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Degradation of Chlorophenols by *Alcaligenes eutrophus* JMP134(pJP4) in Bleached Kraft Mill Effluent

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The ability of *Alcaligenes eutrophus* JMP134(pJP4) to degrade 2,4-dichlorophenoxyacetic acid, 2,4,6-trichlorophenol, and other chlorophenols in a bleached kraft mill effluent was studied. The efficiency of degradation and the survival of strain JMP134 and indigenous microorganisms in short-term batch or long-term semicontinuous incubations performed in microcosms were assessed. After 6 days of incubation, 2,4-dichlorophenoxyacetate (400 ppm) or 2,4,6-trichlorophenol (40 to 100 ppm) were extensively degraded (70 to 100%). In short-term batch incubations, indigenous microorganisms were unable to degrade such of compounds. Degradation of 2,4,6-trichlorophenol by strain JMP134 was significantly lower at 200 to 400 ppm of compound. This strain was also able to degrade 2,4-dichlorophenoxyacetate, 2,4,6-trichlorophenol, 4-chlorophenol, and 2,4,5-trichlorophenol when bleached Kraft mill effluent was amended with mixtures of these compounds. On the other hand, the chlorophenol concentration and the indigenous microorganisms inhibited the growth and survival of the strain in short-term incubations. In long-term (>1-month) incubations, strain JMP134 was unable to maintain a large, stable population, although extensive 2,4,6-trichlorophenol degradation was still observed. The latter is probably due to acclimation of the indigenous microorganisms to degrade 2,4,6-trichlorophenol. Acclimation was observed only in long-term, semicontinuous microcosms.

Chloroaromatic compounds are pollutants, since they are released in large quantities, are toxic and recalcitrant, and accumulate in sediments and biota. Bacterial degradation is the main natural removal mechanism for these pollutants (3, 31). *Alcaligenes* strains are able to degrade a variety of these compounds, such as 2,4-dichlorophenoxyacetate (2,4-D) (6), 3-chlorobenzoate (6, 10), monochlorophenols (33), 4-fluorobenzoate (32), dichlorobenzoates (26), chlorobiphenyls (1, 35), and DDT (27). The strain *A. eutrophus* JMP134(pJP4) has received a lot of attention, since it grows on 2,4-dichlorophenoxyacetate, 3-chlorobenzoate, and other phenoxyacetates (30), phenol (29), and some trihalophenols (4) as the sole carbon and energy source. Several of these catabolic properties are encoded in the broad-host-range plasmid pJP4 (6, 7, 15). Degradation of chloroaromatic compounds by *A. eutrophus* JMP134(pJP4) and other *Alcaligenes* and *Pseudomonas* strains carrying pJP4 or pJP4 derivatives has been reported to occur in soils (8, 17, 18, 34). However, little is known about the abilities of these strains to degrade such compounds in industrial effluents.

During the chlorine bleaching of cellulose, effluents containing low-molecular-weight chlorolignin derivatives, such as chloroguaiacols, chlorophenols, chlorocatechols, and some chloroaliphatics are produced (21, 24). The microbial, aerobic treatment of these bleached kraft mill effluents (BKME) is principally designed to remove most of the biodegradable organic matter, although chloroorganic removal also takes place (12, 14). The study of the role of microorganisms in chloroorganic removal during aerobic treatment of BKME has only recently been attempted (11). Some bacterial strains degrading chloroaliphatics have been reported (9). We have reported that indigenous BKME microorganisms are able to remove lower levels (<20 ppm) of 2,4-D, 2,4-dichlorophenol (2,4-DCP), and 4-chlorophenol (4-CP) in batch incubations (2). With the exception of 2,4-D, about 0.004 to 0.2 ppm of these and other chlorophenols are found in the effluent used in our previous work and in other BKME (2, 12, 21, 23).

The main purpose of the present work was to assess the ability of *A. eutrophus* JMP134(pJP4) to degrade 2,4-D, 2,4,6-trichlorophenol (2,4,6-TCP), or chlorophenol mixtures in BKME microcosms. The efficiency of degradation and the growth and survival of the introduced strain were studied in the presence or absence (sterilized BKME) of indigenous microorganisms, and in the presence of high (untreated BKME) or low (aerobically treated BKME) levels of degradable organic matter.

MATERIALS AND METHODS

Chemicals, culture media, and growth conditions. The sources and purity of the chemicals used have been described previously (4). BKME was obtained from Celulosa Arauco y Constitución and stored at 4°C. Aerobically treated BKME was collected from the outlet of a 12-liter microcosm designed to simulate an aerobic treatment lagoon (2). Removal of biochemical and chemical oxygen demand, absorbable organic halogen, and chlorophenols determined for these microcosms is similar to the removal observed in the mill lagoon (2). Simulation of BKME treatment lagoons has been also used by others (14). The *Alcaligenes* strain, minimal culture medium, and growth conditions have been described previously (4, 6, 13). When 2,4-D (400 ppm) was used as the growth substrate, the final optical density at 660 nm (OD_{660}) and the number of cultivable cells were 0.2 unit and 10^8 CFU/ml, respectively. After 2 days of culture, 2,4-D was completely consumed.

Degradation in BKME aerobic treatment microcosms. Short-term batch incubations were performed with 100-ml Erlenmeyer flasks containing 50 ml of effluent aerated with sterile, humid air (aeration rate, 12.5 ml of air min^{-1} ml of $effluent^{-1}$). Half of the volume consisted of nonsterile or sterile (autoclaved), untreated or aerobically treated BKME. The other half consisted of different dilutions (10^0 to 10^{-6}) of a 2-day culture of strain JMP134 in 2,4-D, prepared in minimal saline medium (13). Chloroaromatic compounds were added from 50

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mM stock solutions prepared in distilled water and filtered. For each condition, the incubations were run in duplicate. Unless otherwise is indicated, batch incubations were aerated for 6 days and 0.7-ml samples were taken on days 0, 3, and 6. In long-term experiments, the microcosm consisted of a 4.9-cm^2, 15-cm tube with a working volume of 40 ml, aerated as indicated above (aeration rate, 31.3 ml min^{-1} ml^{-1}). To start the system, half of the volume consisted of nonsterile, untreated BKME and the other half consisted of a 10^{-2} dilution of a 2-day culture of strain JMP134 in 2,4-D, prepared as above. This system was operated semicontinuously by replacing, every 2 days, one-third of the volume with fresh, nonsterile BKME, amended or not amended with the chloroaromatic.

Analytical methods. The percentage of chloroaromatic degradation was determined in a diode array spectrophotometer (Hewlett-Packard HP 8452-A). Aliquots were extracted with ethyl acetate (1:1), and the organic phase was scanned. The baseline was adjusted with the organic solvent. 2,4,6-Trichlorophenol (2,4,6-TCP) standard solutions prepared in BKME and extracted as above gave linear absorbance readings between 0 and 100 ppm (calculated molar absorbability, 2,690 ± 156.7; maximum OD, 294 nm). Absorbance readings with 2,4-D were linear between 0 and 400 ppm (calculated molar absorbability, 265.3 ± 71.9; maximum OD, 284 nm). Unfortunately, the high level of chloride in BKME prevented us from monitoring degradation through determination of chloride release. However, as complete degradation of 2,4,6-TCP and 2,4-D has been shown previously (4, 6, 29, 30), we think that removal corresponds to true degradation. Selected samples were also analyzed by gas chromatography as described previously (2). A Hewlett-Packard model 5890 gas chromatograph equipped with a model 5972 mass selective detector and a capillary column (HP5; phenylmethylsilicone, 30 m by 0.25 mm) was used, with a temperature gradient of 100 to 250°C, increasing at 4°C per min. Under these conditions, retention times for the acetylated derivatives of 4-CP, 2,4-DCP, 2,4,6-TCP, 2,4,5-TCP, and the internal standard 2,4,6-tribromophenol were 5.7, 8.2, 10.7, 12.1, and 18.3 min, respectively. The detection limit was 4 ppb.

Cultivable counts. Total cultivable counts in effluent (indigenous microorganisms plus strain JMP134) were routinely determined in plates containing nutrient agar, incubated for 3 days at 30°C. *A. eutrophus* JMP134 cultivable counts were determined on 2,4-D (400 ppm) agar plates, prepared in minimal medium, after 5 days of incubation at 30°C. Strain JMP134 colonies were distinguished from background colonies because strain JMP134 colonies were opaque, white to cream colored, circular with a regular edge, convex, and moist whereas most background colonies were transparent or light blue. Colonies scored positive were occasionally checked by being streaked on 2,4-D (400 ppm) plates containing 0.004% bromothymol blue (pH 7.0) (25). Chloride released by dehalogenation of 2,4-D turned this dye to yellow. Colony hybridization assays with a biotin-labeled probe containing part of the *tfdB* gene from pJP4 (16) were also performed, using a bioluminescence detection kit, as recommended by the manufacturer (Gibco BRL, Gaithersburg, Md.). A method for plasmid detection was occasionally used to verify the presence of pJP4 in colonies scored positive (20).

RESULTS

Degradation of 2,4-D by *A. eutrophus* JMP134 in BKME. Strain JMP134 was originally described by its ability to grow on 2,4-D (6). We tested if such a property was expressed in a chloroaromatic-containing industrial effluent. Different numbers of cells of this strain (10^2 to 10^8 cultivable cells/ml) were incubated with a nonsterile, high- (untreated) or low (aerobically treated)- organic-matter-containing BKME, amended with 400 ppm of 2,4-D. Table 1 shows that strain JMP134 was able to remove 45 to 70% of 2,4-D and that there was no degradation in the absence of strain JMP134. The amount of starting cells had little effect on the removal efficiency (Table 1). On the other hand, the strain always reached a stable population of 10^8 and 10^4 cultivable cells/ml with untreated and treated BKME, respectively (Table 1).

Degradation of 2,4,6-TCP by *A. eutrophus* JMP134 in BKME. The ability of *A. eutrophus* JMP134 to grow on 2,4,6-TCP has recently been shown by our group (4). To test the ability of strain JMP134 to degrade 2,4,6-TCP in this effluent, 40 to 400 ppm of this compound was added to either sterile or nonsterile, untreated or aerobically treated BKME. 2,4,6-TCP was degraded in all effluents, and the degradation was strongly dependent on the amount of compound (Table 2). When selected samples were analyzed by gas chromatography, complete degradation of 2,4,6-TCP was also observed (Fig. 1b). With a starting inoculum of 10^8 cells/ml, degradation of 100 ppm of this chlorophenol in aerobically treated BKME was also complete (Table 2, footnote *e*). Further assays in aerobically treated BKME with 10^8 cells/ml were not performed, since we did not want the added strain to exceed the number of indigenous microorganisms. Although Table 2 shows removal values only after 6 days of incubation, rates of degradation were also determined on day 3. Under optimal conditions, that is, 100 ppm of 2,4,6-TCP, higher inocula, and absence of indigenous microorganisms, 85 to 90% of the final degradation was observed on day 3.

TABLE 1. Degradation of 2,4-D in untreated and aerobically treated BKME inoculated with *A. eutrophus* JMP134

Condition	% Degradation[a]	No. of *A. eutrophus* JMP134 cells/ml[b] on:	
		Day 0	Day 6
BKME	68.6	6.7×10^7	2.6×10^8
	64.8	5.6×10^6	3.4×10^8
	61.5	8.4×10^5	3.0×10^8
	52.4	1.0×10^5	4.5×10^8
	53.3	3.1×10^3	4.3×10^8
	55.9	1.9×10^2	3.9×10^8
	1.5	0	0
BKME, AT[c]	56.2	6.6×10^4	6.5×10^3
	63.5	5.4×10^2	3.8×10^4
	44.6	3.0×10^1	4.0×10^4
	0	0	0

[a] Estimated as removal of UV-absorbing material after 6 days of incubation with 400 ppm of 2,4-D. Values are means of duplicate determinations. Standard deviations (SD) were less than 10%.
[b] *A. eutrophus* JMP134 counts were determined in plates with 2 mM 2,4-D as the sole carbon source.
[c] AT, Aerobically treated.

TABLE 2. Degradation of 2,4,6-TCP by *A. eutrophus* JMP134 in untreated and aerobically treated BKME

Condition	2,4,6-TCP concn (ppm)	% Degradation[a]	No. of *A. eutrophus* JMP134 cells/ml[b] on:	
			Day 0	Day 6
BKME, sterile	100	90.4	1.4×10^8	8.1×10^7
BKME	100	92.9	4.0×10^7	3.0×10^7
BKME, sterile	200	12.0	2.3×10^8	2.8×10^7
BKME	200	5.5	2.5×10^8	1.6×10^7
BKME, sterile	400	0	1.2×10^8	1.0×10^5
BKME	400	0	1.0×10^8	9.0×10^3
BKME, sterile	0	0	ND[c]	ND
BKME	0	0	3.7×10^7	4.5×10^7
BKME, AT[d] sterile	40	90.5	8.5×10^4	2.8×10^8
BKME, AT	40	64.3	1.9×10^4	3.5×10^6
BKME, AT, sterile	100	46.9[e]	ND	6.3×10^7
BKME, AT	100	34.0[e]	3.2×10^4	1.5×10^5
BKME, AT, sterile	200	31.5	1.3×10^4	7.5×10^3
BKME, AT	200	10.2	2.1×10^4	2.0×10^3
BKME, AT, sterile	400	10.8	7.5×10^3	3.1×10^1
BKME, AT	400	17.9	5.4×10^4	0
BKME, AT, sterile	0	0	ND	ND
BKME, AT	0	0	1.7×10^4	2.6×10^2

[a] Estimated as removal of UV-absorbing material after 6 days of inoculation with 2,4,6-TCP. Values are means of duplicate determinations. SD were less than 10%.
[b] *A. eutrophus* JMP134 counts were determined in plates with 2 mM 2,4-D as the sole carbon source.
[c] ND, not determined.
[d] AT, aerobically treated.
[e] Complete degradation after 3 days with 10^8 cells/ml as the initial inoculum.

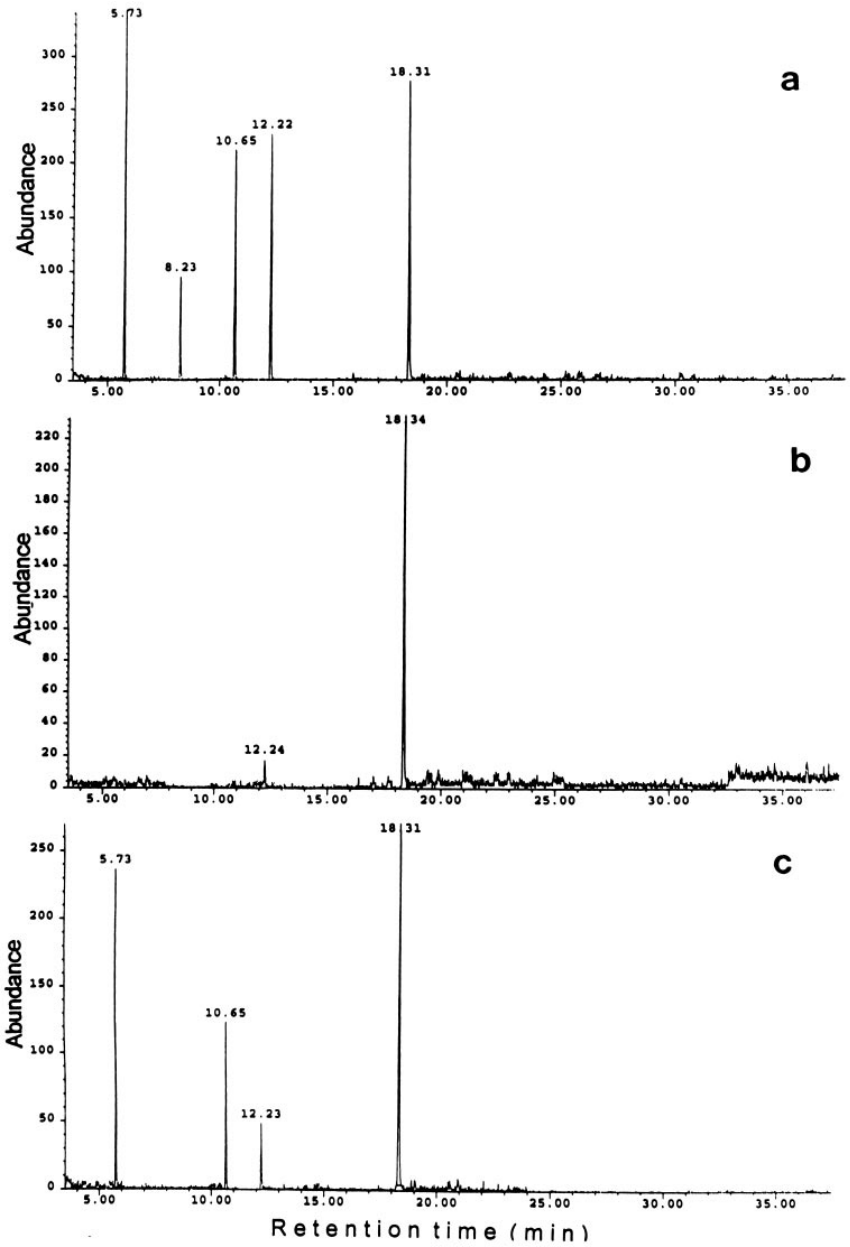

FIG. 1. Gas chromatography profiles of samples from batch microcosms of untreated BKME taken after 0 (a) or 6 (b and c) days of incubation, inoculated (a and b) or not inoculated (c) with 10^8 cells of strain JMP134 per ml. Retention times for acetylated compounds and chromatographic conditions are given in the text. Incubation were performed at an aeration rate of 31.3 ml min^{-1} ml^{-1}.

The effect of different numbers of cells of strain JMP134 on the degradation of 2,4,6-TCP in untreated and aerobically treated BKME was also assessed. The removal efficiency in BKME was only slightly affected by the size of the inoculum (Table 3). The results also showed that there was no significant removal of 2,4,6-TCP in the absence of strain JMP134 (Table 3). Furthermore, experiments with sterile BKME indicated that indigenous microorganisms were not involved in 2,4,6-TCP removal. In untreated BKME, strain JMP134 reached a final level of 10^7 to 10^8 cultivable cells/ml in the absence of native microorganisms but only sustained the starting levels of inocula in their presence. In contrast, in aerobically treated BKME, strain JMP134 reached the same final cell level independent of the initial inoculum or the presence native microorganisms (Table 3).

Degradation of 2,4,6-TCP by *A. eutrophus* JMP134 in BKME amended with mixtures of chloroaromatics. We tested the potential of *A. eutrophus* JMP134 to degrade mixtures of 2,4,6-TCP and 2,4-D in the high-organic-matter-containing BKME. High levels of degradation and survival were observed, except with 400 ppm of both compounds (Table 4). The ability of *A. eutrophus* JMP134 to remove a mixture of 2,4,6-TCP plus other chlorophenols was also studied. 4-CP, 2,4-DCP, 2,4,5-TCP, and 2,4,6-TCP (20 ppm each) were added to either sterile or nonsterile untreated BKME in the presence or absence of 10^8 cultivable cells of strain JMP134 per ml. Gas chromatography

TABLE 3. Degradation of 2,4,6-TCP in untreated and aerobically treated BKME inoculated with *A. eutrophus* JMP134

Condition	% Degradation[a]	No. of *A. eutrophus* JMP134 cells/ml[b] on:	
		Day 0	Day 6
BKME, sterile	90.4	1.8×10^8	7.0×10^7
BKME	92.7	1.2×10^8	1.0×10^7
BKME, sterile	100	1.4×10^6	1.3×10^8
BKME	93.3	1.3×10^6	9.5×10^6
BKME, sterile	100	1.1×10^4	2.1×10^8
BKME	92.8	4.7×10^5	2.0×10^5
BKME, sterile	93.3	7.0×10^3	7.5×10^8
BKME	52.5	9.5×10^3	1.0×10^5
BKME, sterile	70.3	1.4×10^2	1.2×10^7
BKME	66.7	8.0×10^2	2.3×10^3
BKME, sterile	10.8	0	0
BKME	11.0	0	0
BKME, AT,[c] sterile	22.5	2.7×10^5	3.1×10^6
BKME, AT	23.7	1.7×10^5	5.0×10^5
BKME, AT, sterile	31.4	2.2×10^3	4.2×10^6
BKME, AT	11.3	2.5×10^3	1.2×10^5
BKME, AT, sterile	25.7	2.7×10^2	5.1×10^6
BKME, AT	3.5	2.0×10^2	1.4×10^5
BKME, AT, sterile	6.6	0	0
BKME, AT	6.9	0	0

[a] Estimated as removal of UV-absorbing material after 6 days of incubation with 100 ppm of 2,4,6-TCP. Values are means of duplicate determinations. SD were less than 10%.
[b] *A. eutrophus* JMP134 counts were determined in plates with 2 mM 2,4-D as the sole carbon source.
[c] AT, aerobically treated.

analysis indicated that 4-CP, 2,4-DCP, and 2,4,6-TCP were completely removed and that only 6.2% of the 2,4,5-TCP remained after 6 days of incubation (Fig. 1b). However, the removal of 2,4-DCP was completely abiotic (Fig. 1c). Significant volatilization (77.4%) of 2,4,5-TCP was also observed, indicating that only 16.4% of the removal was due to degradation by strain JMP134. Indigenous microorganisms did not remove 2,4,5-TCP (data not shown). With respect to 2,4,6-TCP, 33.5% was removed in abiotic controls (Fig. 1c) and 11% was removed by indigenous microorganisms (results not shown); therefore, 55.5% of the degradation was produced by the addition of strain JMP134. Finally, volatilization and the activity of indigenous microorganisms accounted for 20.6% and 7% of 4-CP removal, respectively, indicating that strain JMP134 degraded about 75% of this chlorophenol. Volatilization is the abiotic removal mechanism, since it was not observed in nonaerated batch incubations. The final level of *A. eutrophus* JMP134 with this mixture in the absence of native microorganisms was 1.5×10^7 cultivable cells/ml, but only 3.3×10^3 cultivable cells/ml were detected in their presence. The total population remained unaltered in incubations with this mixture.

TABLE 4. Degradation of mixtures of 2,4-D and 2,4,6-TCP by *A. eutrophus* JMP134 in untreated BKME

Concn (ppm) of:		% Degradation[a]	No. of *A. eutrophus* JMP134 cells/ml[b] on:	
2,4-D	2,4,6-TCP		Day 0	Day 6
100	100	89	7.3×10^7	7.0×10^7
200	100	87	7.3×10^7	4.0×10^7
400	100	92	7.3×10^7	8.0×10^7
400	400	0	1.5×10^6	2.2×10^3

[a] Estimated as removal of UV-absorbing material after 6 days of incubation. Values are means of duplicate determinations. SD were less than 10%.
[b] *A. eutrophus* JMP134 counts were determined in plates with 2 mM 2,4-D as the sole carbon source.

Long-term survival and degradation of 2,4,6-TCP by *A. eutrophus* JMP134 in BKME. Long-term expression of catabolic activity and survival are important when considering the addition of a degrader strain to a polluted environment. To assess these factors, 2.0×10^6 cultivable cells of *A. eutrophus* JMP134 per ml plus 100 ppm of 2,4,6-TCP were added to a semicontinuous microcosm containing nonsterile, untreated BKME. For the first 6 days, incubation was carried out under batch conditions to allow initial acclimation of strain JMP134 to such a microcosm (Fig. 2). The rate of degradation agreed with that in batch assays (Table 2), with no remaining compound detected after 6 days of incubation (Fig. 2). The viability of *A. eutrophus* JMP134 steadily decreased thereafter, reaching the detection limit after 40 days of incubation (Fig. 2). At this time, positive counts were confirmed by detecting the presence of pJP4 by a miniprep method and by hybridization with a *tfdB* gene probe. Total cultivable counts decreased to 3.0×10^5 cell/ml after 3 weeks of incubation and remained essentially constant thereafter (Fig. 2). When a new load of 100 ppm of 2,4,6-TCP was added to the system at day 46 and the system was again operated under batch conditions for 6 days, 95% of degradation was detected after 4 days (Fig. 2). This percentage was higher than that observed (65%) in batch assays with 10^2 cultivable cells of starting inoculum per ml (Table 3). After a third load of 2,4,6-TCP (day 60), this time under semicontinuous operation, degradation was higher than expected but even lower counts of strain JMP134 were detected (Fig. 2).

Consecutive loads of 20 or 100 ppm of 2,4,6-TCP every 2 days under semicontinuous operation, which now included the compound in the feeding, were readily degraded after 1.5 days, while the level of cultivable cells of strain JMP134 stabilized at 10 cells/ml (Fig. 2). As such a high level of degradation could not be produced by this small number of cells of strain JMP134, a noninoculated microcosm was semicontinuously fed with 100 ppm of 2,4,6-TCP. After 20 days of incubation, the indigenous microorganisms were acclimated to degrade more than 80% of 2,4,6-TCP.

When a microcosm was initially and continuously amended with 100 ppm of 2,4,6-TCP and inoculated with strain JMP134, degradation of the chloroaromatic was always higher than 90%. A steady decline of cultivable counts of strain JMP134 was observed over a 90-day period. After that, 10^3 cells/ml were still detected. Abiotic removal (volatilization) of 2,4,6-TCP in incubation mixtures semicontinuously fed with compound accounted only for about 25% of the removal. Finally, when the same experiment was performed with 400 ppm of 2,4-D, 60 to 70% degradation was observed, with 10^4 to 10^5 cells of strain JMP134 per ml remaining at day 75. In all long-term experiments, the total cultivable counts remained unchanged.

DISCUSSION

The ability of *A. eutrophus* JMP134 to degrade 2,4-D, 2,4,6-TCP, 2,4,5-TCP, and 4-CP when added to an organic matter- and chloroaromatic-containing industrial effluent is reported here for the first time. The inoculum size had little effect on the degradation of 2,4,6-TCP and 2,4-D. The total viable microbial population in this effluent, estimated by epifluorescence microscopy, ranged between 10^7 and 10^8 cells/ml and 10^5 and 10^6 cells/ml for untreated and aerobically treated BKME, respec-

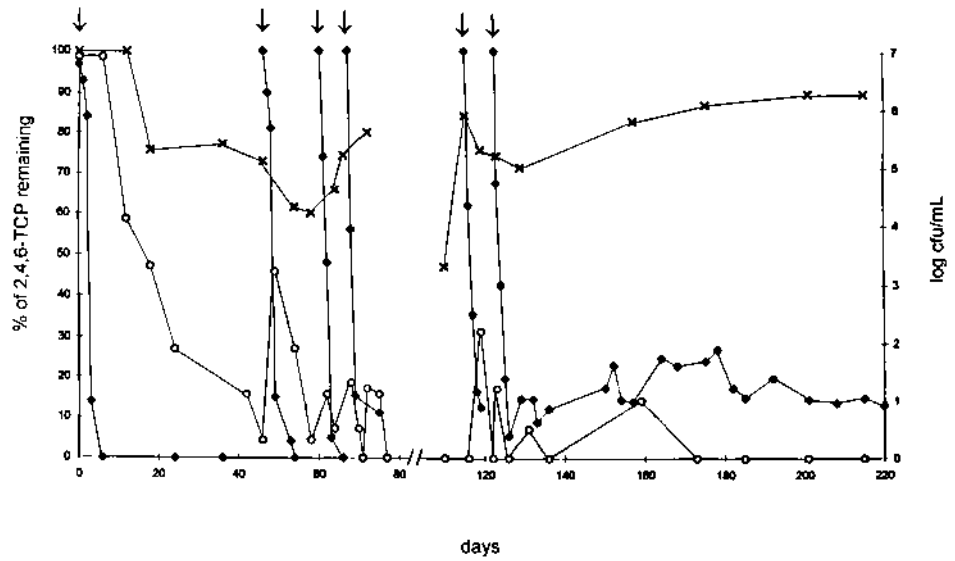

FIG. 2. Long-term survival and 2,4,6-TCP degradation by *A. eutrophus* JMP134 in BKME. ◆; percentage of compound remaining in the effluent; ○, culturable cells of strain JMP134; ✕, total culturable cells. Arrows indicate times when changes in feeding were introduced: 100 ppm of 2,4,6-TCP was added on days 0, 46, and 60, followed by feeding without compound; 20 ppm of 2,4,6-TCP was added on days 67 and 115, followed by feeding with compound; 100 ppm of 2,4,6-TCP was added on day 122, followed by feeding with compound.

tively (2). We also report that degradation of 2,4-D or 2,4,6-TCP took place even if, at the start of the incubation, only 1 of 10^6 cells was strain JMP134.

Degradation of 2,4,6-TCP was strongly influenced by the concentration of compound. High degradation levels were observed with only 40 or 100 ppm of compound. The latter suggests that higher concentrations of 2,4,6-TCP are toxic even in the presence of the carbon sources from BKME. The concentration of 2,4,6-TCP also affected the growth and survival of strain JMP134. 2,4,6-TCP at 400 ppm killed a substantial part of the population of the introduced strain (Table 2). Toxicity of 2,4,6-TCP was also observed in mixtures with 2,4-D (Table 4). Survival of the strain was also inhibited by indigenous microorganisms in untreated BKME in incubations with 2,4,6-TCP (Table 3) or in CP mixtures. The presence of a significant protozoan population (10^3 cells/ml) in BKME may explain this observation. The effect of protozoans in decreasing the survival of *A. eutrophus* AEO106 in lake water microcosms has been shown previously (22). Curiously, survival was not affected by indigenous microorganisms in incubations with aerobically treated BKME (Table 3). We have no good explanation for this observation, except that the activity (but not the number) of protozoa could be lower in aerobically treated BKME than in untreated BKME.

In experiments with 2,4-D, the higher organic matter content of untreated BKME (biochemical oxygen demand, ~300 to 400 mg of O_2/liter) allowed populations of 10^7 to 10^8 cultivable cells/ml, in contrast with that of aerobically treated BKME (biochemical oxygen demand, ~45 to 90 mg of O_2/liter), where strain JMP134 reached only 10^3 to 10^4 cultivable cells/ml (Table 1). However, higher than initial levels of strain JMP134 were found in incubations with aerobically treated BKME amended with 2,4,6-TCP (Table 3). These observations suggest that the lower growth detected with aerobically treated BKME and 2,4-D was probably due to a toxic effect of 400 ppm of this compound on strain JMP134 rather than to carbon limitation. It has recently been reported that the survival of strain JMP134 in seawater microcosms depends on nutrient and substrate amendment (28). However, as the BKME used in the present work is amended with nutrients and contains a significant level of organic matter (2), it seems more probably that survival is controlled primarily by the chloroaromatic concentration and secondarily by the indigenous microorganisms.

On the other hand, 2,4-D was more extensively degraded in mixtures with 2,4,6-TCP than in incubations with 2,4-D alone (compare Tables 1 and 4). Also, when strain JMP134 was grown in batch cultures containing a mixture of 100, 200, or 400 ppm of 2,4-D plus 100 ppm of 2,4,6-TCP, prepared in minimal medium, an increase in growth (73, 18, and 13%, respectively), in comparison with cultures containing 2,4-D alone, was observed. This result suggests that 2,4,6-TCP is also incorporated into biomass, with 2,4-D as the primary carbon source, in minimal medium and BKME.

Long-term experiments suggest that the competitiveness of strain JMP134 in this BKME, although high to allow a constant or increased number of cells under short-term batch conditions, is not high enough to deal with indigenous microorganisms in long-term incubation without chloroaromatic amendement (Fig. 2). Only when 100 ppm of 2,4,6-TCP or 400 ppm of 2,4-D was initially and continuously added did the population of the strain stabilize at significant levels (10^3 to 10^5 cells/ml).

In experiments with 2,4-D (but not with 2,4,6-TCP), the degradative ability relies on the pJP4-encoded *tfd* genes. Then, the presence of the plasmid may provide strain JMP134 with a selective advantage over the native microbial population. This could explain the higher level of strain JMP134 found under conditions of continuous feeding with 2,4-D (10^4 to 10^5 cells/ml) than with 2,4,6-TCP (10^3 cells/ml). The role of plasmid pJP4 in determining the competitiveness of *Pseudomonas cepacia* DBO1(pJP4) in the presence of other 2,4-D degraders lacking the plasmid in the native microbiota of a Kansas prairie soil has been reported previously (19). The low activity of indigenous 2,4-D degraders in BKME (2), which contrasts with the presence of putative 2,4,6-TCP degraders (this work), may also favor the growth and survival of strain JMP134, as has been observed for mixtures of 2,4-D-degrading strains in soil (19).

It can be argued that part of the counts scored as JMP134

could be indigenous microorganisms that had received pJP4 by conjugal transfer, allowing them to grow on 2,4-D plates. Significant transfer of pJP4 in soil has been reported recently (5). However, the morphology of positive colonies was always the same as that of strain JMP134. Furthermore, in incubations with untreated BKME, a reported plasmid rescue procedure (36), with the plasmid-free derivative strain *A. eutrophus* JMP222 as receptor, gave a pJP4 conjugal transfer frequency lower than 10^{-5} cells/ml.

A possible explanation for the high level of 2,4,6-TCP degradation with a very low level of cultivable *A. eutrophus* JMP134 cells (Fig. 2) is that cells of strain JMP134 had lost plasmid pJP4. This implies that the cells were no longer detectable on 2,4-D plates but still could degrade 2,4,6-TCP, which is a chromosomally encoded property (4). However, the high stability of pJP4 observed when strain JMP134 was grown under several nonselective conditions (unpublished results) suggests that this is not the correct explanation. Alternatively, these cells could become noncultivable, but they still were numerous and metabolically active. This possibility has been also suggested by others (5). However, our results strongly suggest that in long-term incubations, the degradation of 2,4,6-TCP can also be carried out by acclimated indigenous microorganisms. In contrast to strain JMP134, such microorganisms expressed this ability only in aerated, long-term continuous BKME incubations. Batch cultures containing 100 ppm of 2,4,6-TCP in minimal saline medium and inoculated with nonacclimated (2) or acclimated (this work) indigenous microorganisms did not allow bacterial proliferation or 2,4,6-TCP degradation.

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