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Bacterial Oxidation of Methyl Bromide in Fumigated Agricultural Soils

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The oxidation of [^{14}C]methyl bromide ([^{14}C]MeBr) to $^{14}CO_2$ was measured in field experiments with soils collected from two strawberry plots fumigated with mixtures of MeBr and chloropicrin (CCl_3NO_2). Although these fumigants are considered potent biocides, we found that the highest rates of MeBr oxidation occurred 1 to 2 days after injection when the fields were tarped, rather than before or several days after injection. No oxidation of MeBr occurred in heat-killed soils, indicating that microbes were the causative agents of the oxidation. Degradation of MeBr by chemical and/or biological processes accounted for 20 to 50% of the loss of MeBr during fumigation, with evasion to the atmosphere inferred to comprise the remainder. In laboratory incubations, complete removal of [^{14}C]MeBr occurred within a few days, with 47 to 67% of the added MeBr oxidized to $^{14}CO_2$ and the remainder of counts associated with the solid phase. Chloropicrin inhibited the oxidation of MeBr, implying that use of this substance constrains the extent of microbial degradation of MeBr during fumigation. Oxidation was by direct bacterial attack of MeBr and not of methanol, a product of the chemical hydrolysis of MeBr. Neither nitrifying nor methane-oxidizing bacteria were sufficiently active in these soils to account for the observed oxidation of MeBr, nor could the microbial degradation of MeBr be linked to cooxidation with exogenously supplied electron donors. However, repeated addition of MeBr to live soils resulted in higher rates of its removal, suggesting that soil bacteria used MeBr as an electron donor for growth. To support this interpretation, we isolated a gram-negative, aerobic bacterium from these soils which grew with MeBr as a sole source of carbon and energy.

Methyl bromide (MeBr) is of environmental concern because of its ability to destroy stratospheric ozone (19, 25, 29). About one-third of the global flux of MeBr to the atmosphere is thought to be derived from anthropogenic sources (13, 22), and fumigation of agricultural soils for the control of nematodes and fungi is the largest use (22, 27). Chloropicrin (i.e., tear gas [CCl_3NO_2]) is commonly added with MeBr to soils for an added biocidal effect (24) and as a warning agent to minimize personal exposure to MeBr.

Emission of commercially applied MeBr from soils to the atmosphere is a function of the method of fumigation and of soil properties, such as moisture, pH, and organic carbon content. Although estimates of the amount of MeBr emitted from fumigated fields vary widely, several studies have reported that significant mineralization of MeBr occurs within soils during fumigation, thereby reducing the outward flux of MeBr (26–28). Mineralization (i.e., degradation) of MeBr is indicated by increased levels of Br^- in soils after fumigation (8, 9, 26–28). Chemical reactions, including methylation of organic matter (1, 7), hydrolysis (6), and nucleophilic exchange with other halides and sulfide (5, 16), may account for some of the mineralization of MeBr in soils. However, the possibility that soil bacteria directly oxidize MeBr and thereby contribute to its destruction during fumigation operations is generally overlooked. This is because both MeBr and its coinjectate chloropicrin are potent biocides and are presumed to kill off or inactivate any soil bacteria, along with the target organisms.

Both methanotrophs and nitrifiers are able to oxidize MeBr, because it is an analog of methane and ammonium (17, 20). However, the very high concentrations of MeBr used during field fumigation may inhibit these bacteria (17). Shorter et al. (21) found that an unidentified bacterial flora present in incubated soils was responsible for removal of extremely low trace levels (~10 parts per trillion) of MeBr from the enclosed atmosphere. In this paper, we report on combined field and laboratory experiments which indicate that most of the bacterial oxidation of MeBr occurs during field fumigation events, when soil MeBr concentrations are very high. Our results demonstrate that this is achieved by bacteria which directly oxidize the MeBr and use it as a substrate to support their growth.

MATERIALS AND METHODS

Field sites. Field investigations were conducted at two commercial strawberry farms in California. Gavilan Berry Farm (GBF), a coastal site in Monterey County comprised of Oceano Loamy Sand (total organic C = 0.8%; pH = 6.8), was sampled from 1 to 8 October 1994 and again on 24 February 1997. Irvine, an interior valley site in Orange County previously studied for MeBr flux (26) and comprised of Sorrento Loam (total organic C = 0.4%; pH = 6.3), was sampled from 14 to 20 August 1995. Commercial fumigation with 67% MeBr and 33% chloropicrin (ratio by weight) occurred on 2 October 1994 at GBF and on 14 August 1995 at Irvine. The amounts of fumigant injected were 280 and 220 mmol of MeBr m^{-2} at GBF and Irvine, respectively. A wing-shaped wedge with multiple nozzles which reduces disruption of the soil surface while injecting the fumigants at a depth of ~30 cm was employed (26–28). The fields were simultaneously covered with sheets of clear, high-density polyethylene (0.0025 cm thick) that remained in place during the fumigation. We refer to the fumigation period as the time from the injection of the fumigant (day 0) to removal of the plastic sheets (day 6).

Physical and chemical properties of soils. Bulk density (grams $milliliter^{-1}$) was determined as the weight of soil sampled by using an aluminum ring of known volume (88.75 ml), and soil water content (milliliters $milliliter^{-1}$) was calculated from the difference in weight between fresh and dried (85°C for 5 days) soils. Particle density (grams $milliliter^{-1}$) was determined by measuring, in a 25-ml volumetric flask, the volume of deionized water displaced by a known weight (5.0 g) of dry soil.

The soil gas content was calculated from the bulk density, particle density, and water content by using the following equation: gas content = [1 − (bulk density/particle density)] − water content. Soil MeBr amounts were calculated from gas concentrations measured in the upper 90 cm of soil (see below) and included the

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amount of gas dissolved in the aqueous phase, which was determined from the average soil water content and gas-water partition coefficients based on solubility data for pure water at 25°C (23).

Soil MeBr measurements. MeBr was collected at various depths from one site at GBF and from three sites located 2 m apart at Irvine before, during, and after the fumigations. Soil gas piezometers (stainless steel tubing; 120-cm length by 0.16-cm inside diameter) were sampled by using gas-tight glass syringes, either through nylon three-way Luer stopcocks (GBF) or through rubber septa (Irvine). Several volumes of the piezometers were flushed at each depth before a sample was collected in the syringe. Syringes were fitted with septa contained in modified Luer needle hubs with the needles removed, and MeBr was analyzed within 1 h by gas chromatography in a mobile field laboratory.

Field experiments. Soil incubations to determine MeBr oxidation rates were initiated several hours after the samples were collected. Soils from discrete depths were collected 1 day before (day −1), during (day 1), and after (day 6) fumigation at GBF and just prior to (day 0), during (day 2), and after (day 6) fumigation at Irvine. Triplicate soil samples (2 to 8 g) from five depths were collected in cut-off glass syringes (2.5 or 5 ml). Heat-killed controls in this and all subsequent experiments consisted of soil that was autoclaved at 121°C and 250 kPa for 1 h. [^{14}C]MeBr diluted in water (0.1 ml; 2.5 μCi; specific activity = 29.7 μCi μmol^{-1}; purity = 100%) (New England Nuclear, Boston, Mass.) was added by syringe through a rubber serum cap affixed to the cut end of the glass syringe and dispersed throughout the sample. The purity of the [^{14}C]MeBr was verified in our laboratory by gas chromatography combined with gas proportional counting (see below), and radioactivity was found only in the eluted peak for MeBr and not in that for methanol (MeOH), the most likely contaminant. The amount of MeBr added with [^{14}C]MeBr in all field experiments was ~16 nmol g^{-1}. Therefore, this addition elevated prefumigation MeBr concentrations, but concentrations during the fumigations were raised less than 30%. Soils were incubated for 2 to 3 days at 23°C and the incubations were terminated by freezing. The frozen soils were extruded into 25-ml conical flasks or 27-ml serum bottles containing 10 ml of saturated NaCl solution. The flasks were capped, acidified below pH 1 with HCl, and shaken overnight before analyses of the $^{14}CO_2$. The lowest detectable [^{14}C]MeBr oxidation rate (fraction oxidized times in situ concentration) was 0.01 nmol g^{-1} day^{-1}.

Soil incubations to determine CH_4 oxidation rates were conducted in the field at Irvine. Soils were collected from five discrete depths (as described above) before, during, and after fumigation, and 15- to 17-g samples were transferred to 27-ml serum bottles (air volume = 17 ml) and crimp sealed with butyl rubber stoppers. $^{14}CH_4$ (3.4 μCi; specific activity = 56 μCi μmol^{-1}; purity = 97.5%) (Amersham Corp.) was added by syringe to the headspaces of triplicate bottles from each depth and of single control bottles, resulting in the addition of 12 nmol of CH_4 g^{-1}. ^{14}C-labeled gases ($^{14}CO_2$ and $^{14}CH_4$) were determined initially and following 2 days of incubation. Final samples were acidified with 0.5 ml of 6 N HCl and equilibrated overnight before determination of headspace gas activities. The lowest detectable CH_4 oxidation rate was 0.02 nmol g^{-1} day^{-1}.

Laboratory experiments. Incubations to determine CH_4 and ammonium (NH_4^+) oxidation rates were conducted in the laboratory with GBF and Irvine soils; GBF soils (20 to 30 cm) were collected 2.5 years (February 1997) after the initial study, and Irvine soils (45 to 55 cm) were stored for 1.5 years at 4°C in glass jars before they were tested. We have readily detected methanotrophic activity in soils from other locations which were stored in this fashion for comparable time periods (17). CH_4 oxidation was determined by the same method as described above. The effect of MeBr on NH_4^+ oxidation rates was determined with 5 g of soil contained in 57-ml serum bottles (air volume = 53 ml) to which 100 μmol of $(NH_4)_2SO_4$ (0.1 ml of a 0.5 M stock) was added. Three bottles from each site were immediately frozen (−40°C) to preserve the contents for determination of initial conditions. Some bottles received MeBr additions (440 nmol g^{-1}), while three control bottles (no added NH_4^+) received 0.1 ml of water and MeBr. Nitrite plus nitrate (NO_2^- plus NO_3^-) was determined after 2 days of incubation at 23°C in the dark. The lowest detectable NH_4^+ oxidation rate was 2 nmol g^{-1} day^{-1}.

Incubations of soils were conducted to determine if the production of $^{14}CO_2$ was the result of (i) oxidation of [^{14}C]MeOH formed as an intermediate product via hydrolysis of [^{14}C]MeBr or (ii) direct oxidation of the added [^{14}C]MeBr. Sufficient MeOH was added to achieve an observable dilution of the amount of $^{14}CO_2$ produced if MeOH was an intermediate substrate; however, the amount of MeOH added (5 or 10 nmol g^{-1}) was kept small in order to avoid inhibitory effects of high concentrations of MeOH. Serum bottles (27 ml) containing 5 g of soil were stoppered and crimp sealed after receiving additions of 5 or 10 nmol of MeOH g of soil^{-1} in 0.05 or 0.1 ml of deionized water. Control bottles received 0.1 ml of water. [^{14}C]MeBr (6.7 nmol of MeBr g^{-1}) was added at the start, and the headspace [^{14}C]MeBr and $^{14}CO_2$ activities were monitored over several days (T = 23°C, dark).

Soil incubations were conducted to determine the effect of added chloropicrin on the oxidation of [^{14}C]MeBr. Soil (5 g) from GBF or Irvine was added to serum bottles, and the bottles were stoppered, as described above. Samples received additions of 0, 10, 100, and 1,000 nmol of chloropicrin g of soil^{-1} along with [^{14}C]MeBr at the start. Headspace gases were analyzed for [^{14}C]MeBr and $^{14}CO_2$. Soils were acidified after 3 days of incubation (T = 23°C, dark) with 2 ml of 1 N HCl, shaken overnight, and analyzed again for [^{14}C]MeBr and $^{14}CO_2$ activities in the headspace. Afterwards, stoppers were removed and the bottles were vented overnight to remove remaining gaseous [^{14}C]MeBr. Aliquots (1.0 ml) of acid slurry were transferred to 2-ml tubes and centrifuged at 10,000 × g for 5 min. Aliquots of the supernatant (0.2 ml) were diluted with 3.0 ml of deionized water, and the ^{14}C activity was determined by liquid scintillation counting. A fraction of the acid supernatant (0.01 ml) was analyzed for $^{14}CO_2$, and none was found. After the remaining acid supernatant was decanted, the soil pellet was washed three times in 0.5 ml of deionized water and resuspended in 0.5 ml of deionized water, and then a fraction (0.1 ml) of the suspension was diluted with 3.0 ml of deionized water and the ^{14}C activity was determined by liquid scintillation counting.

The effects of exogenously supplied electron donors upon the degradation of MeBr by soils was investigated to determine if the destruction of MeBr was linked to a cooxidation phenomenon. Bottled soils (5 g in 27-ml serum bottles) from Irvine and GBF were amended with several different concentrations of trimethylamine, Na formate, NH_3 formate, or methane; injected with [^{14}C]MeBr (1 μCi; equivalent to 6.6 nmol g^{-1}); and analyzed for $^{14}CO_2$ over a time course incubation. A modification of the experiment was also used, in which the rate of loss from the headspace of 2 μmol of added MeBr (400 nmol g^{-1}) in soils amended with trimethylamine, dimethylamine, monomethylamine, ethanol, Na formate, NH_3 formate, glucose, or methanol was examined. The results for the amended samples in both experiments were compared to those for unamended controls. In a separate experiment, the effect of repeated additions of MeBr to live, unamended soils on the rate of MeBr consumption was also examined.

Isolation of bacterial cultures capable of growth on MeBr. Soils which had demonstrated enhanced MeBr uptake upon repeated additions of MeBr were used as the inoculum (200 mg), which was added to sterile 57-ml serum bottles containing 20 ml of the mineral salts medium of Doronina et al. (4a), modified with respect to the trace elements solution (17a). MeBr (9 μmol) was aseptically injected into the air headspace of the bottles by using 0.2-μm-pore-size sterile filters. Bottles were incubated in the dark with orbital shaking (200 rpm) at 30°C for 3 days, at which time MeBr was no longer detectable. Two sequential transfers (1-ml inoculum) of this enrichment were carried out in this fashion at 3-day intervals, the time which corresponded to the complete removal of MeBr. Subsequently, five successive transfers (0.5-ml inoculum) were carried out in 25-ml Balch tubes containing 10 ml of medium. These transfers were conducted at 3-day intervals, which corresponded to the time of complete MeBr depletion.

This initial enrichment was streaked onto petri plates containing the solidified agar mineral salts medium, and the plates were stacked within a glass jar used for anaerobic isolations; however, an air atmosphere was maintained within the jar. MeBr (100 μmol/liter or ~2,500 ppm) was injected through a rubber sampling port; its concentration was monitored daily, and it was replenished as needed. After 10 days of incubation, isolated colonies were picked and inoculated into fresh liquid medium. Growth of this culture revealed the presence of at least two morphologies (rods and cocci) upon microscopic examination, and preliminary experiments (see below) were carried out with this enrichment. Subsequent isolation efforts employed highly purified deionized water (<2 mg of dissolved organic carbon per liter) for medium preparation in order to select against heterotrophic bacteria. The enrichment was serially diluted in liquid medium, and the highest dilution (10^{-5}) exhibiting turbidity and MeBr consumption was streaked onto solid medium which also contained bromthymol blue as a pH indicator (4a). Plates were incubated under air plus MeBr as indicated above, and isolated yellow colonies were picked and placed in liquid medium. The yellow color of the bromthymol blue indicated an acidic pH caused by the accumulation of HBr. Growth of the enrichment culture was monitored by increases in turbidity (A_{680}) and Br^- and loss of MeBr. In a second experiment, after growth was complete, 10 ml of cells was harvested by two cycles of centrifugation (5,000 × g) and resuspension in 10 ml of the mineral salts medium, and the final pellet was resuspended in 5 ml of mineral salts and sealed in a 12-ml serum vial under an air atmosphere. [^{14}C]MeBr (1.8 μCi; final concentration = ~12 μM) was injected, and the cells were left to incubate statically for 3.25 h at 25°C, at which time the incubation was stopped by addition of 0.25 ml of 6 N HCl. After 45 min, the bottle was vortexed and the headspace was analyzed for $^{14}CO_2$. Growth of the pure culture was conducted in 580-ml conical flasks containing 240 ml of medium, sealed under air, and fitted with a sidearm for measurement of absorbance. With one flask, only a single injection of MeBr (80 μmol) was performed, while with a second flask, MeBr was injected into the headspace several times over the course of the incubation following its consumption by the growing culture (cumulative amount of MeBr added = ~1,300 μmol). This was done to avoid employing inhibitory concentrations of MeBr at the outset of the experiment. In addition to measurement of MeBr and Br^-, acridine orange direct counts of cells were performed (9a).

Analytical methods. MeBr collected in the field was determined by flame ionization detection after separation on a column containing Krytox 143A (60/80 mesh) on a Graphpak support (4.6 m by 0.16 cm [inside diameter]; Alltech Associates, San Jose, Calif.). The carrier gas (He) flow rate was 30 ml min^{-1}, and the temperatures of the oven and the detector were 100 and 200°C, respectively. The detection limit for MeBr was 0.1 nmol ml^{-1} (~0.25 ppm) in the gas phase or 0.07 nmol g^{-1} in soil, and the standard error of the mean for three samples collected at each depth was ±50%, which represented the variability of field measurements. For the culture experiments, the dissolved MeBr concentration was calculated from MeBr solubility data and applied to the relative proportions of gas and liquid phases (3, 16). Dissolved MeBr was added to the amount

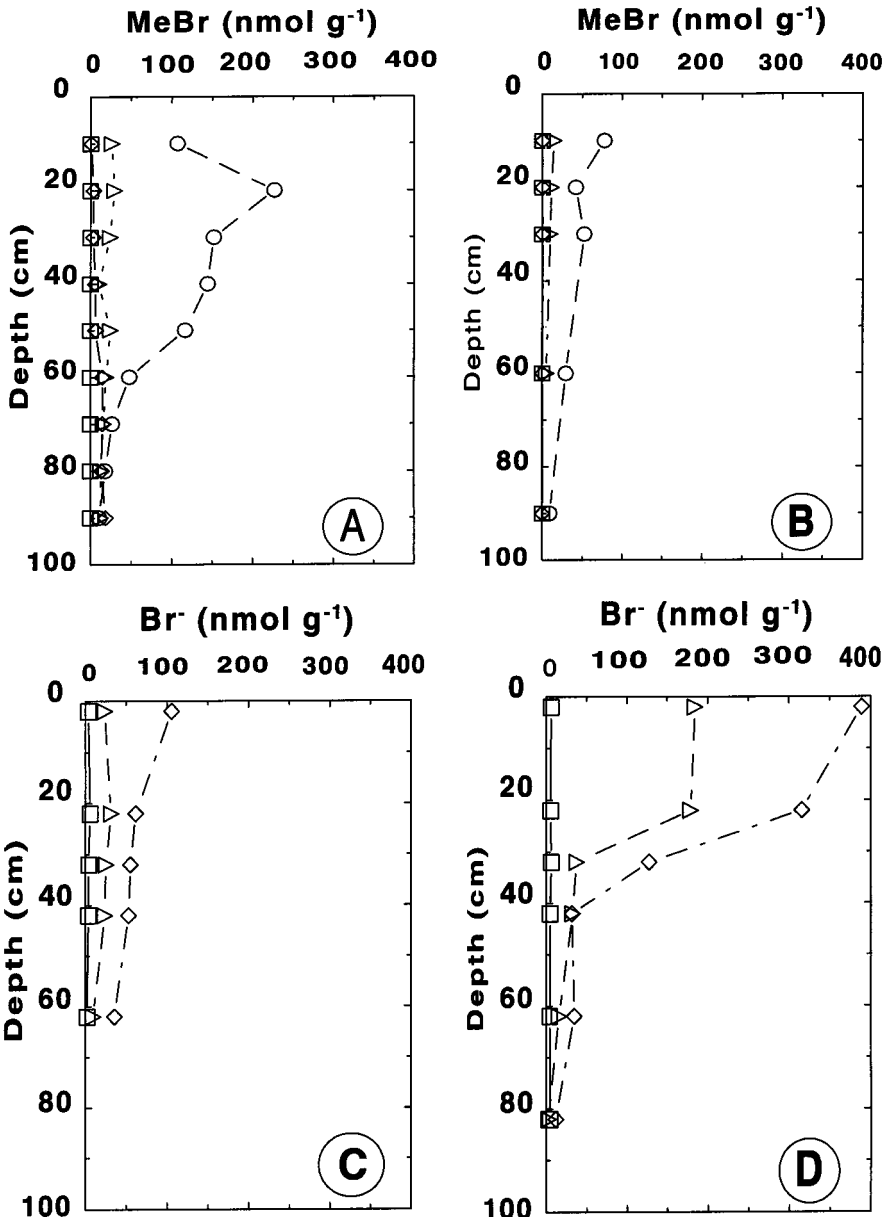

FIG. 1. Vertical profiles of MeBr (A and B) and Br^- (C and D) concentrations in the soil versus depth at GBF (A and C) and Irvine (B and D). Symbols: □, before fumigation (day −1 or 0), ○, during fumigation (day 1); △, during fumigation (day 3); and ◇, after fumigation (day 6).

determined in the gas phase to yield the total quantity of MeBr contained in the culture vessels.

$^{14}CO_2$ produced from [^{14}C]MeBr in the field experiments was quantified by gas proportional counting (4) after chromatographic separation on a Porapak S column (2.4 m by 0.16 cm [inside diameter]), while in laboratory incubations, separations were achieved on a Krytox column (see above). $^{14}CO_2$ produced and $^{14}CH_4$ added during field and laboratory methane oxidation experiments were also quantified by gas proportional counting, but with a Porapak S column (7.3-m length).

Soil Br^- and soil NO_2^- plus NO_3^- produced during NH_4^+ oxidation experiments were extracted from 5 g of soil by using deionized water (25 or 30 ml) and overnight shaking, followed by centrifugation (8,000 × *g* for 15 min) and collection of the supernatant. The supernatant was filtered (0.4-μm-pore-size filter) and stored at 4°C overnight before analysis by liquid ion chromatography for Br^- (15) and colorimetry for NO_2^- plus NO_3^- (12). The detection limit for Br^- was 0.2 μM, corresponding to 1.0 nmol g of dry soil^{-1}. The detection limit for NO_2^- plus NO_3^- was 2 μM, corresponding to 12 nmol g of soil^{-1}.

Total organic carbon was analyzed commercially by combustion under O_2 at 1,500°C, followed by infrared detection (Huffman Labs, Golden, Colo.). Inorganic carbon was determined coulometrically after acidification with 2 N $HClO_4$ and was below the detection limit (0.02% [wt/wt]) in all samples. pH was measured on soil mixed 1:1 (wt/wt) with deionized water.

RESULTS

Bulk densities, particle densities, water contents, and gas contents were similar at the two locations. The soil pH was greater at GBF (pH 6.8) than at Irvine (pH 6.2 [26]). The mean concentration of organic carbon was significantly higher ($P < 0.05$) at GBF (0.8%) than at Irvine (0.4% [26]).

The concentrations of MeBr in the soils before fumigation were below the detection limit at all depths sampled (Fig. 1A and B). During fumigation, concentrations of MeBr increased at all depths. The maximum concentrations of MeBr in the soil were observed in the upper 20 cm on the day after injection of

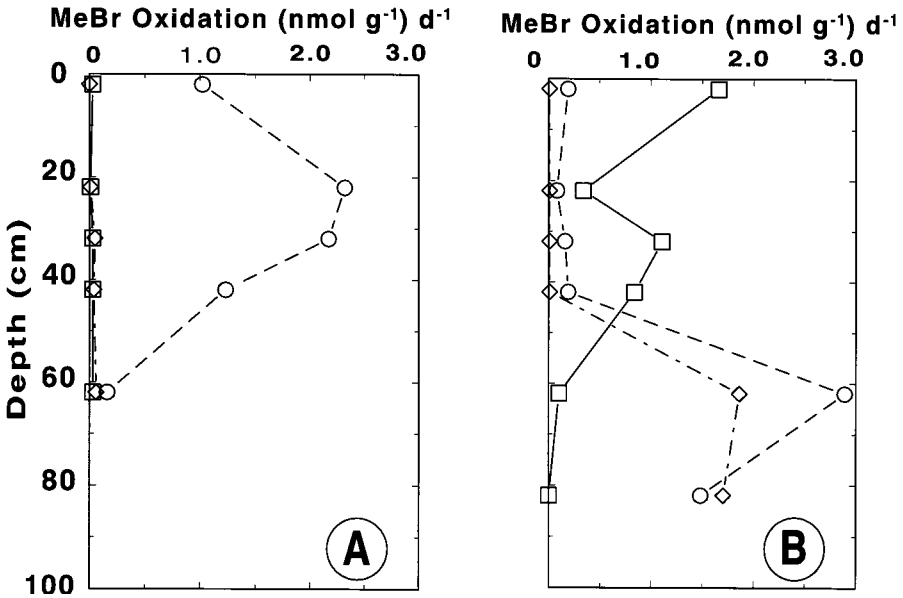

FIG. 2. Vertical profiles of potential MeBr oxidation rates in the soil versus depth at GBF (A) and Irvine (B). Symbols: □, before fumigation (day −1 or 0); ○, during fumigation (day 1 or 2); and ◇, after fumigation (day 6). The mean errors (one standard deviation) for three analyses at each depth are ±28% (A) and ±38% (B).

the fumigant at both GBF and Irvine. MeBr concentrations declined over the following days, but nonetheless, levels were still higher than those determined before fumigation. The concentration of Br^- in the soils increased from low, uniform levels before fumigation to highest levels near the surface after fumigation, following removal of the plastic sheets (Fig. 1C).

Field experiments. A small amount of $^{14}CO_2$ (~0.03 nmol g^{-1} day^{-1}) was produced from added [^{14}C]MeBr at all depths sampled before fumigation at GBF (Fig. 2A [not evident from scale]). Autoclaved soils produced no detectable $^{14}CO_2$ during the same incubation period (not shown). During fumigation (day 1), a 77-fold increase in oxidation rate was observed, with peaks in activity at the 22- and 32-cm depths, which roughly corresponded to the depths of injection and highest MeBr concentration. Vertical integration of the soil column oxidation rates results in an areal estimate of 1.2 mmol m^{-2} day^{-1} during fumigation. After fumigation (day 6), rates of oxidation decreased to near prefumigation levels at all depths sampled.

Significant rates of [^{14}C]MeBr oxidation were also observed during the fumigation at Irvine (Fig. 2B). Before fumigation, there was more $^{14}CO_2$ produced than at GBF, especially at soil depths shallower than 60 cm. During and after fumigation, the oxidation rate decreased in samples collected in the upper 40 cm of soil but was elevated in samples collected from the 60- and 80-cm depths. Vertical integration of the soil column oxidation rates results in an areal estimate of 1.3 mmol m^{-2} day^{-1} during fumigation. Rates of oxidation of CH_4 determined in field incubations of Irvine soil were below the limit of detection (data not shown). Field rates were not determined at GBF.

Laboratory experiments. Oxidation of CH_4 was not detected in any of the assayed soil samples. No $^{14}CO_2$ was produced from $^{14}CH_4$ under any incubation condition (not shown). MeBr inhibited nitrification in both GBF and Irvine soils (Table 1). Production of NO_2^- plus NO_3^- over 2 days in uninhibited soils accounted for up to 3.3% of the NH_4^+ added.

Loss of [^{14}C]MeBr from the headspace during incubations of soil from both sites was unaffected by MeOH (Fig. 3). All of the added [^{14}C]MeBr was consumed in 3 days, while no consumption or $^{14}CO_2$ production occurred in heat-killed controls. MeOH did not inhibit the formation of $^{14}CO_2$ in any of the samples, and in the case of GBF, addition of 10 nmol of MeOH g^{-1} actually enhanced the formation of $^{14}CO_2$ (Fig. 3B). Low levels of chloropicrin (10 nmol g^{-1}) caused a slight lag in MeBr oxidation as determined by both [^{14}C]MeBr loss and $^{14}CO_2$ production (Fig. 4). However, additions of 10- and 100-fold-greater amounts of CCl_3NO_2 completely inhibited both [^{14}C]MeBr loss and $^{14}CO_2$ production.

Biological activity enhanced the incorporation of ^{14}C label into the soil matrix. This is illustrated by ^{14}C mass balances taken from the previous experiment with chloropicrin (Fig. 5). The amount of ^{14}C label recovered as $^{14}CO_2$ in uninhibited soils was greater than 50%. The remaining ^{14}C activity was distributed between the acid-soluble fraction (1 to 5%) and the soil fraction (26 to 34%). In heat-killed controls, less than 2% of added [^{14}C]MeBr was recovered as $^{14}CO_2$ (data not shown), and most of the activity remained as [^{14}C]MeBr (79 to 97%) or was associated with the soil fraction (14 to 22%).

The addition of the exogenous electron donor trimethylamine, formate, or methane to soils neither enhanced the rate

TABLE 1. Effect of MeBr on nitrification of NH_4^+ in soils from GBF and Irvine[a]

Condition	Net NO_3^- + NO_2^- (μmol g^{-1}) produced[b] at:	
	GBF	Irvine
Without MeBr	0.66 ± 0.06	0.22 ± 0.17
With MeBr[c]	0.02 ± 0.04	−0.07 ± 0.11

[a] Soil was incubated for 2 days with 20 μmol of $(NH_4)_2SO_4$ added g^{-1}.
[b] Values are means ± standard deviations for three soil samples. Net production = final concentration − initial concentration; initial values were 0.14 ± 0.02 and 0.60 ± 0.11 μmol g^{-1} at GBF and Irvine, respectively.
[c] Four hundred forty nanomoles of MeBr was added per g of soil (900 ppm).

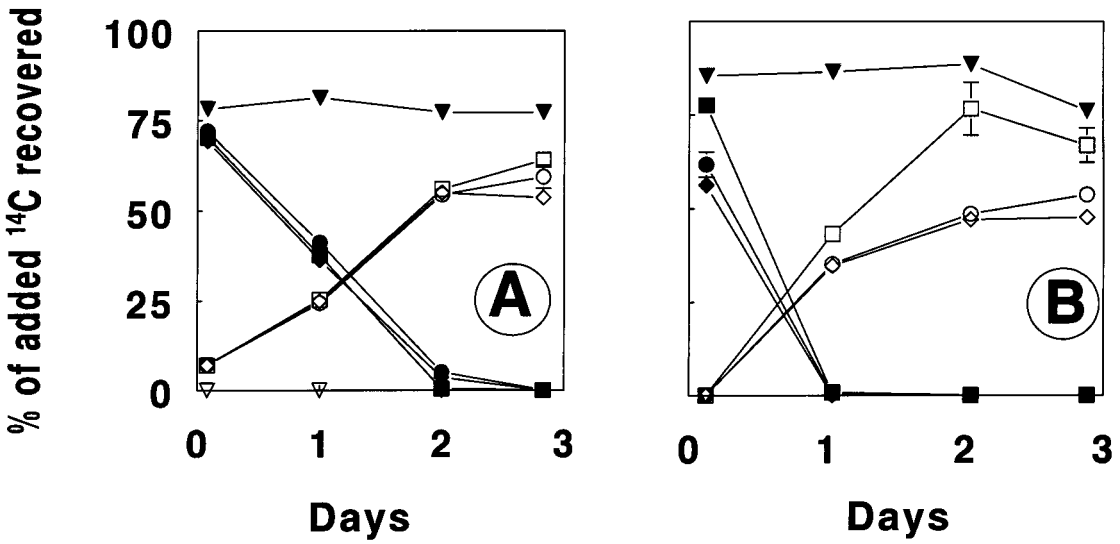

FIG. 3. Percentages of added [^{14}C]MeBr recovered as [^{14}C]MeBr (closed symbols) and as $^{14}CO_2$ (open symbols) in laboratory incubations with GBF (A) and Irvine (B) soils and various additions of MeOH. Symbols: ○, no additions; ◇, MeOH at 5 nmol g^{-1}; □, MeOH at 10 nmol g^{-1}; ▽, heat-killed sample (single analysis). Error bars indicate ±1 standard deviation of the mean for triplicate analyses. The absence of a bar indicates that the error was smaller than the symbol size.

of [^{14}C]MeBr oxidation over the incubation period (not shown) nor affected the extent of [^{14}C]MeBr oxidation in Irvine soil compared to that for unamended controls (Table 2). The same results were obtained for soils from GBF (not shown). Similarly, the addition of various electron donors to soils did not enhance the rate of MeBr consumption (not shown), and with the exception of glucose, the extents of MeBr consumption were similar for all these additions compared to those for unamended controls (Table 3). Glucose, however, strongly inhibited MeBr consumption, which was comparable to that achieved by autoclaving.

Repeated additions of MeBr to live Irvine soil resulted in a more rapid rate of its consumption (Fig. 6). After a 3-day lag during which the rate of loss corresponded to that displayed by autoclaved controls, live soils demonstrated an enhanced rate of MeBr removal. With the addition of more MeBr to these soils, the rate of removal increased by about threefold, and this result was reproduced by a second addition of MeBr.

Bacterial cultures. The enrichment culture demonstrated increased turbidity with time, which corresponded to consumption of MeBr (not shown). A culture sealed with 4 μmol of MeBr (6,700 ppm) completely consumed it after 2 days of incubation and achieved an A_{680} of 0.06 and a recovery of 2.5 μmol of Br^- in the medium. A second tube received repeated additions (seven in all) of MeBr as MeBr was depleted from the headspace. A total of 40 μmol of MeBr was added over a 13-day incubation, and a final A_{680} of 0.11 was achieved, with a recovery of 43 μmol of Br^- in the spent medium. The final pH of the medium had dropped from 7.1 at the outset to 6.4 at the end, indicating the production of HBr. No growth was observed in tubes initially containing 16 μmol (~27,000 ppm) of MeBr sealed in the headspace. The final washed cell sus-

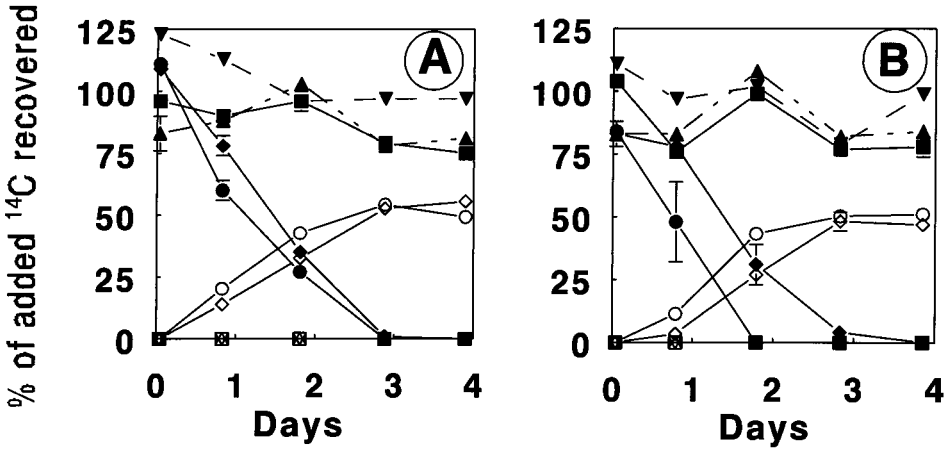

FIG. 4. Percentages of added [^{14}C]MeBr recovered as [^{14}C]MeBr (closed symbols) and as $^{14}CO_2$ (open symbols) in laboratory incubations with GBF (A) and Irvine (B) soils and various additions of CCl_3NO_2. Symbols: ○, no additions; ◇, CCl_3NO_2 at 10 nmol g^{-1}; □, CCl_3NO_2 at 100 nmol g^{-1}; △, CCl_3NO_2 at 1,000 nmol g^{-1}; ▽, heat-killed sample (single analysis). Error bars indicate ±1 standard deviation of the mean for triplicate analyses. The absence of a bar indicates that the error was smaller than the symbol size.

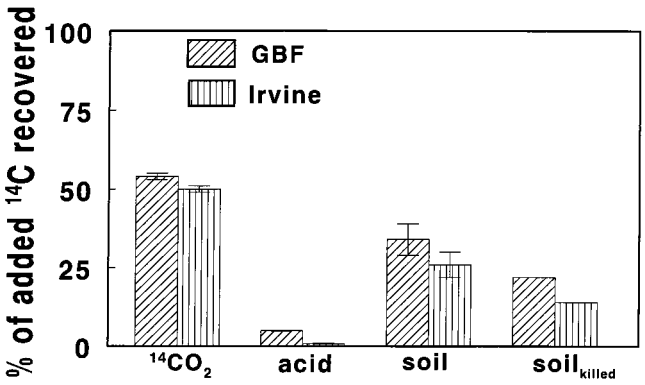

FIG. 5. Percentages of added [^{14}C]MeBr recovered as ^{14}C counts in gas ($^{14}CO_2$), liquid (acid), and solid (soil) phases following acidification of GBF and Irvine soils incubated for 4 days as for the case of no CCl_3NO_2 addition in Fig. 4. Also shown is the percentage of added [^{14}C]MeBr recovered as ^{14}C counts in the solid (soil) phase of heat-killed controls (single analyses) of the same experiment. Error bars indicate ±1 standard deviation of the mean for triplicate analyses.

pensions had an A_{680} of 0.08 and oxidized 47% of the added [^{14}C]MeBr (1.78 μCi) to $^{14}CO_2$ (0.83 μCi) in 3.25 h.

The pure culture consisted of motile, gram-negative rods (length = 1.2 to 1.4 μm and width = 0.5 to 0.6 μm as determined by scanning electron microscopy). After 3 days of incubation in medium initially containing 78 μmol of MeBr, all the MeBr was gone and there was a recovery of 82 μmol of Br^- in the medium. Cell counts during this period had increased from 4×10^6 cells ml^{-1} to 4×10^7 cells ml^{-1} (not shown). In the growth experiment in which there was repeated addition of MeBr to the medium over the course of the incubation, there was a 1:1 stoichiometry of MeBr consumed and recovery of Br^- in the medium, which coincided with increases in cell counts (Fig. 7). Cell densities reached 4×10^8 cells ml^{-1} after consumption of ~1,200 μmol of MeBr, and the pH dropped from 7.05 at the start to 6.03 by 20 days (not shown). No growth occurred in controls containing live cells without added MeBr (Fig. 7) or in sterile medium containing MeBr (not shown).

DISCUSSION

Estimates of the amount of MeBr applied to surface soils which escapes to the atmosphere during fumigation are highly variable (14, 26–28). This may reflect differences in both fumigation practices and soil characteristics which result in different degrees of retention and subsequent mineralization of MeBr. Retention occurs by reversible processes, including dissolution in the aqueous phase and sorption onto dry soil surfaces (2). Mineralization refers to chemical and/or biological reactions which result in the liberation of bromide (Br^-). Release of Br^- from MeBr can occur by nucleophilic exchange (5) or by hydrolysis of MeBr to form methanol (6, 9). Although these chemical degradative reactions for MeBr mineralization have been invoked as the basis for previously observed increases in soil Br^- after fumigation (26–28), chemical reactions were never actually proven to be the causative agents of the reported mineralization. Our detection of [^{14}C]MeBr oxidation to $^{14}CO_2$ in live soils, but not in killed controls, indicates that biological mineralization occurred at our two study sites (Fig. 2). Our field observations are the first to show that bacteria in agricultural soils oxidize [^{14}C]MeBr to $^{14}CO_2$ in the presence of fumigation levels (e.g., >100 nmol g^{-1}) of MeBr.

TABLE 2. Effect of exogenously supplied one-carbon electron donors on the oxidation of [^{14}C]MeBr to $^{14}CO_2$ by Irvine soils

Expt	Addition[a] (5 μmol)	% MeBr oxidized[b]
1	Na formate	63 ± 6
	None	63 ± 3
2	NH_3 formate	46 ± 2
	None	44 ± 3
3	Trimethylamine	68 ± 3
	None	69 ± 5
4	Methane	40 ± 3
	None	40 ± 6

[a] With addition of [^{14}C]MeBr to 5 g of soil as follows: Na formate, 0.6 μCi (2.0 nmol g^{-1}); NH_3 formate, 1.0 μCi (3.6 nmol g^{-1}); trimethylamine, 1.3 μCi (2.3 nmol g^{-1}); and methane, 1.1 μCi (3.9 nmol g^{-1}).

[b] Amount of [^{14}C]MeBr oxidized over 42 to 48 h of incubation; results represent the means ± standard deviations for three soil samples.

TABLE 3. Effect of various potential electron donors on the consumption of MeBr by Irvine soils[a]

Addition (amt [μmol]) or treatment	MeBr consumed (nmol g^{-1})[b]	% MeBr consumed
None	400 ± 50	100
Trimethylamine (10)	430 ± 10	100
Dimethylamine (10)	420 ± 20	100
Methylamine (10)	390 ± 40	100
MeOH (0.1)	420 ± 10	100
Na formate (10)	470 ± 10	100
NH_3 formate (10)	440 ± 20	100
Ethanol (10)	390 ± 40	100
Glucose (10)	86 ± 30	20
Autoclaving	84	16

[a] Five-gram soil samples received 2 μmol of MeBr and were incubated for 162 h.

[b] Results represent means ± standard deviations for three soil samples. The autoclaved control consisted of only a single soil sample.

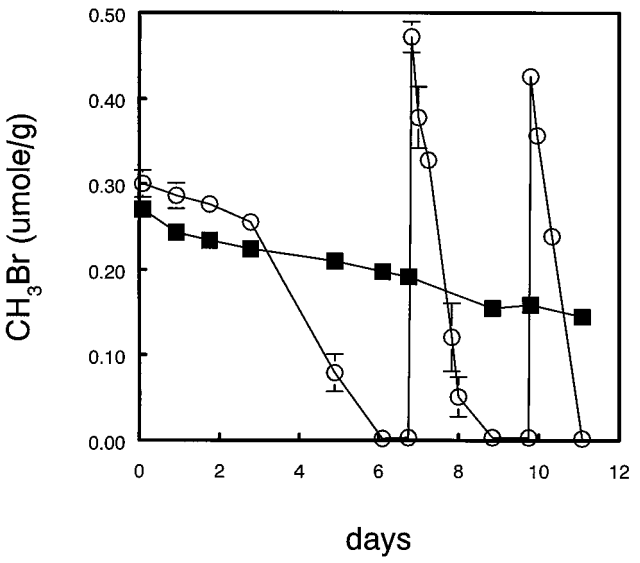

FIG. 6. Consumption of MeBr by live (○) and autoclaved (■) Irvine soils. Live soils received additional MeBr injections when it was apparent that bacteria had completely consumed the amount previously enclosed. Results represent the means for three soil samples, and error bars indicate ±1 standard deviation. The absence of a bar indicates that the error was smaller than the symbol.

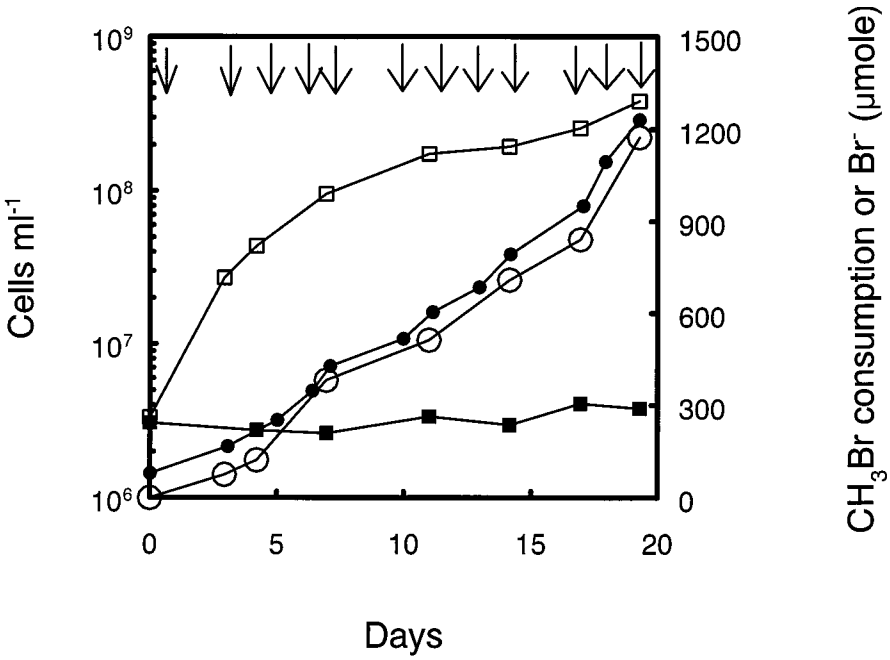

FIG. 7. Growth of the pure culture on MeBr. MeBr consumption is the cumulative quantity consumed from 12 discrete additions (80 to 150 μmol of MeBr each) (arrows) made over the course of the incubation after it was determined before each addition that MeBr was absent from the gas phase. Symbols: □, cell counts; ●, MeBr, ○, bromide; ■, cell counts in control incubated without MeBr.

We observed significant increases in soil Br^- during and after fumigation with MeBr (Fig. 1C and D). Using estimates of the integrated soil Br^- amounts before and after fumigation, we calculate that roughly 60 and 130 mmol of MeBr m^{-2} (20 and 50% of the MeBr added) were mineralized at GBF and Irvine, respectively. Vertical integration of soil MeBr oxidation rates resulted in areal estimates of 1.2 and 1.3 mmol m^{-2} day^{-1} at GBF and Irvine, respectively, or about 0.5% of the initially injected MeBr per day. With the assumption that these rates were constant over the 6-day fumigation period, bacterial oxidation was responsible for a minimum of 6% of the total mineralization. These estimates may represent the lower limit for oxidation because sampling was not done during the first 24 to 48 h from the time of fumigant injection. Alternatively, it is also possible that the level of chloropicrin during this initial period was inhibitory and that degradation was severely constrained (see below). Regardless of which scenario actually occurred, the fact that we detected biological oxidation of MeBr in the early stages of the fumigation period is significant.

MeBr was completely removed in 2 to 3 days in laboratory incubations with live soils. Because autoclaving inhibited MeBr consumption, most (>90%) of this activity was attributable to biological mechanisms, whereas chemical processes were only a minor factor (Fig. 3, 4, and 6; Table 3). Chloropicrin is a potent biocide (23); however, its effect upon bacterial degradation of MeBr has not been considered previously (17, 20). We found that when added at concentrations of greater than 10 nmol g^{-1} (fumigation level = 30 nmol g^{-1}), chloropicrin inhibited the oxidation of [^{14}C]MeBr (Fig. 4). The distribution of chloropicrin in soils after fumigant injection may not be the same as that of MeBr, and hence, soil zones containing higher levels of chloropicrin may diminish MeBr oxidation and alter its degradative patterns both temporally and spatially (Fig. 2B).

Since MeBr can be slowly hydrolyzed in soils to form MeOH, the question arises as to whether the oxidation we observed was a result of direct bacterial attack on the MeBr itself or on any MeOH produced by hydrolysis. If bacteria oxidized [^{14}C]MeOH instead of [^{14}C]MeBr, then addition of a pool of unlabeled MeOH would result in a smaller amount of evolved $^{14}CO_2$. Based on the number of atoms of ^{14}C added as [^{14}C]MeBr (9.1×10^{15} atoms of C), the amount of added MeOH (1.6×10^{16} or 3.1×10^{16} atoms of C) should have been sufficient to result in 66 to 77% diminishment of $^{14}CO_2$ evolved if MeOH was integral to the oxidation pathway. We found no evidence of inhibition of $^{14}CO_2$ evolution by MeOH and conclude that the bacteria oxidized the added [^{14}C]MeBr directly. Indeed, MeOH appeared to have slightly enhanced $^{14}CO_2$ evolution in Irvine soils (Fig. 3B). We recently reported that bacterial populations present in the water column of Mono Lake, Calif., were also unaffected by MeOH, and we concluded that they oxidized [^{14}C]MeBr directly (3).

[^{14}C]MeBr was completely removed from the headspace of unamended controls during the incubations with methanol and chloropicrin described above. However, only about half of the added ^{14}C was recovered as $^{14}CO_2$. The remainder was predominantly associated with the soil itself (Fig. 5), suggesting that methylation of soil organic matter (7) or incorporation into microbial cells may be a significant mineralization pathway. The finding that smaller amounts of ^{14}C were associated with soils that were heat killed suggests that at least some facet of the methylation process is under biological control.

Aerobic bacterial oxidation of MeBr has been considered to be a cometabolism phenomenon carried out by methanotrophs and/or nitrifying bacteria (10, 17, 18, 20). However, because CH_4 oxidation was not detected in either GBF or Irvine soils, it is unlikely that methanotrophs were the causative agents. Indeed, addition of methane to soils had no effect on the oxidation of MeBr (Table 2). Furthermore, nitrification was strongly inhibited by MeBr (Table 1), which means that nitrifiers were not active during these incubations. Oremland et al.

(17) found that addition of CH_3F (an inhibitor of methanotrophs and nitrifiers) to methanotrophic soils resulted in only a partial inhibition of the oxidation of MeBr, suggesting that there was a significant contribution of other types of bacteria to the observed MeBr oxidation. It was recently reported that trimethylamine-using methylotrophs were important agents of the oxidation of MeBr in Mono Lake, Calif. (3). Therefore, it is possible that methylotrophs were active in oxidizing MeBr in our soils. However, with the exception of one experiment with methanol (Fig. 3B), we did not observe any enhancement of MeBr oxidation with a variety of electron donors, including trimethylamine as well as other one-carbon substrates (Tables 2 and 3). This suggests that the microbes responsible for MeBr oxidation were not carrying out the reaction as a non-energy-yielding cometabolism linked to the oxidation of a primary substrate. Although the responsible organisms could still be methylotrophs, they did not require the presence of a C_1 substrate to drive the reaction forward.

The fact that glucose strongly inhibited MeBr consumption (Table 3) argues that the microbes responsible for MeBr oxidation were overgrown by those capable of metabolizing this juicy sugar. Since the extent of inhibition of MeBr consumption caused by glucose was equivalent to that achieved by autoclaving, this shows that the heat treatment did not seriously disrupt the soil matrix with regard to its ability to absorb MeBr. This further underscores the biological, rather than chemical, nature of the MeBr degradation process.

The enhanced rates of MeBr removal observed with repeated MeBr additions (Fig. 6) suggest that the microbes responsible for MeBr oxidation were actually capable of utilizing this substance as an electron donor for energy and as a carbon source for growth. Whereas only about half of the MeBr added ($\sim$0.13 μmol g^{-1}) was lost in the heat-killed controls due to chemical processes over 11 days, the live samples consumed a total of $\sim$1.15 μmol g^{-1} during this period, and the removal rates tripled with repeated MeBr addition. Although it is often argued that autoclaving will alter some aspects of the delicate fabric of soil chemistry, there is no reasonable chemical explanation for the increased rates of MeBr consumption in the live samples. This can best be explained by the growth of a soil microbial population adapted to the use of MeBr as a substrate, and as this cell population increases, the rates of MeBr oxidation also increase with repeated additions. The fact that two soil methylotrophic bacteria which can grow on methyl chloride were recently isolated (4a) led us to hypothesize that similar bacteria must also be responsible for the MeBr oxidation we observed in these agricultural soils.

To prove this point, we obtained an enrichment culture and ultimately isolated a pure culture which grows aerobically on MeBr in mineral salts medium. Washed cells of the enrichment oxidize MeBr to CO_2, while growing cultures of the enrichment demonstrate an equivalence of Br^- accumulation and MeBr consumption. The latter point was also shown for the pure culture (Fig. 7) and argues that the growth is achieved via a simple reaction: $CH_3Br + 1.5O_2 \rightarrow HCO_3^- + Br^- + 2H^+$. It will be of interest to determine whether populations of these bacteria are larger in fumigated than in nonfumigated soils. Our preliminary investigations indicate that the isolate is a facultative methylotroph (3a).

Since the nature of bacterial MeBr oxidation in these fumigated soils has now been identified, it might be possible to enhance the activity of these organisms during the tarping period of fumigation, thereby reducing or eliminating MeBr escape to the atmosphere. Stimulation of nitrification has been used to enhance MeBr removal in laboratory experiments (18), but we have found that the high levels of MeBr applied during field fumigation will likely inhibit nitrifiers (Table 1). Indeed, the general absence of both methanotrophy and nitrification in these soils argues that repeated application of MeBr will severely constrain or eliminate the soil populations of these bacteria, a result which contrasts with our earlier studies on nonfumigated, methanotrophic soils (17).

Lowering the amount of chloropicrin used during fumigation would stimulate the bacterial oxidation of MeBr, since it is clear that this substance strongly inhibits MeBr oxidation (Fig. 4). It is possible that during the initial day of fumigation, concentrations of chloropicrin are too high to allow for MeBr oxidation, but as these gases spread out in the soil column, bacterial oxidation of MeBr may come into play. Recent studies using soil columns suggest that decreased gas diffusivity as a result of surface compaction (8) or irrigation (11) could result in reduced emission of MeBr to the atmosphere. Such scenarios could increase the residence time of MeBr in soils by localizing their concentrations; however, such manipulations would also constrain bacterial MeBr oxidation by keeping the soil concentration of chloropicrin too high. With this information, the challenge will be to devise fumigant mixtures and application procedures which optimize MeBr mineralization while effectively eliminating the target pest organisms.

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REFERENCES

1. **Arvieu, J. C.** 1983. Some physico-chemical aspects of methyl bromide behaviour in soil. Acta Horticult. **152:**267–274.
2. **Brown, B. D., and D. E. Rolston.** 1980. Transport and transformation of methyl bromide in soils. Soil Sci. **130:**68–75.
3. **Connell, T. L., S. B. Joye, L. G. Miller, and R. S. Oremland.** 1996. Bacterial oxidation of methyl bromide in Mono Lake, California. Environ. Sci. Technol. **33:**1489–1495.

3a. **Connell, T. L., and R. S. Oremland.** Unpublished data.

4. **Culbertson, C. W., A. J. B. Zehnder, and R. S. Oremland.** 1981. Anaerobic oxidation of acetylene by estuarine sediments and enrichment cultures. Appl. Environ. Microbiol. **41:**396–403.

4a. **Doronina, N. V., A. P. Sokolov, and Y. A. Trotsenko.** 1996. Isolation and initial characterization of aerobic chloromethane-utilizing bacteria. FEMS Microbiol. Lett. **142:**179–183.

5. **Elliott, S., and F. S. Rowland.** 1993. Nucleophilic substitution rates and solubilities for methyl halides in seawater. Geophys. Res. Lett. **20:**1043–1046.
6. **Elliott, S., and F. S. Rowland.** 1995. Methyl halide hydrolysis rates in natural waters. J. Atmos. Chem. **20:**229–236.
7. **Gan, J., S. R. Yates, M. A. Anderson, W. F. Spencer, F. F. Ernst, and M. V. Yates.** 1994. Effect of soil properties on degradation and sorption of methyl bromide in soil. Chemosphere **29:**2685–2700.
8. **Gan, J., S. R. Yates, D. Wang, and W. F. Spencer.** 1996. Effect of soil factors on methyl bromide volatilization after soil application. Environ. Sci. Technol. **30:**1629–1636.
9. **Gentile, I. A., L. Ferris, and S. Crespi.** 1989. The degradation of methyl bromide in some natural freshwaters. Pest. Sci. **25:**261–272.

9a. **Hobbie, J. E., R. L. Daley, and S. Jaspar.** 1977. Use of Nuclepore filters for counting bacteria for fluorescence microscopy. Appl. Environ. Microbiol. **33:**1225–1228.

10. **Hyman, M. R., and P. M. Wood.** 1984. Bromocarbon oxidations by *Nitrosomonas europaea*, p. 49–52. *In* R. L. Crawford and R. S. Hanson (ed.), Microbial growth on C_1 compounds: proceedings of the 4th international symposium. American Society for Microbiology, Washington, D.C.
11. **Jin, Y., and W. A. Jury.** 1995. Methyl bromide diffusion and emission through soils under various management techniques. J. Environ. Qual. **24:**1002–1009.
12. **Johnson, K. S., and R. L. Petty.** 1983. Determination of nitrate and nitrite in seawater by flow injection analysis. Limnol. Oceanogr. **28:**1260–1266.
13. **Khalil, M. A. K., R. A. Rasmussen, and R. Gunawardena.** 1993. Atmospheric

methyl bromide: trends and global mass balance. J. Geophys. Res. **98:**2887–2896.

14. **Majewski, M. S., M. M. McChesney, J. E. Woodrow, J. H. Pruger, and J. N. Sieber.** 1995. Aerodynamic measurements of methyl bromide volatilization from tarped and non-tarped fields. J. Environ. Qual. **24:**742–752.
15. **Oremland, R. S., and C. W. Culbertson.** 1992. Evaluation of methyl fluoride and dimethyl ether as inhibitors of aerobic methane oxidation. Appl. Environ. Microbiol. **58:**2983–2992.
16. **Oremland, R. S., L. G. Miller, and F. E. Strohmaier.** 1994. Degradation of methyl bromide in anaerobic sediments. Environ. Sci. Technol. **28:**514–520.
17. **Oremland, R. S., L. G. Miller, C. W. Culbertson, T. L. Connell, and L. Jahnke.** 1994. Degradation of methyl bromide by methanotrophic bacteria in cell suspensions and soils. Appl. Environ. Microbiol. **60:**3640–3646.

17a. **Oremland, R. S., J. Switzer Blum, C. W. Culbertson, P. T. Visscher, L. G. Miller, P. Dowdle, and F. E. Strohmaier.** 1994. Isolation, growth, and metabolism of an obligately anaerobic, selenate-respiring bacterium, strain SES-3. Appl. Environ. Microbiol. **60:**3011–3019.

18. **Ou, L.-T., P. J. Joy, J. E. Thomas, and A. G. Hornsby.** 1997. Stimulation of degradation of methyl bromide in soil during oxidation of an ammonia fertilizer by nitrifiers. Environ. Sci. Technol. **31:**717–722.
19. **Prather, M. J., M. B. McElroy, and S. C. Wofsy.** 1984. Reduction in ozone at high concentrations of stratospheric halogens. Nature **312:**227–231.
20. **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.
21. **Shorter, J. H., C. E. Kolb, P. M. Crill, R. A. Kerwin, R. W. Talbot, M. E. Hines, and R. C. Harriss.** 1995. An effective soil surface sink for atmospheric methyl bromide. Nature **377:**717–719.
22. **Singh, H. B., and M. Kanakidou.** 1993. An investigation of the atmospheric sources and sinks of methyl bromide. Geophys. Res. Lett. **20:**133–136.
23. **Wilhelm, E., R. Battino, and R. J. Willcock.** 1977. Low-pressure solubility of gases in liquid water. Chem. Rev. **77:**219–262.
24. **Wilhelm, S., R. C. Storkan, and J. E. Sagen.** 1961. Verticillium wilt of strawberry controlled by fumigation of soil with chloropicrin and chloropicrin-methyl bromide mixtures. Phytopathology **51:**744–748.
25. **Wofsy, S. C., M. B. McElroy, and Y. L. Yung.** 1975. The chemistry of atmospheric bromine. Geophys. Res. Lett. **2:**215–218.
26. **Yagi, K., J. Williams, N.-Y. Wang, and R. J. Cicerone.** 1993. Agricultural soil fumigation as a source of atmospheric methyl bromide. Proc. Natl. Acad. Sci. USA **90:**8420–8423.
27. **Yagi, K., J. Williams, N.-Y. Wang, and R. J. Cicerone.** 1995. Atmospheric methyl bromide (CH_3Br) from agricultural soil fumigations. Science **267:** 1979–1981.
28. **Yates, S. R., J. Gan, F. F. Ernst, A. Mutziger, and M. V. Yates.** 1996. Methyl bromide emissions from a covered field. I. Experimental conditions and degradation in soil. J. Environ. Qual. **25:**184–192.
29. **Yung, Y. L., J. P. Pinto, R. T. Watson, and S. P. Sander.** 1980. Atmospheric bromine and ozone perturbations in the lower stratosphere. J. Atmos. Sci. **37:**339–353.