Nitrite, chloride, and protein assays. The nitrite level was determined colorimetrically as described previously (17). The chloride level was determined colorimetrically as described previously (5). Protein concentrations were determined by the biuret assay (16) after solubilization of cell protein in aqueous 3 N NaOH (30 min at 60°C) and sedimentation of insoluble material by centrifugation (14,000 × *g*, 5 min).

RESULTS

Inhibition of ammonia-oxidizing activity by TCE. The effects of inhibitors of ammonia oxidation can be observed continuously and nearly instantaneously by monitoring NH_4^+-dependent O_2 uptake with an O_2 electrode. A typical response of cells to the addition of a range of TCE concentrations during steady-state ammonia oxidation is shown in Fig. 1. The electrode traces can be separated into three phases. First, the

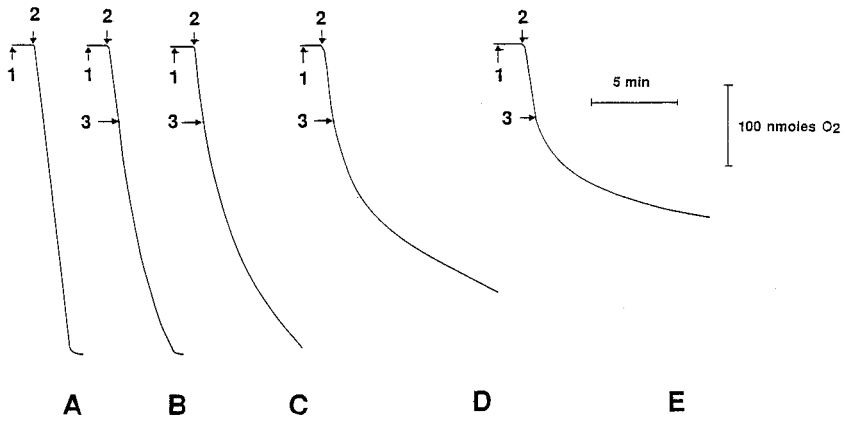

FIG. 1. Ammonium-dependent O_2 uptake by *N. europaea* in the presence of TCE. O_2 uptake was monitored as described in Materials and Methods. The numbers next to each trace indicate the times of addition of ammonia (added as NH_4Cl, 10 mM) (addition 1), cells (40 μg of protein) (addition 2), and TCE (addition 3). TCE was added in the traces to the final concentrations indicated in parentheses: trace A (0 μM), trace B (23 μM), trace C (46 μM), trace D (92 μM), and trace E (184 μM).

addition of NH_4Cl (10 mM) to the reaction mixture resulted in a steady rate of O_2 uptake. Second, the addition of TCE (up to 184 μM) resulted in an immediate decrease in the rate of O_2 uptake. The extent of the decrease was dependent on the TCE concentration, and this effect represents an immediate inhibitory effect of TCE on ammonia-oxidizing activity. The third phase was characterized by a progressive loss of the remaining ammonia-dependent O_2 uptake activity. This phase represents the time-dependent inactivation of ammonia-oxidizing activity which occurs as a result of the cometabolic oxidation of TCE by AMO. The rate of decrease in the O_2 uptake activity was also dependent on the TCE concentration present during the incubation.

The effects of a range of TCE concentrations on ammonia-dependent O_2 uptake rates were examined to estimate the effectiveness of TCE as a rapid-equilibrium inhibitor of ammonia oxidation. Rates of ammonia-dependent O_2 uptake were measured for cells incubated in the presence of 1, 2.5, or 10 mM NH_4Cl. TCE was added to the electrode chamber at several concentrations up to 184 μM. The O_2 uptake traces obtained in the presence of TCE were nonlinear (Fig. 1), thereby preventing a direct measurement of the initial inhibitory effect of TCE. To estimate the initial inhibitory effect, the residual rates of O_2 uptake were determined at 1-min intervals after the addition of TCE. When these rates were plotted in semilog form versus time, the plots were nearly linear ($R^2 \geq 0.983$) but did not extrapolate to 100% activity at time zero. The initial inhibitory effect of each TCE concentration was then determined by extrapolating the semilog plots to time zero and calculating the difference between the initial activity (100%) and the extrapolated initial activity. The reciprocals of these extrapolated TCE-inhibited rates of ammonia-dependent O_2 uptake versus the concentration of TCE added to the reaction mixture were plotted in a Dixon plot (Fig. 2). The plot shows that increasing concentrations of TCE led to increased inhibition of O_2 uptake for cells incubated with all concentrations of NH_4Cl. The intercept in the upper left-hand quadrant of Fig. 2 is characteristic of a competitive interaction (6), and this plot provides an estimate of the K_i for TCE of 30 μM.

Inactivation of ammonia- and hydroxylamine-oxidizing activity by TCE. The inactivation of ammonia-oxidizing activity resulting from TCE oxidation was quantified by two separate approaches. In one approach, cells were first exposed to TCE for 10 min. The cells were washed to remove the TCE, and then residual ammonia- and hydrazine-oxidizing activities were determined. Both ammonia- and hydrazine-oxidizing activities were inactivated during the exposure to TCE, although the extents of the inactivation were different for the two activities (Fig. 3A). The inactivation of ammonia-oxidizing activity was dependent on the TCE concentration, and greater than 95% of the activity was lost in the presence of 4.2 μmol of TCE (1.15 mM TCE in solution) during the 10-min incubation. In contrast, HAO-dependent hydrazine-oxidizing activity was inactivated by approximately 50% in the presence of 0.21 μmol of TCE (57.5 μM TCE in solution) but the residual activity changed very little with further additions of up to 7.6 μmol of TCE (2.08 mM TCE in solution). However, not all of the inactivation of HAO-dependent hydrazine-oxidizing activity was dependent on the presence of active AMO or TCE oxidation. Treatment of cells with acetylene results in the complete inactivation of ammonia-oxidizing activity without effects on

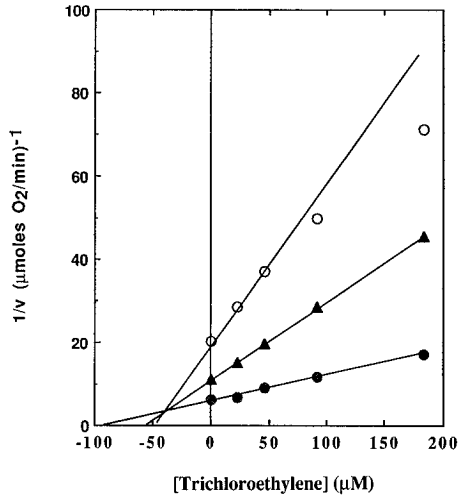

FIG. 2. Competitive inhibition of NH_4^+-dependent O_2 uptake by TCE. O_2 uptake was monitored as described in Materials and Methods. The rates of O_2 uptake immediately after the addition of TCE were determined as described in Results. The reciprocal of the extrapolated rate of NH_4^+-dependent O_2 uptake immediately after the addition of TCE is plotted against the concentration of TCE added to cells incubated with 1 mM (○), 2.5 mM (▲), and 10 mM (●) NH_4^+. v, rate of NH_4^+-dependent O_2 uptake.

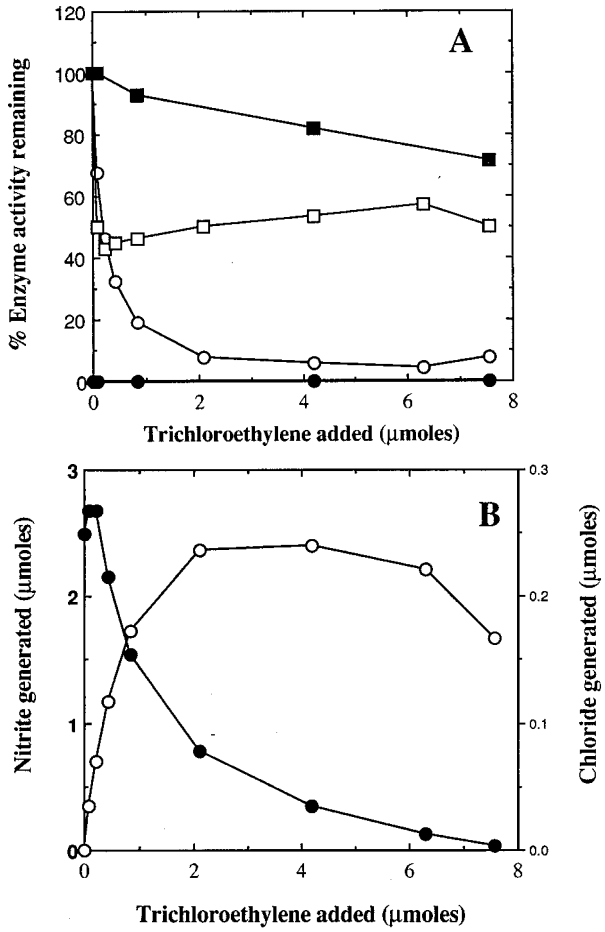

FIG. 3. Inactivation of ammonia- and hydrazine-oxidizing activities after exposure of cells to TCE. Cells were exposed to TCE, and O_2 uptake measurements were made as described in Materials and Methods. (A) Residual NH_4^+-dependent (circles) and hydrazine-dependent (squares) O_2 uptake rates for active (○ and □) and acetylene-treated (1% [vol/vol] for 30 min) (● and ■) cells incubated with NH_4^+ (5 mM) and the indicated amounts of TCE. (B) Amounts of nitrite (●) and chloride (○) ions generated in the same incubation conditions with active cells.

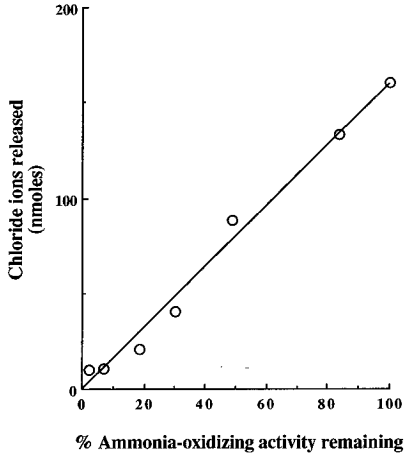

FIG. 4. TCE-dependent chloride ion release by cells after treatment with light. Ammonia-oxidizing activity in cells was inactivated to various degrees by exposing cells to light from a projector bulb. The residual ammonia- and hydrazine-dependent O_2 uptake activities were then determined, as described in Materials and Methods. The cells were then incubated in 1-ml reaction volumes (see Materials and Methods) in the presence of 4.2 μmol of TCE (1.15 mM TCE in solution). After a 10-min incubation, the cells were harvested from the TCE-containing incubation mixtures and the supernatant was analyzed for the presence of chloride ions. Complete inactivation of ammonia-dependent O_2 uptake activity was confirmed by O_2 uptake measurements. The amount of TCE-dependent chloride ion released is plotted against the residual ammonia-dependent O_2 uptake activity after treatment of the cells with light.

hydrazine-oxidizing activity (24). When acetylene-treated cells were incubated with TCE, hydrazine-dependent O_2 uptake activity was inactivated although the extent of inactivation was proportional to the concentration of TCE (Fig. 3A).

The experimental approach described above allowed the relationship between TCE degradation and inactivation of ammonia-oxidizing activity to be quantified. The amounts of chloride and nitrite ions produced during each of the incubations described in Fig. 3A are shown in Fig. 3B. Chloride ion release resulting from TCE oxidation was directly associated with the extent of loss of ammonia-oxidizing activity. No further chloride ion release was observed after ammonia-oxidizing activity was completely (>95%) inactivated. The results in Fig. 3B also demonstrate that increasing concentrations of TCE led to a concomitant decrease in the amount of nitrite ions generated from ammonia. No nitrite or chloride ions were observed for the same incubation conditions with acetylene-treated cells (data not shown). Together, these observations indicate that all chloride ion release from TCE is due to ammonia-oxidizing activity. Accordingly, the amount of chloride ions released after ammonia-oxidizing activity is completely inactivated reflects the maximum quantity of TCE that can be degraded by cells during these short-term incubations. Assuming that TCE is completely dehalogenated (32), the *N. europaea* cells used in this experiment oxidized 58 nmol of TCE per mg of protein before complete inactivation of ammonia-oxidizing activity.

The amount of TCE required to inactivate AMO should vary with the total amount of AMO present in a given cell mass (i.e., the specific AMO activity), and the specific AMO activity of cells is dependent on factors such as the phase of the growth cycle when the cells were harvested. Indeed, the amount of TCE oxidation required to fully inactivate AMO varied from 30 nmol of TCE per mg of protein for cells harvested in late stationary phase, with low specific rates of ammonia-oxidizing activity, to 105 nmol of TCE per mg of protein for cells harvested during logarithmic growth which exhibited high specific rates of ammonia-oxidizing activity.

A further experiment was conducted to confirm that a direct relationship exists between the level of ammonia-oxidizing activity and the amount of TCE which can be oxidized before all ammonia-oxidizing activity is inactivated. The ammonia-oxidizing activity of cells was decreased by known amounts by illuminating the cells with light from a projector bulb. Light is a specific inactivator of AMO activity (19), and the time-dependent effects of light can be stopped by simply removing the source of illumination. Cells with various specific ammonia-oxidizing activities generated by illumination were incubated for 10 min with sufficient TCE to completely inactivate (>99%) the remaining ammonia-oxidizing activity. Chloride ion release from TCE oxidation was then determined. The results of this experiment (Fig. 4) confirm that there is a linear relationship ($R^2 = 0.987$) between the level of TCE-dependent chloride ion release and the level of ammonia-oxidizing activity remaining after light inactivation. In this experiment, nonilluminated control cells oxidized 63 nmol of TCE per mg of protein prior to complete inactivation of ammonia-oxidizing activity.

Additional experiments similar to those described in the

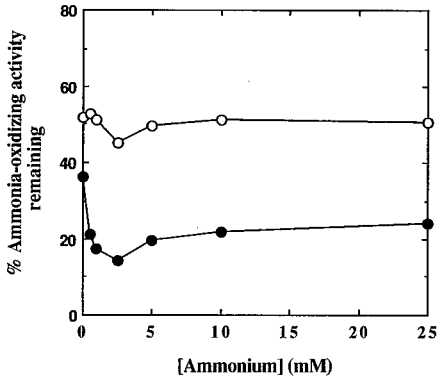

FIG. 5. Effect of NH_4^+ concentration on inactivation of ammonia-oxidizing activity by TCE. Cells were exposed to TCE, and O_2 uptake measurements were made as described in Materials and Methods. The figure shows the residual NH_4^+-dependent O_2 uptake rates for cells incubated for 10 min with 0.21 (○) and 0.84 (●) μmol of TCE (57.5 and 230 μM TCE in solution, respectively) in the presence of the indicated concentrations of NH_4^+.

legend to Fig. 3 were also conducted to examine whether the presence of ammonia protected the cells against TCE-dependent inactivation of ammonia-oxidizing activity. Cells were exposed for 10 min to one of two initial quantities of TCE (0.21 or 0.84 μmol [58 or 232 μM in solution, respectively]) in the presence of various concentrations of $(NH_4)_2SO_4$ (0 to 12.5 mM). The cells were then washed to remove the TCE and were assayed for residual ammonia-oxidizing activity. The results (Fig. 5) show that the maximal extent of TCE-dependent inactivation of ammonia-oxidizing activity occurred in the presence of 2.5 mM NH_4^+. Only slightly less inactivation was observed at lower and higher NH_4^+ concentrations. The same trend was confirmed by examining the quantity of chloride ions released during each incubation (data not shown).

The recovery of ammonia-oxidizing activity in TCE-treated cells. The third response of cells to TCE cometabolism considered in this study was the rate of recovery of ammonia-oxidizing activity after the exposure of cells to TCE. Cells in which ammonia- and hydrazine-oxidizing activities have been partially inactivated by TCE cometabolism can fully recover ammonia-dependent nitrite-generating activity over the course of several hours (19, 32). This recovery of enzyme activity is inhibited by both rifampin and chloramphenicol and is therefore thought to involve de novo protein synthesis (19, 32). In this study, we compared the rate of recovery of ammonia-oxidizing activity for cells treated with TCE and that for cells treated with light. This experiment enabled us to compare the effects of an AMO-specific inactivating treatment (light inactivation) with the less-specific effects resulting from TCE cometabolism.

Ammonia-oxidizing activity in cells was inactivated to various degrees by exposure to either TCE (as for Fig. 3) or light (as for Fig. 4). The cells were then washed before being inoculated into fresh medium. The time-dependent accumulation of nitrite in the medium was monitored and was used as an estimate of the extent of recovery of ammonia-oxidizing activity. The results indicate that even cells in which ammonia-oxidizing activity had been completely inactivated with light (Fig. 6A) were able to fully recover ammonia-dependent nitrite-generating activity within 4 h. In contrast, TCE-treated cells showed a more complex behavior. At the low levels of inactivation of ammonia-oxidizing activity, TCE-treated cells were able to recover quickly. However, with increased levels of inactivation the rate of recovery was much lower than that for

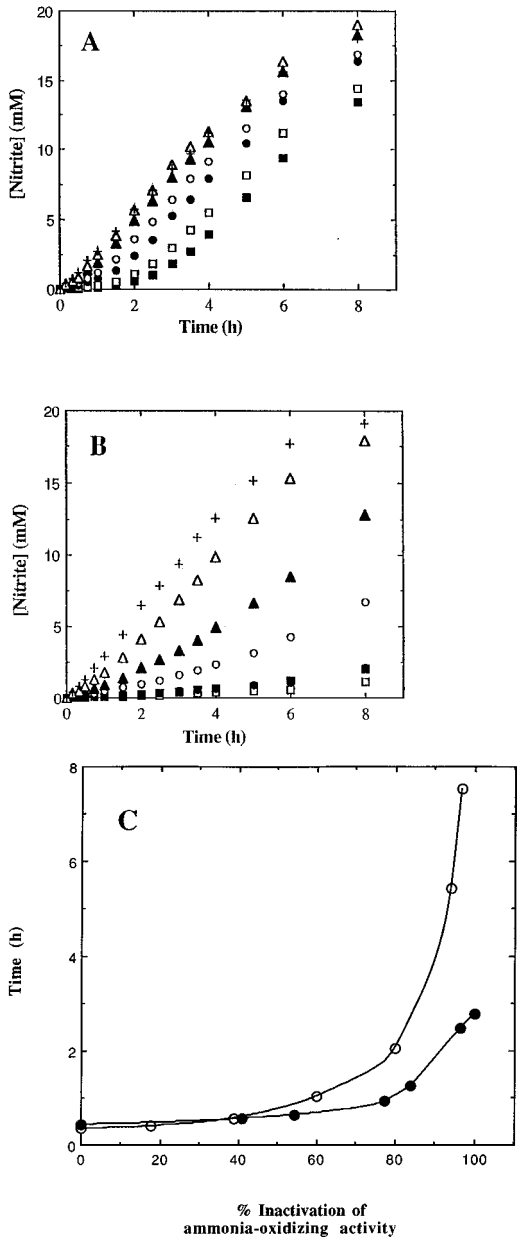

FIG. 6. Time course for recovery of ammonia-oxidizing activity in cells after treatment with TCE or light. Ammonia-oxidizing activity in cells was inactivated to various extents by TCE (as for the experiment for Fig. 3) and light (as for that for Fig. 4), as described in Materials and Methods. The inactivated cells were then washed and were added to 20 ml of growth medium containing 50 mM NH_4^+ in 75-ml glass flasks at a protein concentration of 290 μg/ml. (A) Time course of nitrite accumulation in the medium for cells with 100 (+), 59.1 (△), 45.5 (▲), 22.7 (○), 16 (●), 3.4 (□), and 0 (■)% residual NH_4^+-dependent O_2 uptake remaining after treatment with light. (B) Time course of nitrite accumulation in the medium for cells with 100 (+), 82.5 (△), 61.2 (▲), 40 (○), 20 (●), 5.8 (□), and 3.2 (■)% residual NH_4^+-dependent O_2 uptake remaining after treatment with TCE. (C) Plot of the time taken for cells treated with light (●) and TCE (○) to generate 1 mM nitrite versus the initial extent of inactivation of NH_4^+-dependent O_2 uptake activity.

light-inactivated cells. A direct comparison of the effects of light and TCE treatment is provided by comparing the times taken for each treatment to generate a fixed concentration of nitrite (1 mM) in the reaction medium. An example of this analysis is shown in Fig. 6C. These results demonstrate that the

rates of recovery for cells inactivated up to approximately 40% by light and TCE were equivalent. However, at elevated levels of inactivation there was an increasing disparity between the faster recovery from light inactivation and the slower recovery from TCE-dependent inactivation.

DISCUSSION

The data presented in this study have aimed to separate and quantify three important components of the response of *N. europaea* to the cometabolism of TCE. The three effects we have considered are (i) the inhibition of ammonia-oxidizing activity by TCE, (ii) the inactivation of ammonia-oxidizing activity by TCE, and (iii) the recovery of ammonia-oxidizing activity after the exposure of cells to TCE. These effects are discussed individually below.

(i) Inhibition of ammonia-oxidizing activity by TCE. Our results (Fig. 2) indicate that TCE is a competitive inhibitor of ammonia oxidation by *N. europaea*. The detection of competitive inhibition by TCE is surprising, since most C_2 compounds examined exhibit noncompetitive inhibition of ammonia oxidation (26) and only two alternative AMO substrates, methane (22) and ethylene (23), exhibit classical competitive behavior. Some of these unusual characteristics of TCE might be explained by the apparently high affinity AMO has for this compound. For example, the K_i for TCE (30 μM) we have estimated is low compared with those for the other alternative substrates known for AMO (22, 23, 26) and is similar to the K_m for NH_3 (approximately 40 μM) for AMO in whole cells (22). In the special case that a competitive inhibitor of an enzyme is also a substrate for that enzyme, the K_i for the compound as an inhibitor is the equivalent of the K_m for that compound as a substrate (6). This result indicates that the K_m for TCE for AMO is almost equivalent to that for the enzyme's natural substrate, ammonia. These close values may partly explain the limited protective effect which ammonia provides cells against inactivation of ammonia-oxidizing activity by TCE.

The K_i for TCE for *N. europaea* is comparable to the values obtained with other TCE-cometabolizing bacteria and enzymes. Fox et al. (15) determined a K_m of 35 μM for TCE for the purified soluble methane monooxygenase obtained from *Methylosinus trichosporium* OB3b, and a K_m for TCE of 145 ± 61 μM (29) was obtained with methanotroph cultures. The lowest K_i for TCE so far determined, 3 μM, was observed with toluene-oxidizing bacteria (14).

(ii) Inactivation of ammonia-oxidizing activity by TCE. In this study, we have demonstrated that chloride ion release from TCE oxidation is a saturable process and that it is closely associated with a TCE-dependent inactivation of ammonia-oxidizing activity. The quantitative measurement of chloride ion release from TCE has enabled us to calculate the amount of TCE which *N. europaea* can oxidize before ammonia-oxidizing activity is completely inactivated. For the majority of cells used in this study, these values were near 60 nmol/mg of protein.

It is important to consider three assumptions inherent in our calculations. First, the quantity of TCE oxidation required to inactivate ammonia-oxidizing activity can change as a function of the growth stage of the cells. We have measured values as high as 105 nmol of TCE per mg of protein, but values obtained for batch cells grown to early stationary phase averaged 60 nmol of TCE per mg of protein. These differences probably reflect changes in the actual amounts of active AMO per cell rather than changes in the number of active cells (19).

The second important assumption we have made is that three chlorine atoms are released as chloride ions after oxidation of each TCE molecule. A stoichiometry of three chloride ions released for each TCE molecule degraded has been demonstrated for *N. europaea* (32). However, the generation of incompletely dechlorinated products would increase the maximal amount of TCE which is transformable by resting cells. The third assumption we have made is that de novo protein synthesis (recovery) does not occur to any significant extent during the short-term (10-min) incubations of cells with TCE. A lack of de novo protein synthesis under these conditions indicates that our estimate of the TCE-degrading capability of *N. europaea* should be regarded as the minimum amount of TCE degradation which can be achieved by a fixed amount of bacteria.

The TCE-degrading capability of *N. europaea* discussed above has some similarity to the "transformation capacity" for TCE degradation by methanotrophic bacteria (2). This capacity was most recently defined as the maximum mass of cometabolized compound (contaminant) that can be transformed per unit of mass of resting cells (3). There are, however, significant differences between the assumptions and calculations involved in determining the two values. For example, our value is based on the amount of TCE which can be degraded by nongrowing cells during short-term incubations. In contrast, the transformation capacity for methanotrophs is usually determined in longer-term incubations (3 h) in the presence of formate as an electron donor (3). These incubation conditions may allow cells to replace a significant portion of TCE-inactivated methane monooxygenase activity through de novo protein synthesis.

In this study, we have also observed that ammonium concentration has little effect on the extent of TCE-dependent inactivation of ammonia-oxidizing activity (Fig. 5). This result is in contrast to the strong protective effect that ammonium provides against inactivation of AMO by other competitive inactivators such as acetylene (24). The reason why increased ammonium concentration does not prevent TCE-dependent inactivation is not fully apparent. The most significant practical aspect of this observation is that the extent of TCE-dependent inactivation cannot be controlled by manipulation of ammonium concentration and is therefore almost solely determined by TCE concentration and the rate of TCE oxidation.

(iii) Recovery of ammonia-oxidizing activity after exposure of *N. europaea* to TCE. The third effect we have examined in relation to TCE cometabolism by *N. europaea* is the ability of cells to recover from the inactivating effects resulting from TCE oxidation. In a previous study, we have shown that the recovery of ammonia-oxidizing activity after light inactivation requires de novo protein synthesis. The high rate of recovery relative to the long generation time of these microorganisms (8 to 12 h) and the lack of an increase in either HAO-dependent hydrazine-oxidizing activity or protein concentration during these recovery experiments (19) suggest that this recovery process is due to resynthesis of new proteins within preexisting cells rather than uncharacteristically rapid division of a subset of noninactivated cells (19). Furthermore, we have also established that the rate of nitrite generation is an accurate measure of the quantity of active AMO present in cells recovering from inactivating treatments (19). In the present study, we have extended these observations and have demonstrated that the rate of recovery from either light- or TCE-dependent inactivation of ammonia-oxidizing activity is dependent on the initial level of inactivation of ammonia-oxidizing activity (Fig. 6). In summary, these data demonstrate that a greater extent of inactivation of ammonia-oxidizing activity entails a longer recovery phase. However, it is difficult to establish a quantitative measurement of the complex recovery process from our data

because the kinetics are independently influenced by several factors. The two most important factors are discussed below.

The first factor influencing the rate of recovery is the extent of inactivation of ammonia-oxidizing activity. We have already established that recovery from both TCE inactivation and light inactivation of ammonia-oxidizing activity is dependent on protein synthesis (19, 32). Protein synthesis is energy intensive, and ammonia oxidation is the primary and most significant source of energy available to *N. europaea* for biosynthetic purposes. It follows that an inverse relationship should exist between the energy-generating capacity, measured as residual ammonia-oxidizing activity, and the observed rate of recovery of inactivated ammonia-oxidizing activity. This relationship is confirmed by our data, which demonstrate that cells with the greatest loss of ammonia-oxidizing activity exhibit the lowest rate of recovery of nitrite-generating activity (Fig. 6A). A further important point is that the rate of recovery should not be constant over time. As more proteins required to reestablish ammonia-oxidizing activity are synthesized, the capacity for energy generation should increase and the subsequent rate of recovery should also increase. This effect is also confirmed by our data. In the limiting case in which complete inactivation of ammonia-oxidizing activity has occurred, the only initial source of energy for protein synthesis is respiration supported by the oxidation of endogenous energy reserves. Although these endogenous energy sources are limited, even a low level of de novo synthesis of proteins required to reinstate ammonia-oxidizing activity will facilitate ATP synthesis, which in turn will power further synthesis of required proteins.

The second factor is the number of cellular constituents which must be resynthesized before full recovery of ammonia-oxidizing activity can be achieved. We have previously demonstrated that the inactivating effects of light are directed solely at AMO (19) and that the recovery from light inactivation of AMO requires the de novo synthesis of a limited number of polypeptides, including the 27-kDa active site-containing polypeptide of AMO (19). In contrast, we have also previously shown that numerous polypeptides, in addition to the 27-kDa polypeptide, are covalently radiolabeled after exposure of active cells of *N. europaea* to [^{14}C]TCE (32). These data complement our current kinetic data which suggest that in addition to AMO, other enzymes (e.g., HAO) are inactivated during the short-term exposures to high concentrations of TCE. Taken together, these results suggest that full recovery from TCE inactivation requires the resynthesis of a larger number of proteins than that required to recover from light-dependent inactivation of AMO. Given that the rate of protein synthesis is limited by the rate of energy generation (either from ammonia oxidation or endogenous substrates), then the rate of recovery will also be influenced by the number of proteins which need to be resynthesized to fully reinstate the original ammonia-oxidizing activity. Accordingly, we would expect TCE-inactivated cells to recover at a lower rate than light inactivated cells. This effect is also confirmed in our experiments (Fig. 6C) and is most noticeable when TCE-dependent inactivation of AMO is greater than 60% of the original activity. The increasing disparity between the rates of recovery of TCE- and light-inactivated cells as a function of the extent of inactivation of ammonia-oxidizing activity may reflect the differing energy requirements for full recovery from each inactivating treatment.

A final important consideration concerning recovery of ammonia-oxidizing activity is that resynthesis of the components required for ammonia-oxidizing activity could significantly increase the total amount of TCE oxidation possible by a given quantity of cells. Our current estimate of the amount of TCE that can be degraded by *N. europaea* prior to complete inactivation of ammonia-oxidizing activity is based on a single exposure to TCE. However, one approach to increasing the amount of TCE degraded by each cell would be to expose cells to TCE and then allow these cells to resynthesize the inactivated components required for ammonia-oxidizing activity before again exposing the cells to TCE. When TCE degradation capabilities are calculated on the basis of total protein, then any recovery of TCE-degrading capability (without an increase in total biomass) will result in an increase in the estimate of TCE that can be degraded by a given mass of cells. An alternative approach would be to establish a steady-state reaction in which the rate of inactivation of ammonia-oxidizing activity equals the rate of protein resynthesis. To achieve this would require a carefully balanced mixture of TCE and ammonia and would probably be most successful in resting cells whose maximal diversion of energy could be directed into continuous TCE oxidation and cellular repair rather than cell growth and division. The data presented here do not allow prediction of this optimum mixture and would require longer-term experiments conducted under steady-state conditions with considerably reduced concentrations of TCE. Experimental results and the development and testing of a predictive mathematical model for the responses of cells of *N. europaea* to TCE under quasi-steady-state conditions have been conducted and are described in full elsewhere (9, 10).

ACKNOWLEDGMENTS

Funding for this study was provided by the Department of Energy under a grant (OS 91-7) through the USEPA-supported Western Region Hazardous Substance Research Center and by the Oregon Agricultural Experiment Station.

REFERENCES

1. **Alvarez-Cohen, L., and P. L. McCarty.** 1991. Effects of toxicity, aeration, and reductant supply on trichloroethylene transformation by a mixed methanotrophic culture. Appl. Environ. Microbiol. **57:**228–235.
2. **Alvarez-Cohen, L., and P. L. McCarty.** 1991. Product toxicity and cometabolic competitive inhibition modelling of chloroform and trichloroethylene transformation by methanotrophic resting cells. Appl. Environ. Microbiol. **57:**1031–1037.
3. **Alvarez-Cohen, L., and P. L. McCarty.** 1991. A cometabolic biotransformation model for halogenated compounds exhibiting product toxicity. Environ. Sci. Technol. **25:**1381–1387.
4. **Arciero, D., T. Vannelli, M. Logan, and A. B. Hooper.** 1989. Degradation of trichloroethylene by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. Biochem. Biophys. Res. Commun. **159:**640–643.
5. **Bergmann, J. G., and J. Sanik.** 1957. Determination of trace amounts of chlorine in naphtha. Anal. Chem. **29:**241–243.
6. **Cornish-Bowden, A.** 1979. Fundamentals of enzyme kinetics, p. 82. Butterworths, London.
7. **Dabrock, B., J. Riedel, J. Bertram, and G. Gottschalk.** 1992. Isopropylbenzene (cumene)—a new substrate for the isolation of trichloroethylene-degrading bacteria. Arch. Microbiol. **158:**9–13.
8. **Dalton, H., and D. I. Stirling.** 1982. Co-metabolism. Philos. Trans. R. Soc. Lond. B **297:**481–496.
9. **Ely, R. L., M. R. Hyman, D. J. Arp, R. B. Guenther, and K. J. Williamson.** A cometabolic biodegradation model incorporating enzyme inhibition, inactivation, and recovery. 2. Trichloroethylene degradation experiments. Biotechnol. Bioeng., in press.
10. **Ely, R. L., K. J. Williamson, R. B. Guenther, M. R. Hyman, and D. J. Arp.** A cometabolic biodegradation model incorporating enzyme inhibition, inactivation, and recovery. 1. Model development, analysis, and testing. Biotechnol. Bioeng., in press.
11. **Ensign, S. A., M. R. Hyman, and D. J. Arp.** 1992. Cometabolic degradation of chlorinated alkenes by alkene monooxygenase in a propylene-grown *Xanthobacter* strain. Appl. Environ. Microbiol. **58:**3038–3046.
12. **Ensley, B. D.** 1991. Biochemical diversity of trichloroethylene metabolism. Annu. Rev. Microbiol. **45:**283–299.
13. **Ewers, J., D. Frier-Schroder, and H.-J. Knackmuss.** 1990. Selection of trichloroethylene (TCE) degrading bacteria that resist inactivation by TCE. Arch. Microbiol. **154:**410–413.
14. **Folsom, B. R., P. J. Chapman, and P. H. Pritchard.** 1990. Phenol and trichloroethylene degradation by *Pseudomonas cepacia* G4: kinetics and interactions between substrates. Appl. Environ. Microbiol. **56:**1279–1285.

15. **Fox, B. G., J. G. Borneman, L. P. Wackett, and J. D. Lipscomb.** 1990. Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and environmental implications. Biochemistry **29:**6419–6427.
16. **Gornall, A. G., C. J. Bardawill, and M. M. David.** 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177:**751–766.
17. **Hageman, R. H., and D. P. Hucklesby.** 1971. Nitrate reductase from higher plants. Methods Enzymol. **23:**491–503.
18. **Horvath, A. L.** 1982. Halogenated hydrocarbons: solubility-miscibility with water. Marcel Dekker, Inc., New York.
19. **Hyman, M. R., and D. J. Arp.** 1992. $^{14}C_2H_2$ and $^{14}CO_2$-labeling studies of the *de novo* synthesis of polypeptides by *Nitrosomonas europaea* during recovery from acetylene and light inactivation of ammonia monooxygenase. J. Biol. Chem. **267:**1534–1545.
20. **Hyman, M. R., I. B. Murton, and D. J. Arp.** 1988. Interaction of ammonia monooxygenase from *Nitrosomonas europaea* with alkanes, alkenes, and alkynes. Appl. Environ. Microbiol. **54:**3187–3190.
21. **Hyman, M. R., A. W. Sansome-Smith, J. H. Shears, and P. M. Wood.** 1985. A kinetic study of benzene oxidation to phenol by whole cells of *Nitrosomonas europaea* and evidence for the further oxidation of phenol to hydroquinone. Arch. Microbiol. **143:**302–306.
22. **Hyman, M. R., and P. M. Wood.** 1983. Methane oxidation by *Nitrosomonas europaea*. Biochem. J. **212:**31–37.
23. **Hyman, M. R., and P. M. Wood.** 1984. Ethylene oxidation by *Nitrosomonas europaea*. Arch. Microbiol. **137:**155–158.
24. **Hyman, M. R., and P. M. Wood.** 1985. Suicidal inactivation and labelling of ammonia monooxygenase by acetylene. Biochem. J. **227:**719–725.
25. **Infante, P. F., and T. A. Tsongas.** 1987. Mutagenic and oncogenic effects of chloromethanes, chloroethanes and halogenated analogs of vinyl chloride. Environ. Sci. Res. **25:**301–327.
26. **Keener, W. K., and D. J. Arp.** 1993. Kinetic studies of ammonia monooxygenase inhibition in *Nitrosomonas europaea* by hydrocarbons and halogenated hydrocarbons in an optimized whole-cell assay. Appl. Environ. Microbiol. **59:**2501–2510.
27. **Love, O. T., Jr., and R. G. Eilers.** 1982. Treatment of drinking water containing trichloroethylene and related industrial solvents. J. Am. Water Works Assoc. **74:**413–425.
28. **Malachowsky, K. J., T. J. Phelps, A. B. Teboli, D. E. Minnikin, and D. C. White.** 1994. Aerobic mineralization of trichloroethylene, vinyl chloride, and aromatic compounds by *Rhodococcus* species. Appl. Environ. Microbiol. **60:**542–548.
29. **Oldenhuis, R., J. Y. Oedzes, J. J. van der Waarde, and D. B. Janssen.** 1991. Kinetics of chlorinated hydrocarbon degradation by *Methylosinus trichosporium* OB3b and toxicity of trichloroethylene. Appl. Environ. Microbiol. **57:** 7–14.
30. **Rasche, M. E., R. E. Hicks, M. R. Hyman, and D. J. Arp.** 1990. Oxidation of monohalogenated ethanes and *n*-chlorinated alkanes by whole cells of *Nitrosomonas europaea*. J. Bacteriol. **172:**5368–5373.
31. **Rasche, M. E., M. R. Hyman, and D. J. Arp.** 1990. Biodegradation of halogenated hydrocarbon fumigants by nitrifying bacteria. Appl. Environ. Microbiol. **56:**2568–2571.
32. **Rasche, M. E., M. R. Hyman, and D. J. Arp.** 1991. Factors limiting aliphatic chlorocarbon degradation by *Nitrosomonas europaea*: cometabolic inactivation of ammonia monooxygenase and substrate specificity. Appl. Environ. Microbiol. **57:**2986–2994.
33. **Truesdale, G. A., and A. L. Downing.** 1954. Solubility of oxygen in water. Nature (London) **173:**1236.
34. **Vannelli, T., M. Logan, D. M. Arciero, and A. B. Hooper.** 1990. Degradation of halogenated aliphatic compounds by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. Appl. Environ. Microbiol. **56:**1169–1171.
35. **Vogel, T. M., and P. L. McCarty.** 1985. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. Appl. Environ. Microbiol. **49:**1080–1083.
36. **Wackett, L. P., G. A. Brusseau, S. R. Householder, and R. S. Hanson.** 1989. Survey of microbial oxygenases: trichloroethylene degradation by propane-oxidizing bacteria. Appl. Environ. Microbiol. **55:**2960–2964.
37. **Wackett, L. P., and D. T. Gibson.** 1988. Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. Appl. Environ. Microbiol. **54:**1703–1708.
38. **Westrick, J. J., J. W. Mello, and R. F. Thomas.** 1984. The groundwater supply survey. J. Am. Water Works Assoc. **76:**52–59.
39. **Wood, P. M.** 1986. Nitrifcation as a bacterial energy source, p. 39–62. *In* J. I. Prosser (ed.), Nitrification. IRL Press, Oxford.