Applied and Environmental Microbiology, Oct. 1988, p. 2424–2431 Vol. 54, No. 10
0099-2240/88/102424-08$02.00/0
Copyright © 1988, American Society for Microbiology

Degradation of 2-Methylisoborneol by Aquatic Bacteria

GEORGE IZAGUIRRE,* ROY L. WOLFE, and EDWARD G. MEANS III

Water Quality Division, Metropolitan Water District of Southern California, La Verne, California 91750

Received 4 January 1988/Accepted 28 June 1988

2-Methylisoborneol (MIB) is a musty- or muddy-smelling compound which occurs in some natural waters and which is difficult to remove by conventional water treatment methods. Bacterial degradation of MIB was examined in batch culture experiments. Cultures able to metabolize MIB were enriched in a mineral salts medium supplemented with milligram-per-liter levels of the compound and were inoculated with water and sediment samples from reservoirs where MIB is seasonally produced. Bacteria from degrading cultures were isolated on R2A agar and identified as predominantly *Pseudomonas* spp. Degradation occurred only in cultures consisting of three or more different bacteria. MIB supported growth as the sole added carbon source at 1 to 6.7 mg/liter. MIB was also degraded at microgram-per-liter levels in sterile filtered lake water inoculated with washed bacteria and in synthetic medium supplemented with various sugars or acetate. Complete degradation of MIB took from 5 days to more than 2 weeks. Enrichment with isoborneol, a structural analog of MIB, failed as a preenrichment for MIB degraders. Isoborneol at 20 to 40 mg/liter readily supported bacterial growth, whereas MIB at 12 to 20 mg/liter took months to degrade. The relative recalcitrance of MIB compared with isoborneol may be a result of the additional methyl group in MIB.

A common and recurrent problem in drinking water supplies is the formation of earthy-musty tastes and odors. These off flavors are primarily the result of two compounds, geosmin and 2-methylisoborneol (MIB), which are produced by some cyanobacteria (15, 34) and actinomycetes (10, 27). Both compounds are resistant to chemical oxidation and are difficult to remove by conventional water treatment methods (8, 18). Biodegradation may offer an alternative means of removal. This paper will focus on the degradation of MIB.

MIB is a bridged cycloalkanol (molecular weight, 168.3) with the structure shown in Fig. 1. It was reported as a natural metabolite of actinomycetes in 1969 by Medsker et al. (21) and Gerber (9) and independently in 1970 by Rosen et al. (27). MIB is characterized by an earthy-musty or camphorous odor and a muddy flavor. The compound has an extremely low average threshold odor concentration of 29 ng/liter (24), but it can be detected at even lower levels by many people. Episodes of MIB-tainted drinking water have been reported from various areas in the United States (15, 20, 27), the Netherlands (25), and Japan (40). MIB has also been found in muddy-flavored fish in Canada (41) and the United States (19).

One of the water supply systems that has been plagued by MIB episodes belongs to the Metropolitan Water District of Southern California, a major wholesaler serving about 13 million people in six counties. Metropolitan receives its water from the Colorado River and from Northern California via the California State Water Project. Three reservoirs in the Metropolitan system have been significantly affected by MIB occurrences. All three are in Riverside County, Calif., but they impound water from different sources. Lake Mathews, containing Colorado River water, has yearly occurrences of benthic (epipelic and epilithic) cyanobacteria that release MIB in areas around the shoreline (20). Lake Skinner contains a variable blend of Colorado River and State Project water; this lake also has MIB problems from attached cyanobacteria, but they are not as predictable as those in Lake Mathews. Lake Perris receives only State Project water, which is less saline (total dissolved solids of 200 mg/liter versus 530 mg/liter for Colorado River water), and periodically experiences MIB episodes in late summer and fall. The MIB is produced by both planktonic and benthic cyanobacteria (G. Izaguirre et al., Proc. Am. Water Works Assoc. Wat. Qual. Technol. Conf. 1983, p. 319–334).

Interest in biodegradation of MIB resulted from two observations. In September 1981, significant MIB levels developed in Lake Perris, reaching approximately 100 ng/liter and then dropping to about 2 ng/liter by early November. This suggested possible biodegradation of the compound along with volatilization or reduced production. In addition, a study in this laboratory (unpublished) showed that MIB levels in lake water samples declined at ambient temperature and not in mercuric chloride-preserved controls.

A laboratory study was undertaken in 1981 to examine the biodegradation of MIB in samples of Lake Perris water. The results of that study indicated that MIB at 0.4 to 1.0 μg/liter was degraded in vitro within 12 days by organisms in the water at 28°C (E. G. Means et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, Q36, p. 266). The degrading organisms appeared to be bacterial but were not identified.

To our knowledge, the only other published report of MIB biodegradation was that of Namkung and Rittmann (22), who reported the removal of MIB by a biofilm under continuous-plug-flow conditions. The biofilm was grown on peat fulvic acid and fed MIB at 100 and 1,000 μg/liter for 2 to 3 h; removal percentages were 44 and 17%, respectively. Other papers dealing with MIB biodegradation, primarily in sand or carbon filters, were presented at a recent symposium on off flavors in drinking water (K. Ashitani, Water Sci. Technol., in press; B. V. Lundgren, H. Boren, A. Grimvall, and R. Sävenhed, Water Sci. Technol., in press; H. Sumitomo, S. Ito, Y. Ishihara, and M. Nuno, Water Sci. Technol., in press; M. Yagi, S. Nakashima, and S. Muramoto, Water Sci. Technol., in press). The biodegradation of two related compounds, camphor (4) and borneol (26), has also been documented.

The primary objective of the present study was to isolate and identify bacterial MIB degraders from the Metropolitan

* Corresponding author.

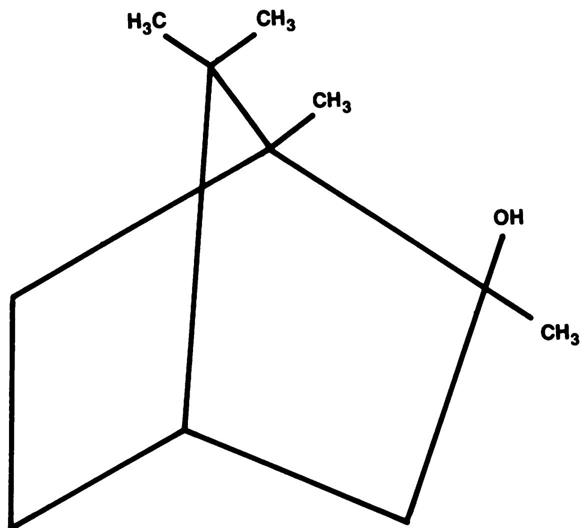

FIG. 1. Structure of MIB.

source-water reservoirs. A further objective was to examine MIB degradation in the presence and absence of other nutrients and to examine the kinetics of the process under those conditions.

MATERIALS AND METHODS

Chemicals. Solid MIB, prepared by synthesis from camphor (39) in this laboratory, was 99% MIB with 1% camphor by weight. Additional MIB was obtained from Wako Pure Chemicals, Ltd., Osaka, Japan. Sterile MIB spiking solutions (4.0 to 40 μg/ml) were prepared by dissolving MIB in purified water, which had been passed through a Super-Q system (Millipore Corp., Bedford, Mass.) and an ORGANICpure ultraviolet irradiation system (Sybron/Barnstead, Boston, Mass.). This solution was then filtered through a sterile 0.2-μm-pore-size Acrodisc (Gelman Instrument Co., Ann Arbor, Mich.) into a sterile tube. The membrane had previously been rinsed with purified water to remove any leachable substances.

Isoborneol was obtained from ICN Pharmaceuticals, Inc. (Plainview, N.Y.), as a crystalline mixture of 85% isoborneol and 15% borneol, the endoisomer. D-Glucose was obtained from Difco Laboratories (Detroit, Mich.). Reagent-grade D-fructose and sodium acetate were obtained from J. T. Baker Chemical Co. (Phillipsburg, N.J.). Sucrose was obtained from Sigma Chemical Co. (St. Louis, Mo.).

All glassware used in this study was carefully washed and rinsed three times in purified water. Pipettes were washed and then oven sterilized at 180°C for at least 3 h.

Analysis of MIB. The absence of the earthy-musty odor characteristic of MIB was considered a presumptive indication of MIB disappearance. Confirmation of MIB concentration was performed by salted closed-loop stripping and gas chromatographic analysis as described previously (14, 17). Culture samples were diluted in 900 ml of organic-free water (irradiated with UV light for 1 h) in a closed-loop stripping bottle with 8.0% sodium sulfate. This solution was then air-stripped for 1.5 h to purge the semivolatile organics, which were concentrated on carbon filters. The filters were removed and extracted with carbon disulfide, and the extracts were analyzed on either a Carlo Erba 5300 high-resolution gas chromatograph (with on-column injection coupled with a flame ionization detector) or a Carlo Erba 4160 gas chromatograph coupled with a Hewlett Packard 5970 mass selective detector. Alternatively, some analyses were performed on a Finnigan model 4023 gas chromatograph-mass spectrometer-computer system. All analyses for the same experiment were done on the same instrument. Uninoculated controls were also analyzed for comparison. Under the conditions of these analyses, the detection limit for MIB was approximately 17 ng/liter, and the recovery efficiency was 67%.

Enrichment procedures. Water samples were collected in sterile bottles from Lake Perris. Portions of the water samples were placed in sterile 125-ml screw-cap flasks and spiked with MIB at 20 to 25 μg/liter to screen the samples for the presence of MIB degraders. In the first instance, the control was Lake Perris water, with MIB and 42 mg of $HgCl_2$ per liter; in the second instance, the control was autoclaved Lake Perris water with MIB. Sediment samples were collected by scuba divers from the shallows of Lake Mathews and Lake Skinner.

Enrichment for bacteria able to degrade MIB was performed in a mineral salts medium (MSM) with the following composition (milligrams per liter of purified water): NH_4Cl, 50; $MgSO_4 \cdot 7H_2O$, 50; $CaCl_2 \cdot 2H_2O$, 20; K_2HPO_4, 100; $FeCl_3 \cdot 6H_2O$, 1. The medium also contained 0.5 ml of trace element solution. The trace element solution was that used in BG-11 medium for cyanobacteria (31). The pH of the medium was 7.6.

Cultures of lake water that had degraded MIB were used to inoculate 100 ml of MSM in 300-ml bottles, or 50 ml in 250-ml flasks, with MIB at 12 and 20 mg/liter, respectively. In all experiments, the vessels were tightly capped to prevent loss of MIB through volatilization. For the cultures with sediment samples, 1.0 ml of the supernatant of vigorously shaken samples was added to the same medium containing 15 to 20 μg of MIB per liter or, in some cases, low milligram-per-liter levels. The cultures were incubated at 28°C for up to 4 weeks. Cultures that showed disappearance of MIB, as determined by gas chromatography, were used to inoculate the enrichment cultures. MIB-degrading cultures derived from Lake Perris water were subcultured in MSM with MIB at 1.0 to 2.0 mg/liter over the course of the study.

To ensure that the organic carbon content of the mineral medium before spiking was very low, a medium blank and a sample of the water used to prepare the medium were analyzed for total organic carbon by the persulfate-UV method (1) with a Dohmann carbon analyzer (Xertex Corp., Santa Clara, Calif.). The detection limit by this method is 50 μg of total organic carbon per liter.

In some cultures, isoborneol, a structural analog of MIB, was used as a pre-enrichment for MIB degraders. Isoborneol differs from MIB in having a hydrogen instead of the methyl group at the hydroxyl carbon. This compound was used as a surrogate for MIB because preliminary work had shown that isoborneol could support bacterial growth at milligram-per-liter levels and because isoborneol is commercially available in much larger quantities. The isoborneol was added to the MSM at 20 or 40 mg/liter, and the cultures were inoculated with lake-water samples and sediments and with a few nonlake samples (soil and sludge) for comparison. Some samples or cultures were retested at 1 to 4 mg of isoborneol per liter. Degradation of isoborneol was evidenced by disappearance of the camphorous odor typical of the compound and by the development of visible turbidity. Cultures that grew on isoborneol were tested for the ability to degrade MIB by transferring 1.0 or 2.0 ml of culture to new sterile

medium with MIB. In addition, some MIB-degrading cultures were tested for the ability to degrade isoborneol to compare the degradability of the two compounds by the same bacteria.

Isolation of MIB degraders. Enrichment cultures degrading MIB were streaked onto R2A agar (Difco), or dilutions of the cultures were spread plated onto R2A, and the plates were incubated at 28°C for 7 to 10 days. Colonies were picked, suspended in 0.85% sterile saline, and plated onto the agar at least twice for purification. Bacteria were enumerated by spread plating samples of the cultures onto R2A agar in duplicate or triplicate.

To determine whether MIB degraders could be recovered from agar plates, representative colonies were picked and recombined in MSM that contained MIB. In two instances, a streak was made across an agar plate instead of picking individual colonies, and the mixture of bacteria was transferred to sterile liquid medium. The cultures were incubated for up to 30 days.

For isolation of MIB degraders with MIB as the sole carbon source, the MSM was supplemented with 1% agarose (Eastman Kodak Co., Rochester, N.Y.) and autoclaved. After cooling, MIB was added at 1.0 mg/liter, and the medium was poured into 53-mm plastic plates. The plates were inoculated (by spread plating or streaking) with various cultures that had degraded MIB and were then incubated inside small bottles to minimize desiccation and keep the MIB from volatilizing. The control plates consisted of the same medium without MIB. In one instance, various cultures were streaked onto the agarose medium without MIB in 50-mm dishes with tight lids (Becton Dickinson Labware, Oxnard, Calif.). Sterile cellulose pads (Millipore) were placed inside the lids and saturated with a sterile MIB solution (20 μg/ml). The plates were inverted over the lids, tightly sealed, and incubated at 28°C. Colonies that developed on the MIB plates and not on the control plates were picked and purified on R2A agar.

Identification of bacteria. Purified isolates from the various cultures were identified using the Rapid NFT system for nonfermenters (Analytab Products, Plainview, N.Y.). In the few instances when this system was unsatisfactory, the identification system of Ward et al. (37) was used.

Kinetic experiments. MSM (100 ml) containing MIB at 2.0 mg/liter was inoculated with 2.0 ml of a culture derived from a Lake Skinner sediment enrichment that had been grown on MIB as the sole carbon source. The initial bacterial density was 1.2×10^6 cells per ml. A second 100-ml volume of medium with MIB served as an uninoculated control. To determine growth on MIB as compared with growth without MIB, a third culture was inoculated as above but contained no MIB. The first culture was analyzed for MIB at the beginning and at 3-day intervals thereafter until day 20; the control was analyzed at days 0 and 24. Cultures 1 and 3 were also plated in duplicate onto R2A agar to enumerate the bacteria.

A second kinetic experiment, with MIB at approximately 290 ng/liter, was prepared similarly to the aforementioned experiment, except that the inoculum was a suspension of washed bacteria from a previous culture rather than a sample of a culture. The parent culture contained MIB at 2.0 μg/liter to minimize carryover of carbon. Three 14-ml portions of the culture were centrifuged at $1,260 \times g$ for 20 min, the liquid was decanted, the pellets were suspended, and the suspension was added to 400 ml of MSM. The objective of this experiment was to achieve MIB degradation at environmentally relevant levels rather than, as in the previous experiment, to assess growth at the expense of MIB.

Cometabolism experiments. Since degradation of many organic compounds occurs cometabolically, the ability of four easily metabolized substrates to support MIB degradation was investigated. A mixed culture of Lake Perris bacteria that had previously degraded MIB was used for this experiment. The MSM was supplemented with sucrose, glucose, fructose, or sodium acetate at 20 mg/liter (supplying 8.4, 8.0, 8.0, and 5.9 mg of carbon per liter, respectively) and MIB at 20 μg/liter. Two more bottles received only MIB at 20 μg/liter; one was the uninoculated control. A seventh bottle contained MIB at 20 mg/liter (15.7 mg of carbon per liter). The solutions were inoculated with a suspension of washed cells from a culture that contained D-fructose, at 20 mg/liter, as the carbon source. A 150-ml sample of the culture was washed twice by centrifugation ($1,160 \times g$ for 25 min). The pellet was suspended to 15 ml with sterile phosphate buffer (pH 7.0). Each bottle was inoculated with 1.0 ml of this suspension, providing about 3.3×10^5 cells/ml. The cultures were analyzed at days 0, 6, and 12, with the exception that the two cultures with only MIB were analyzed again at day 18. The cultures were also plated onto R2A agar for bacterial enumeration.

After this experiment, some cultures derived from this Lake Perris culture line were grown in MSM with sodium acetate at 20 to 50 mg/liter and various MIB concentrations (usually 10 μg/liter). This was to maintain higher cell densities than were possible on MIB alone. In some cases, cultures without acetate were run in parallel.

MIB degradation in lake water. Our objective was to determine whether laboratory-cultivated MIB degraders could degrade MIB when reintroduced into lake water. Cultures derived from both Lake Perris and Lake Skinner enrichments were employed.

In the experiment with a Lake Perris mixed culture, a 15-ml portion of culture (containing sodium acetate at 40 mg/liter) was centrifuged twice at $1,260 \times g$ for 15 min; the liquid was then poured off, and the pellet was suspended in 0.3 ml of the liquid that remained. Approximately 0.12 ml of this suspension was added to each of two bottles containing 100 ml of filter-sterilized Lake Perris water with 10 μg of MIB per liter. A third bottle served as the uninoculated control. The water was sterilized to control for degradation by organisms already in the water. The water was specifically filter sterilized because autoclaving of Lake Perris water led to a musty, "woody" odor that masked the odor of MIB. The cultures were analyzed after disappearance of the earthy odor.

The experiment using Lake Skinner bacteria was performed similarly, with the exception that the bacterial suspension was added to autoclaved Lake Mathews water (previously passed through a glass-fiber filter) containing MIB at 10 μg/liter.

RESULTS

Enrichment of MIB degraders. Both samples of Lake Perris water spiked with MIB at 20 and 25 μg/liter showed presumptive disappearance of MIB within 11 to 16 days. The first water sample was analyzed after 13 days, and no MIB was detected. The enrichment cultures derived from these samples, with MIB at 12 and 20 mg/liter, degraded MIB slowly and developed slight turbid growth. The culture starting at 12 mg of MIB per liter had 0.36 mg/liter after 86 days, and some growth was evident after about 63 days. The

TABLE 1. Samples or cultures tested for growth on isoborneol

Sample or culture	Growth on isoborneol[a]
Lake Perris water	+
Sediment samples	+[b]
Lake Skinner MIB enrichment (from sediment)	+
Soil MIB enrichment	+
Soil from potted plant	+
Water treatment plant sludge discharge	−
Five-member Lake Perris consortium	+
Pure cultures from isoborneol enrichments	+[c]

[a] Isoborneol concentration ranged from 4.0 to 40 mg/liter. +, Turbid growth at the expense of isoborneol; −, no growth.
[b] Of the four samples tested, three yielded a positive result.
[c] Of the 12 cultures tested, only 1 yielded a positive result.

TABLE 2. MIB-degrading cultures derived from bacteria on R2A agar plates

Culture[a]	Inoculum	MIB dose (μg/liter)	Time of analysis (days)	Final MIB concn (μg/liter)
A	4 colonies	100	28	<0.1
B	3 colonies	100	5	<0.019
C	Streak[b]	20	17	<0.09
D	Streak[b]	400	10	<0.018

[a] Cultures A, B, and C represent Lake Perris bacteria; culture D was from a soil enrichment.
[b] Cultures C and D were inoculated with growth taken by streaking a loop across a plate one or more times.

enrichment culture containing MIB at 20 mg/liter showed no decrease in MIB level after 18 days; it was not analyzed again until 91 days after inoculation, at which time no MIB was detected. This culture also developed slight turbid growth.

New cultures prepared from the one containing MIB at 12 mg/liter did not continue to degrade MIB and were discarded. The culture initially containing MIB at 20 mg/liter was the parent culture for other enrichments at various MIB levels and also served for isolation of bacteria. One of these cultures, spiked with MIB at 1.0 mg/liter, had less than 90 μg of MIB per liter after 17 days, which represented a reduction of more than 90%.

Nine of 11 sediment samples tested from Lake Mathews and Lake Skinner degraded MIB, but some of these cultures did not do so upon transfer to fresh sterile medium. One enrichment that did degrade MIB upon subculture was derived from a Lake Skinner sediment sample. The initial MIB dose was 0.7 mg/liter, but analysis after 13 days showed less than 0.2 μg/liter. The first subculture, with MIB initially at 11 mg/liter but later diluted to 5.5 mg/liter with sterile MSM, contained <0.4 μg of MIB per liter after 22 days of incubation. The disappearance of MIB was accompanied by turbid growth (1.6×10^7 CFU/ml). The next subculture, spiked with MIB at 6.7 mg/liter, produced similar results: bacterial growth paralleled the gradual loss of the MIB odor, which was undetectable at 17 days. This culture line eventually lost the ability to degrade MIB as the sole carbon source. However, MIB degradation was sustainable in sterile lake water.

The samples enriched or tested with isoborneol as the sole or principal carbon source are listed in Table 1. The cultures designated as positive degraded isoborneol and produced turbid growth, most within 8 days. Most of the cultures degrading MIB also degraded isoborneol. The potting-soil sample (chosen because of its strong earthy odor) showed degradation of isoborneol but not MIB. Instrumental analysis confirmed that the disappearance of the odor corresponded to the absence of isoborneol. In general, cultures previously grown on isoborneol could degrade 20 mg of isoborneol per liter in 5 days. However, none of those cultures initially enriched on isoborneol was able to degrade MIB. The only pure culture able to grow on isoborneol was also unable to degrade MIB, either as the sole carbon source, in the presence of isoborneol as a cosubstrate, with yeast extract, or in sterile lake water. This organism, identified as *Pseudomonas vesicularis*, was isolated from a Lake Mathews sediment sample.

Isolation of MIB degraders. The degradation of MIB by cultures inoculated with bacteria taken from agar plates is summarized in Table 2. In three cases, growth was removed from primary plates of the liquid cultures rather than from purified isolates. Culture A was inoculated with four colonies from an agar plate of a culture, which in turn was derived from a plate inoculated with a Lake Perris enrichment. This culture will hereafter be referred to as "the Lake Perris consortium." Culture B was prepared by combining three different colonies of bacteria isolated from the Lake Perris cultures. The degraders were recoverable from R2A plates (Table 2). Unfortunately, all attempts to obtain MIB degradation by pure cultures and most attempts with reconstituted consortia from purified isolates were unsuccessful; degradation occurred only in consortia derived from primary plates, with the exception of culture B.

The bacteria isolated from culture A were identified as *Pseudomonas aeruginosa*, *P. paucimobilis*, *P. pseudoalcaligenes*, *P. mendocina*, and a red-pigmented bacterium placed in Centers for Disease Control group IVE. After more than 20 months in culture (in the presence of MIB), this consortium has remained stable; i.e., the four species continue to appear when the liquid cultures are plated on agar, and the cultures continue to degrade MIB. Culture B consisted of *P. aeruginosa*, *P. alcaligenes*, and *P. vesicularis*.

Bacteria isolated from the Lake Skinner cultures were identified as *P. aeruginosa*, *P. vesicularis*, *P. diminuta*, *Moraxella osloensis*, and a *Pseudomonas* sp. These species represented the predominant colony types.

On the agarose plates, growth was very slow, taking several weeks. Some colonies developed that were not found on the corresponding control plates, but when these colonies were isolated, purified, and introduced into liquid medium, they failed to degrade MIB either as pure cultures or in combination. The results were the same when multiple colonies were transferred to the medium. Most of the control plates produced small, colorless colonies.

Kinetic experiments. The degradation of MIB as a sole carbon source, with an initial dose of 2.0 mg of MIB per liter, is shown in Fig. 2A. The MIB concentration was not significantly reduced for the first 10 days, but it declined by more than 99% within the next 7 days. Less than 9 μg of MIB per liter was detected at day 17.

The change in bacterial numbers with and without MIB as the sole carbon source is shown in Fig. 2B. A 60-fold increase in numbers over the control was evident by day 17, when the MIB level had begun to decrease. In the control, bacterial numbers gradually declined.

The degradation of MIB at 290 ng/liter is shown in Fig. 3. In this culture, the lag in MIB disappearance was only about 4 days, showing that even at this low MIB level degradation was not immediate. The final MIB concentration of 17 ng/liter represented a 94% decrease over an 11-day period.

The total organic carbon analyses gave results of <50 μg

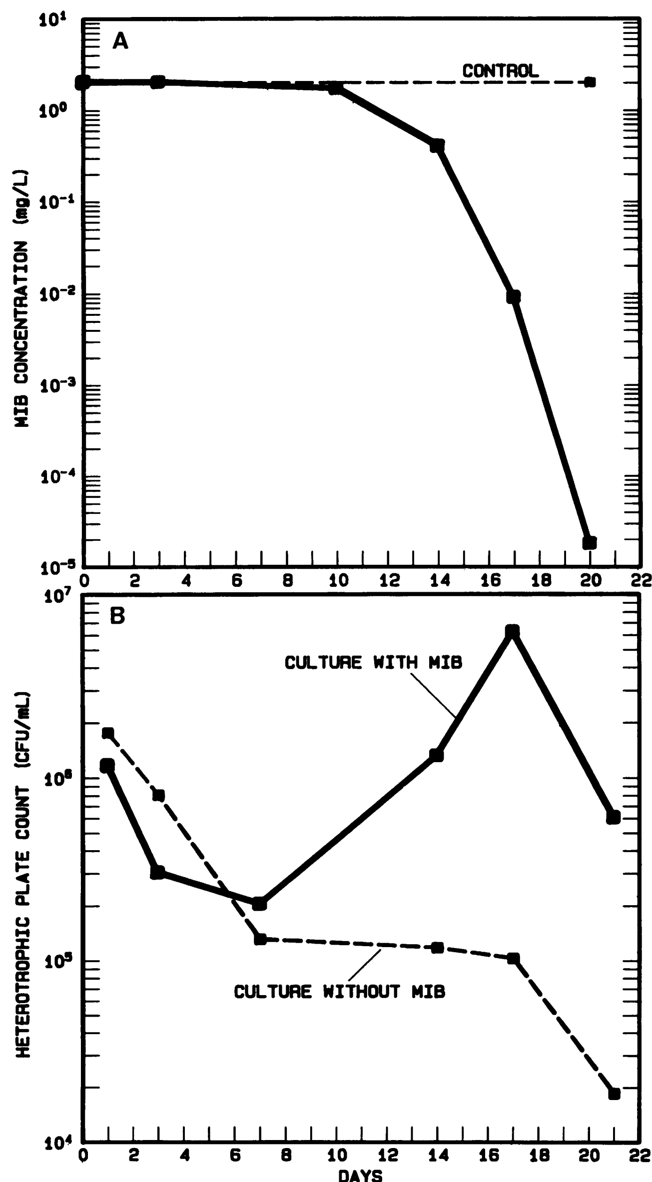

FIG. 2. Degradation of 2.0 mg of MIB per liter as a sole carbon source at 28°C. The inoculum was a mixed culture derived from a Lake Skinner sediment enrichment. (A) Decrease in MIB concentration. (B) Effect of MIB on bacterial growth in MSM.

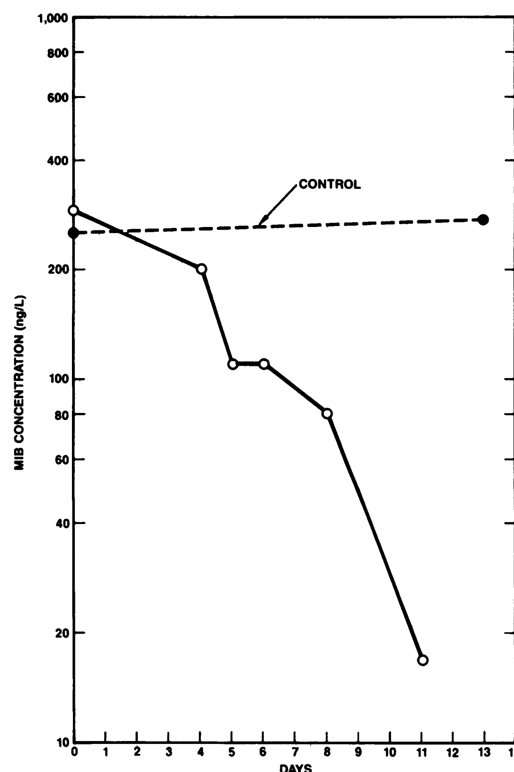

FIG. 3. Degradation of 290 ng of MIB per liter in MSM. The inoculum was a suspension of washed bacteria from a Lake Skinner mixed culture.

of total organic carbon per liter (below detection limit) for the medium without added carbon and for the water used to prepare the medium. Because of the relatively high detection limit for total organic carbon in relation to the MIB level in the latter experiment, the presence of other carbon sources at low levels cannot be ruled out.

MIB degradation in cometabolism experiments. MIB was degraded in the presence of sucrose, glucose, fructose, and sodium acetate (Table 3). In the presence of the sugars or acetate, MIB was degraded in periods ranging from 6 to 12 days; in the control with only 20 μg of MIB per liter, degradation took place between days 12 and 18. Turbid growth was evident after 1 day with glucose, fructose, and acetate and after 2 days with sucrose. Bacterial numbers peaked around day 6 (at 5.0×10^7 CFU/ml). In the presence of the sugars, there was a lag of at least 6 days before any measurable MIB reduction occurred.

MIB degradation in sterile lake water. MIB at 10 μg/liter in sterile Lake Perris and Lake Mathews water was degraded within 7 days. The final MIB concentrations were 18 and <18 ng/liter for the two Lake Perris cultures and 18 ng/liter for both Lake Mathews cultures. These values represent at least a 99.8% reduction in MIB. There was no significant change in MIB level in the controls. Bacterial levels were not determined for this experiment.

DISCUSSION

To the best of our knowledge, this is the first published report of MIB degradation as a sole carbon source or of

TABLE 3. MIB degradation in presence of other substrates[a]

Substrate	MIB concn (μg/liter) on day:		
	6	13	18
Sucrose	17	<0.05	ND[b]
Glucose	21	0.4	<0.02
Fructose	20	<0.05	ND
Sodium acetate	ND	<0.4	ND
MIB only	20	19[c]	<0.05
MIB only	23,000		22,000
Uninoculated control			22

[a] Inoculated with washed cells from a Lake Perris culture. Substrate concentration was 20 mg/liter; initial MIB concentration was 20 μg/liter.
[b] ND, Not determined.
[c] Analyzed on day 12.

bacterial growth on MIB. Although MIB is not encountered in nature as a sole carbon source or at levels sufficient to support bacterial growth, the fact that MIB can be metabolized in this way suggests extensive breakdown of the molecule under favorable conditions, rather than simply a transformation to a similar but odorless compound. Definitive evidence for mineralization of MIB is lacking; this usually involves radioisotopic studies, and radiolabeled MIB was not available for this study. In the absence of mineralization data, the degradation of MIB in defined medium and the apparent enhancement of bacterial growth provide indirect, suggestive evidence for uptake and mineralization. Whether MIB is mineralized at the concentrations usually found in freshwater (nanogram-per-liter levels) is unknown. It has been shown that some organic compounds mineralized at high levels may not be mineralized at low levels (3) and that those mineralized at low levels may be cometabolized at higher levels (36).

A lag in MIB degradation was observed consistently in all cultures, although the lag was of unequal duration from one culture to another. The reason for this lag is unclear; it may represent the time required for the growth of degraders to levels sufficient for degradation to occur, as was recently proposed for the mineralization of 2,4-dichlorophenoxyacetic acid (2,4-D) in lake water and sewage (38). Although this may provide a partial explanation, it is not entirely consistent with the data. In cultures containing sugars or other easily metabolizable substrates growth was rapid, but the lag in MIB degradation was still evident. This presumes that the bacteria growing were not the ones involved in MIB degradation. The rate of degradation was generally not proportional to the inoculum even at roughly comparable MIB levels. Another explanation for the lag in MIB disappearance may be that the necessary enzymes are inducible and that induction under the experimental conditions requires several days. Whether this lag would be observed under continuous culture conditions is uncertain.

In the study by Namkung and Rittmann involving biodegradation of four compounds (22), a plug-flow type of reactor was used for maintaining a biofilm on glass beads. In short-term (2- to 3-h) tests for removal of MIB, the removal percentages for 100 and 1,000 μg/liter were 44 and 17%, respectively. Assuming that this removal was primarily a result of biodegradation, the removals were rapid compared with those observed in this study. However, because the experimental conditions and the time frame were very different from those used in the present study, it is difficult to make meaningful comparisons. Although MIB degradation was slower in the batch-culture experiments, the final MIB levels were usually below the average threshold odor concentration of 29 ng/liter, which was not achieved in the biofilm study. The latter study did show the potential for MIB removal by a biofilm.

In this study, MIB degradation was not proportional to the concentration of the compound. For example, the disappearance of MIB at 290 ng/liter took approximately half the time it took at 2.0 mg/liter (11 days for 290 ng/liter compared with 20 days for 2.0 mg/liter). These results are consistent with the finding of Hoover et al. that the mineralization of *p*-nitrophenol and nitrilotriacetic acid at 100 ng/liter or 2.0 μg/liter of 2,4-D in lake water was significantly slower than at higher levels (13). At 290 ng of MIB per liter, the substrate concentration was lower than that generally considered capable of supporting microbial growth (29). It has been shown that at nanogram-per-liter substrate levels, however, mineralization can occur without carbon assimilation into cell material (33). In a freshwater reservoir, degradation of trace levels of MIB would proceed in the presence of other organic compounds; at trace substrate levels, mineralization in lake water is sometimes enhanced by other nutrients (28).

The enrichment of bacteria on isoborneol before exposure to MIB was intended as a preenrichment or "priming" step, similar to the initial enrichment of polychlorinated biphenyl degraders on biphenyl (2) or the enrichment on methylcyclohexane of bacteria able to grow on a wide range of alicyclic compounds (35). Unfortunately, this strategy was unsuccessful for the enrichment of MIB degraders. The inability of isoborneol-grown cultures to degrade MIB suggests that the degradative pathways for the two compounds are dissimilar. Dagley (7) mentioned several instances in which compounds with single substituents were degraded by different pathways than the unsubstituted parent compound—for example, 4-chlorobenzoate as compared with benzoate. With regard to MIB, the bacteria grown on isoborneol may not metabolize MIB as simply a methyl-substituted isoborneol.

Because MIB is degraded in days to weeks instead of hours, even at microgram-per-liter levels, it can be considered somewhat resistant to bacterial attack. This resistance is surprising for a low-molecular-weight, naturally occurring compound. Cycloalkanols are reported to be readily biodegraded, and bacteria able to utilize them as sole carbon sources are easily isolated from water, mud, and soil (35). In view of the rapid degradation of isoborneol at milligram-per-liter levels, the presence of the additional methyl group in MIB probably accounts for the lesser biodegradability of MIB. Whether the methyl group at carbon 2 exerts its effect through the known steric hindrance of the hydroxyl group (21) or some other mechanism is uncertain. The resistance of MIB to chemical oxidation as used in water treatment is thought to result from its tertiary alcohol structure. This study suggests that the relative resistance of MIB to bacterial oxidation may derive from the same structural feature.

Despite the relative recalcitrance of MIB, degradation occurred in cultures representing a variety of lake-water and sediment samples, indicating widespread presence of potential MIB degraders in the reservoirs in which MIB is produced. Thus, the potential exists for in situ degradation of MIB by the indigenous bacteria in these reservoirs. How this process might be enhanced is a practical question that has yet to be answered.

In the majority of MIB enrichments, degradation was not sustainable upon subculture in MSM—i.e., after the first or second transfer. For example, degradation in the Lake Skinner mixed culture used for the kinetic experiments eventually ceased with MIB as the sole carbon source and could only be achieved in sterile lake water spiked with MIB. These observations suggest that MIB degradation requires other unidentified organic or inorganic nutrients. The loss of degrading ability may also result from diluting out of particulate matter from the inoculum that might have enhanced degradation. This would be especially relevant to the sediment-derived cultures, in which attachment could be important.

A related observation was that MIB degradation occurred only in mixed cultures. This is not surprising, as it is well known that some organic compounds are more easily degraded by mixed populations than by pure cultures (30). If MIB degradation occurs only in mixed populations, then the die-off of one or more strains necessary for the degradation could result in the loss of degrading ability in the culture as a whole. The short-lived degradation of MIB in many of the

cultures may reflect instability of their constituent populations.

The difficulty in achieving MIB degradation in consortia consisting of recombined pure cultures suggests the loss of degrading ability after growth on complex, nonselective media. This loss of degrading ability (for 2,4,5-trichlorophenoxyacetic acid and naphthalenesulfonic acid) has been reported in two recent papers (5, 16), both involving a pure strain of *Pseudomonas* sp.

The bacteria isolated from the MIB-degrading cultures were predominantly members of the genus *Pseudomonas*, a group that is well known for its metabolic versatility (6, 32). Pseudomonads have been shown to grow on a wide variety of organic compounds. In particular, *P. aeruginosa* can utilize a wide range of substrates (32), although natural strains of the species are not known to possess degradative plasmids (11). *P. aeruginosa* is common in fresh water and sediments (12, 23). It is significant, therefore, that this species was isolated from the populations derived from both Lake Perris and Lake Skinner.

Clearly, further work is needed to elucidate the degradative pathways of MIB. Perhaps then the differences in the degradation of MIB and isoborneol can be understood. Another area worthy of investigation is the effect on MIB degradation of particulates and inert surfaces, both of which are present in the natural environment and in water treatment processes. Much remains to be learned about the microbial degradation of this interesting natural compound.

ACKNOWLEDGMENTS

We are deeply indebted to Stuart W. Krasner, Melissa S. Dale, Margaret S. Moylan, Cordelia J. Hwang, and Carmen Taran for the many hours they spent analyzing the cultures. We thank Karen E. Scott, Suzanne M. Teague, and Debra L. Clark for identifying the bacterial isolates. We are grateful for the expert help of Robert F. La Londe in preparing the graphics and Peggy Kimball in editing the text.

LITERATURE CITED

1. **American Public Health Association.** 1985. Standard methods for the examination of water and wastewater, 16th ed. American Public Health Association, Washington, D.C.
2. **Bedard, D. L., R. E. Wagner, M. J. Brennan, M. L. Haberl, and J. F. Brown, Jr.** 1987. Extensive degradation of Arochlors and environmentally transformed polychlorinated biphenyls by *Alcaligenes eutrophus* H850. Appl. Environ. Microbiol. **53:**1094–1102.
3. **Boethling, R. S., and M. Alexander.** 1979. Effect of concentration of organic chemicals on their biodegradation by natural microbial communities. Appl. Environ. Microbiol. **37:**1211–1216.
4. **Bradshaw, W. H., H. E. Conrad, E. J. Corey, I. C. Gunsalus, and D. Lednicer.** 1959. Microbiological degradation of (+)-camphor. J. Am. Chem. Soc. **81:**5507.
5. **Brilon, C., W. Beckmann, M. Hellwig, and H.-J. Knackmuss.** 1981. Enrichment and isolation of naphthalenesulfonic acid-utilizing pseudomonads. Appl. Environ. Microbiol. **42:**39–43.
6. **Clarke, P. H.** 1982. The metabolic versatility of pseudomonads. Antonie van Leeuwenhoek J. Microbiol. Serol. **48:**105–130.
7. **Dagley, S.** 1978. Determinants of biodegradability. Quant. Rev. Biophys. **11:**577–602.
8. **Gauntlett, R. B., and R. F. Packham.** 1973. The removal of organic compounds in the production of potable water. Chem. Ind. **17:**812–817.
9. **Gerber, N. N.** 1969. A volatile metabolite of actinomycetes, 2-methylisoborneol. J. Antibiot. **22:**508–509.
10. **Gerber, N. N., and H. A. Lechevalier.** 1965. Geosmin, an earthy-smelling substance isolated from actinomycetes. Appl. Microbiol. **13:**935–938.
11. **Haas, D.** 1983. Genetic aspects of biodegradation by pseudomonads. Experientia **39:**1199–1213.
12. **Hoadley, A. W.** 1968. On the significance of *Pseudomonas aeruginosa* in surface waters. J. N. Engl. Water Works Assoc. **82:**99–111.
13. **Hoover, D. G., G. E. Borgonovi, S. H. Jones, and M. Alexander.** 1986. Anomalies in mineralization of low concentrations of organic compounds in lake water and sewage. Appl. Environ. Microbiol. **51:**226–232.
14. **Hwang, C. J., S. W. Krasner, M. J. McGuire, M. S. Moylan, and M. S. Dale.** 1984. Determination of subnanogram-per-liter levels of earthy-musty odorants in water by the salted closed-loop stripping method. Environ. Sci. Technol. **18:**535–539.
15. **Izaguirre, G., C. J. Hwang, S. W. Krasner, and M. J. McGuire.** 1982. Geosmin and 2-methylisoborneol from cyanobacteria in three water supply systems. Appl. Environ. Microbiol. **43:**708–714.
16. **Kilbane, J. J., D. K. Chatterjee, J. S. Karns, S. T. Kellogg, and A. M. Chakrabarty.** 1982. Biodegradation of 2,4,5-trichlorophenoxyacetic acid by a pure culture of *Pseudomonas cepacia*. Appl. Environ. Microbiol. **44:**72–78.
17. **Krasner, S. W., C. J. Hwang, and M. J. McGuire.** 1983. A standard method for quantification of earthy-musty odorants in water, sediments, and algal cultures. Water Sci. Technol. **15:**127–138.
18. **Lalezary, S., M. Pirbazari, and M. J. McGuire.** 1986. Oxidation of five earthy-musty taste and odor compounds. J. Am. Water Works Assoc. **78:**62–69.
19. **Martin, J. F., C. P. McCoy, W. Greenleaf, and L. Bennett.** 1987. Analysis of 2-methylisoborneol in water, mud, and channel catfish (*Ictalurus punctatus*) from commercial culture ponds in Mississippi. Can. J. Fish. Aquat. Sci. **44:**909–912.
20. **McGuire, M. J., S. W. Krasner, C. J. Hwang, and G. Izaguirre.** 1981. Closed-loop stripping analysis as a tool for solving taste and odor problems. J. Am. Water Works Assoc. **73:**530–537.
21. **Medsker, L. L., D. Jenkins, J. F. Thomas, and C. Koch.** 1969. Odorous compounds in natural waters: 2-*exo*-hydroxy-2-methylbornane, the major odorous compound produced by several actinomycetes. Environ. Sci. Technol. **3:**476–477.
22. **Namkung, E., and B. E. Rittmann.** 1987. Removal of taste- and odor-causing compounds by biofilms grown on humic substances. J. Am. Water Works Assoc. **79:**107–112.
23. **Pellett, S., D. V. Bigley, and D. J. Grimes.** 1983. Distribution of *Pseudomonas aeruginosa* in a riverine ecosystem. Appl. Environ. Microbiol. **45:**328–332.
24. **Persson, P.-E.** 1979. Notes on muddy odour. III. Variability of sensory response to 2-methylisoborneol. Aqua Fenn. **9:**48–52.
25. **Piet, G. J., B. C. J. Zoeteman, and A. J. A. Kraayeveld.** 1972. Earthy smelling substances in surface waters of the Netherlands. Water Treat. Exam. **21:**281–286.
26. **Pitter, P.** 1976. Determination of biological degradability of organic substances. Water Res. **10:**231–235.
27. **Rosen, A. A., C. I. Mashni, and R. S. Safferman.** 1970. Recent developments in the chemistry of odour in water: the cause of earthy-musty odour. Water Treat. Exam. **19:**106–119.
28. **Rubin, H. E., and M. Alexander.** 1983. Effect of nutrients on the rates of mineralization of trace concentrations of phenol and *p*-nitrophenol. Environ. Sci. Technol. **17:**104–107.
29. **Schmidt, S. K., M. Alexander, and M. L. Shuler.** 1985. Predicting threshold concentrations of organic substrates for bacterial growth. J. Theor. Biol. **114:**1–8.
30. **Slater, J. H., and D. Lovatt.** 1984. Biodegradation and the significance of microbial communities. *In* D. T. Gibson (ed.), Microbial degradation of organic compounds. Marcel Dekker, Inc., New York.
31. **Stanier, R. Y., R. Kunisawa, M. Mandel, and G. Cohen-Bazire.** 1971. Purification and properties of unicellular blue-green algae (order *Chroococcales*). Bacteriol. Rev. **35:**171–205.
32. **Stanier, R. Y., N. J. Palleroni, and M. Douderoff.** 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43:**159–271.
33. **Subba-Rao, R. V., H. E. Rubin, and M. Alexander.** 1982. Kinetics and extent of mineralization of organic chemicals at trace levels in freshwater and sewage. Appl. Environ. Micro-

biol. **43**:1139–1150.

34. **Tabachek, J. L., and M. Yurkowski.** 1976. Isolation and identification of blue-green algae producing muddy odor metabolites, geosmin and 2-methylisoborneol, in saline lakes in Manitoba. J. Fish. Res. Board Can. **33**:25–35.
35. **Trudgill, P. W.** 1984. Microbial degradation of the alicyclic ring. *In* D. T. Gibson (ed.), Microbial degradation of organic compounds. Marcel Dekker, Inc., New York.
36. **Wang, Y.-S., R. V. Subba-Rao, and M. Alexander.** 1984. Effect of substrate concentration and organic and inorganic compounds on the occurrence and rate of mineralization and cometabolism. Appl. Environ. Microbiol. **47**:1195–1200.
37. **Ward, N. R., R. L. Wolfe, C. A. Justice, and B. H. Olson.** 1986. The identification of gram-negative, nonfermentative bacteria from water: problems and alternative approaches to identification. Adv. Appl. Microbiol. **31**:293–365.
38. **Wiggins, B. A., S. H. Jones, and M. Alexander.** 1987. Explanations for the acclimation period preceding the mineralization of organic chemicals in aquatic environments. Appl. Environ. Microbiol. **53**:791–796.
39. **Wood, N. F., and V. L. Snoeyink.** 1977. 2-Methylisoborneol, improved synthesis and a quantitative gas chromatographic method for trace concentrations producing odor in water. J. Chromatogr. **132**:405–420.
40. **Yagi, M., M. Kajino, V. Matsuo, K. Ashitani, T. Kita, and T. Nakamura.** 1983. Odor problems in Lake Biwa. Water Sci. Technol. **15**:311–321.
41. **Yurkowski, M., and J. L. Tabachek.** 1980. Geosmin and 2-methylisoborneol implicated as a cause of muddy odor and flavor in commercial fish from Cedar Lake, Manitoba. Can. J. Fish. Aquat. Sci. **37**:1449–1450.