Appl Microbiol Biotechnol (1996) 45:383–390

ORIGINAL PAPER

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Mechanism of microbial utilization of biphenyl sorbed to polyacrylic beads

Received: 23 May 1995/Received revision: 17 October 1995/Accepted: 30 October 1995

Abstract A microbial consortium mineralized biphenyl sorbed to polyacrylic beads faster than the slow rate at which much of the compound was desorbed. Pure cultures of bacteria isolated from the consortium mineralized biphenyl in solution but not the sorbed compound. However, combinations of two strains did degrade biphenyl. The consortium did not reduce the surface tension in media containing sorbed biphenyl or biphenyl in solution, and addition of synthetic and microbially produced surfactants to pure cultures did not result in utilization of sorbed biphenyl by isolates able to use the soluble molecule. Cells from the consortium that were attached to continuously washed beads degraded the substrate. We suggest that bacteria may act on sorbed compounds without the necessity of an initial desorption and that the mechanism may involve cells attached to the particles rather than the excretion of a surfactant.

Introduction

Hydrophobic pollutants are characteristically sorbed by soils. Because many of these compounds are both toxic and persistent, the consequences of this sorption are of special importance. It is sometimes assumed that the sorbed compound is unavailable for biodegradation (Sabljic 1989). Indeed, evidence exists that hydrophobic compounds that become sorbed to soil are more resistant to biodegradation than the molecules in solution (Manilal and Alexander 1991; Weissenfels et al. 1992). More attention has been given to the effect of sorption of charged than nonionic compounds on their availability to microorganisms. Thus, Dashman and Stotzky (1986) showed that binding of amino acids to clays slowed their utilization by microorganisms, Gordon and Millero (1985) found that sorption of organic acids by hydroxyapatite decreased their bioavailability and Shimp and Young (1988) reported that dodecyltrimethylammonium chloride sorbed by sediments was not degraded.

Nevertheless microorganisms can degrade sorbed compounds. For example, Wszolek and Alexander (1979) found that the rate of degradation of amines complexed to clays exceeded the desorption rate, and Araujo (1991) showed that biphenyl sorbed to polymeric beads was mineralized by microorganisms in sediments. Although it is often assumed that the rate-limiting step in the degradation of a sorbed molecule is its spontaneous desorption (Ogram et al. 1985; Shimp and Young 1988), Guerin and Boyd (1992) suggested that the utilization of naphthalene sorbed to soil by a strain of *Pseudomonas* is associated with its attachment to soil particles.

Because of the interest in bioremediating soils containing hydrophobic compounds and their retention by soil particles, it is important to know the means by which microorganisms acquire potential substrates that are bound to particulate matter. This information should be useful in establishing rate-limiting steps in biodegradation and possible means of enhancing the conversion. Hence, a study was conducted to establish how microorganisms acquire an organic nutrient that is initially bound to particulate matter. For this purpose, biphenyl and polyacrylic beads were chosen as model substrate and solids.

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Materials and methods

The inorganic salts solution contained 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 1 mg $FeCl_3$ and 100 mg each of $MgSO_4 \cdot 7H_2O$, $(NH_4)_2SO_4$, and $CaCl_2$ per liter. *Bacillus licheniformis* was grown in

a medium containing 10 g glucose, 2.8 g $NaNO_3$, 0.5 g KCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 2 ml 84% H_3PO_4, 20 mg $CaCl_2 \cdot H_2O$, 200 mg EDTA, 0.8 mg $ZnSO_4 \cdot 7H_2O$, 0.2 mg $MnSO_4 \cdot H_2O$, 100 μg $CuSO_4 \cdot 5H_2O$ and $CoCl_2 \cdot 6H_2O$, 60 μg H_3BO_3 and 20 μg $Na_2MoO_4 \cdot 2H_2O$ per liter. The water in all media was passed through a series of deionizing columns (Milli-Q, Millipore Corp., Bedford, Mass.) before use. Glassware was soaked for at least 4 h in concentrated sulfuric acid containing 1.5% Nochromix oxidant (Godax Laboratories, New York, NY, USA) and then rinsed extensively with distilled water.

The surfactants used were Triton X-100 (octylphenyl ethyoxylate) and Tween 80 (a nonionic surfactant that contains approximately 75% oleic acid) from Aldrich Chemical Co. (Milwaukee, Wis., USA), Tergitol 15-S-3 (alkyloxypolyethylene oxyethanol) from Union Carbide Chemicals and Plastics Co. (Danbury, Conn., USA), Alfonic 810-60 [$C_nH_{2n+1}(OCH_2CH_2)_xOH$; $n = 8$–14, average $x = 4.5$] from Vista Chemical Co. (Austin, Tex., USA), Enordet LXS-814 ($C_nH_{2n-1}C_8H_8SO_3Na$; $n = 8$–15) and Neodol 25–3S [$C_nH_{2n-1}(OCH_2CH_2)_xOSO_3Na$; $n = 12$–15, average $x = 3$] from Shell Development Co. (Houston Tex., USA), taurocholic acid ($C_{26}H_{44}NO_7SNa$) and dimethyldioctadecylammonium bromide, benzethonium chloride ($C_{27}H_{42}NO_2Cl$) and palmityl sulfobetaine from Sigma Chemical Co. (St. Louis, Mo., USA). A surfactant was also present in the culture filtrate of *Bacillus licheniformis*, which was provided by Dr. M. J. McInerney (University of Oklahoma, Norman, Okla., USA). To obtain a crude surfactant preparation from *B. licheniformis*, the bacterium was grown for 48 h at 30°C on a rotary shaker in the glucose/salts medium described above, and the filtrate from a turbid culture was obtained by removing the cells by passage of the culture through a 0.2-μm membrane filter. The surface tensions of the original medium and the filtrate from the stationary-phase culture were 647 ± 40 μN/cm and 243 ± 70 μN/cm respectively, indicating the presence of a surfactant in the filtrate.

Counts of viable cells were determined by the drop method (Hoben and Somasegaran 1982) after a 2-day incubation on trypticase/soy/agar or nutrient agar (BBL Microbiology Systems, Becton Dickinson Co., Cockeysville, Md., USA) and 4–5 days on minimal agar medium. The minimal agar medium contained 7 μg biphenyl, 100 μg casamino acids and 1 mg yeast extract/ml, and isolates from the consortium were obtained by plating on that medium.

Reagent-grade biphenyl and diethyl phthalate were purchased from Aldrich Chemical Co. The [U-^{14}C] biphenyl (specific activity, 15.4 mCi/mmol; more than 97% pure) was purchased from Sigma Chemical Co. Radiolabeled and unlabeled forms of biphenyl were dissolved in reagent-grade dichloromethane.

SM-7 Biobeads, (BioRad Inc., Richmond, Calif., USA) are neutral porous beads made of acrylic polymers. They are used to sorb nonpolar substances or surface-active agents from aqueous solutions. The beads have diameters of 0.27–0.89 mm, an average pore diameter of 9 nm and a surface area of 300 m^2/g. The beads were washed six times with deionized water, combusted at 150°C for 4 h to remove traces of organic materials and autoclaved for 15 min in the culture flasks before the addition of biphenyl. According to the manufacturer, the beads can withstand heating to 250°C.

The biphenyl-degrading enrichment was derived from 7.0 g Edwards muck amended with 0.7 g unlabeled biphenyl, 2 g Biobeads and 1 ml of activated sludge from the water-treatment plant of Ithaca, N.Y., USA. After 14 days of incubation at 30°C, 1-ml samples were transferred to flasks containing 20 ml inorganic salts solution and 100 μg unlabeled biphenyl supplemented with labeled biphenyl and either 0.2 g beads or 1 g sterile soil. The enrichment (designated SBP-1) was maintained for almost 3 years by transferring every 2 weeks to a medium containing sorbed biphenyl.

Pseudomonas paucimobilis, which uses biphenyl in solution but not sorbed biphenyl as a sole carbon source for growth, was characterized by the Biolog Microplate system (Biolog Inc., Hayward, Calif., USA).

The mineralization of biphenyl was determined in duplicate by trapping the evolved $^{14}CO_2$ in 2 ml 0.5 M NaOH contained in the side-arm of 250-ml biometer flasks (Bellco Glass Inc., Vineland, N.J., USA). The NaOH was transferred to scintillation vials at each assay time and replaced with fresh NaOH. Liquiscint scintillation cocktail (3.5 ml, National Diagnostics Inc., Somerville, N.J., USA) was added to the NaOH, and the radioactivity was counted with a liquid scintillation counter, model LS7500 (Beckman Instruments, Inc., Irvine, Calif., USA).

To determine mineralization by the enrichment culture, a mixture of unlabeled and labeled biphenyl (approximately 100 000 dpm) was added to the bottom of a dry, sterile 250-ml biometer flask containing 0.2 g beads. The dichloromethane in which the biphenyl was dissolved was allowed to evaporate, and 20 ml inorganic salts solution was added to the flask. The side-arm received 2 ml 0.5 M NaOH. The flask was inoculated with 0.1 ml liquid and 0.05 cm^3 beads from an enrichment that was actively mineralizing sorbed biphenyl. The cultures were incubated for 24 h at 30°C on a rotary shaker operating at 112 rpm, and 5-ml portions of the aqueous phase were then removed and the radioactivity counted. When equilibrium was reached, that is, when the concentration of biphenyl in solution did not change in subsequent samplings, the flasks were inoculated with 0.1 ml liquid and 0.05-cm^3 beads taken from an enrichment culture actively mineralizing sorbed biphenyl.

To measure continuous abiotic desorption with minimum loss of biphenyl, 0.2 g beads with 415 μg biphenyl and 1.5 μC_i ^{14}C was placed in 20 ml inorganic salts solution for 24 h and then washed twice with phosphate buffer and transferred to a sterile dialysis membrane with a 3.5-kDa cut-off. The membrane was sealed and placed in a 2-l two-necked round-bottom flask with ground-glass openings that were closed with stopcocks. The solution was stirred constantly with an uncoated magnetic stirrer. Portions (5.0 ml) were removed at intervals to determine radioactivity, and 5.0 ml fresh solution was added using stainless-steel cannulas and glass syringes.

To determine surface tension, samples of the filtrate were passed through 0.2-μm nylon filter units or syringe filters, and duplicate measurements were made with a Surface Tensiomat 21 tensiometer (Fisher Scientific Co., Rochester, N.Y., USA). Deionized distilled water was used to calibrate the tensiometer at 0.7 mN/cm.

To test the effects of synthetic surfactants, two bacteria capable of using biphenyl in solution but not sorbed biphenyl were isolated from the enrichment. The surfactants were added to cultures of the bacteria in biometer flasks containing either 400 μg biphenyl sorbed to 0.2 g beads or 20 μg soluble biphenyl/ml.

In one experiment, beads from a 50-h enrichment in biometer flasks were aseptically washed six times with sterile 10 mM phosphate buffer (pH 7.2). After each washing, the buffer was removed aseptically. That liquid had no detectable biphenyl. Fresh inorganic salts solution was added to the flasks, and the mineralization of sorbed biphenyl was measured. The mineralization by the consortium of sorbed biphenyl on unwashed beads was also determined.

In a study in which columns containing 0.2 g beads were continuously washed, the columns were autoclaved and assembled under aseptic conditions (Fig. 1). Inorganic salts solution from a 10-l reservoir was added continuously through Teflon tubing to the modified distilling receiver at a rate of 0.5 ml/min. A stainless-steel 18-gauge, 10-cm-long cannula was inserted into a silicone rubber stopper that covered the opening of the modified distilling receiver with a side-arm slanted at 120°. The stopper had been wrapped in Teflon tape. Another cannula was inserted into the stopper covering the distilling receiver for the entry of compressed air. The solution flowed into the distilling receiver, which had been modified to retain only 7 ml, and the excess flowed out through a side-arm (which was attached by a 24/40 ground-glass joint to one neck of a round-bottom three-neck flask). A second neck was closed with a silicone rubber stopper wrapped with Teflon tape. The stopper was also wrapped tightly to the neck with Teflon tape, and through this stopper were inserted 18-gauge cannulas for sampling. The third neck was covered with a ground-glass stopper. Beads from an active consortium culture were added to a bag made from a 5-cm^2 piece of Nitex bolting cloth, 37 μm mesh size, which was threaded around the edges to create drawstrings. This bag was placed aseptically into the distilling receiver, and the drawstrings then were pulled shut to

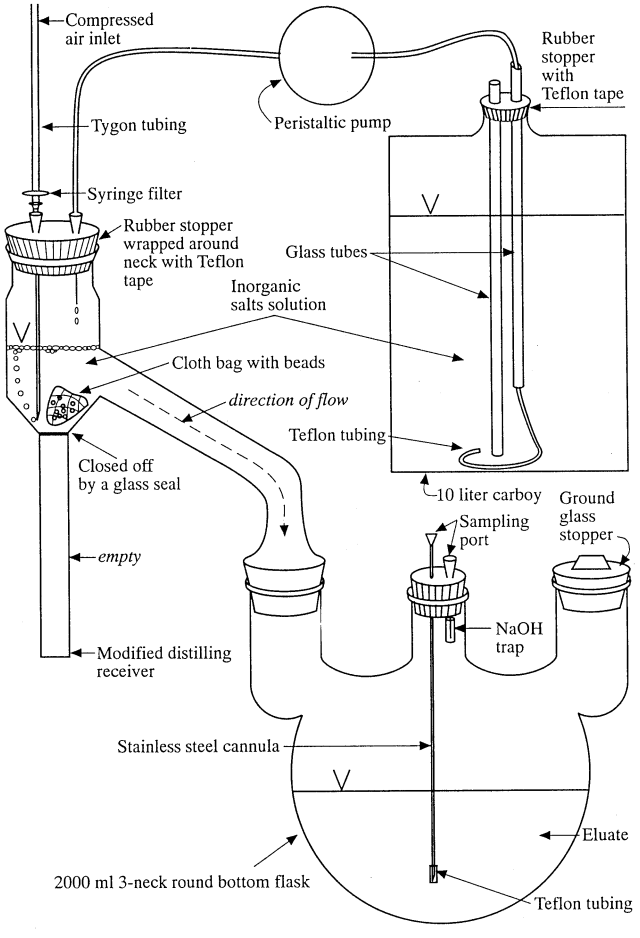

Fig. 1 Experimental unit for continuously washing beads

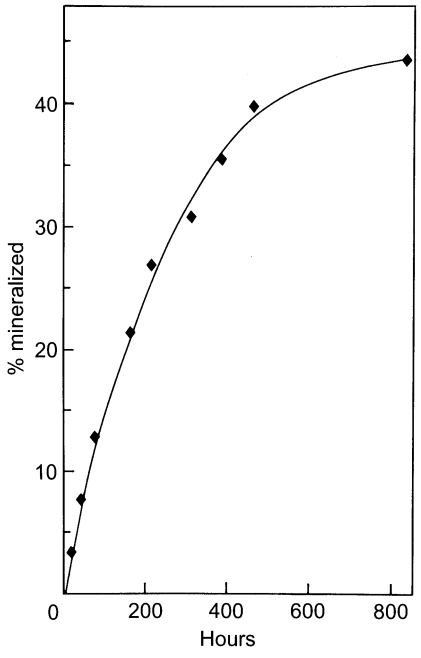

Fig. 2 Metabolism of 400 μg sorbed biphenyl by the consortium

retain the beads. Liquid from the enrichment cultures was added to the distilling receiver, and the liquid was passed over the beads and collected in the round-bottom flask. The flow rate through the receiver was 28 ml/h, which provided for a complete exchange of the solution in the receiver every 15 min. At each assay time, the eluate was acidified to pH 2.0 with trichloroacetic acid to a final concentration of 5.0% and bubbled with air for 2 min to drive off $^{14}CO_2$, which was trapped in 0.5 M NaOH contained in a vial suspended within the flask. The acidified eluate contained no detectable radioactivity, so no labeled product or biphenyl was eluted from the beads.

Results

The rates of abiotic desorption of 400 μg biphenyl from 0.2 g beads were 1.88, 0.019 and 0.004 μg/h for the periods of 0–1, 3–72 and 142–264 h, on the basis of linear plots for these periods. The initial value probably is an underestimate because no measurements were made before 1 h, but the fact that the rate from 3 h to 72 h was linear suggests that most of the readily desorbable compound was desorbed at 0.019 μg/h.

Measurements were also made of the rate of mineralization by consortium SBP-1 of 400 μg biphenyl sorbed to 0.2 g beads. The rate was essentially linear at 0.66 μg/h for approximately the first 100 h before declining (Fig. 2). The rate then decreased to 0.24 μg/h assuming linear kinetics for the period from 170 h to 450 h. If the rate of abiotic desorption at 0–1 h represents release from the micropores at the surface of the beads and the subsequent and sustained rate represents the release from the underlying but still accessible micropores within the beads, the biodegradation rate was slower than the initial desorption but considerably faster than the subsequent release of the chemical to the aqueous phase.

Consortium SBP-1 mineralized 80% of 120 μg biphenyl sorbed to the beads (Fig. 3). Twelve bacterial isolates with different colony morphologies on biphenyl/casamino acids/agar were obtained from the consortium. Each mineralized biphenyl in solution without beads, but none mineralized sorbed biphenyl. The data for one isolate are shown in the figure.

The surface tension was measured in cultures grown on different initial amounts of biphenyl with and without beads. The surface tension of consortium SBP-1 was not significantly lower than the surface tension of uninoculated media when the consortium was grown for 164 h with or without beads containing 0.12 and 4.1 mg biphenyl, for 158 h with or without beads containing 10 mg biphenyl or for 44, 96 and 335 h with beads containing 1.6 mg biphenyl.

Duplicate flasks containing 20 ml medium were inoculated with 1 ml 7-day-old enrichment derived from activated sewage sludge. The inoculated flasks were supplemented with 2.0 mg diethyl phthalate/ml and

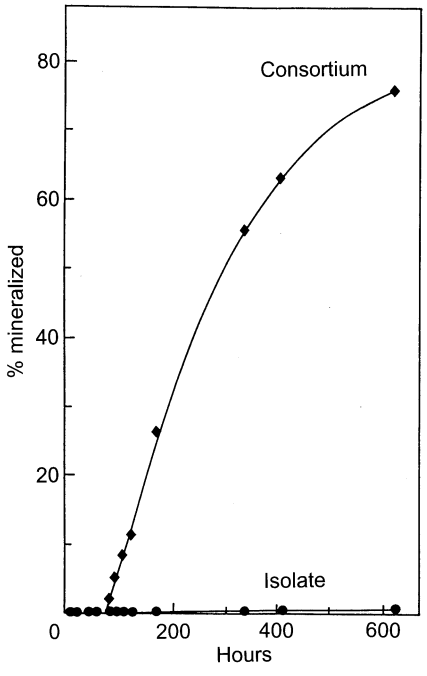

Fig. 3 Mineralization of 120 μg sorbed biphenyl by an enrichment culture and by strain FR

incubated at 30°C for 264 h on a rotary shaker operating at 112 rpm. The surface tension was significantly less than that of the uninoculated medium. The filtrate from the culture, which had a surface tension of 488 μN/cm, was passed through a 0.2-μm nylon filter and added to a culture of *P. paucimobilis*, a biphenyl-degrading bacterium isolated from consortium SBP-1. The inoculum contained 4.2×10^5 or 8.4×10^5 cfu/ml in flasks with 400 μg biphenyl sorbed to 0.2 g beads or with 20 μg biphenyl/ml in solution. The latter range was chosen to include the concentrations found in solution when the beads were shaken for 120 h at 112 rpm under aseptic conditions. After 10 days of incubation at 30°C, the bacterium had mineralized 4.6% and 5.3% of the biphenyl in solution without and with the culture filtrate, values that were not significantly different ($P = 0.05$). In contrast, 0.04% and 0.07% of the sorbed biphenyl was mineralized in cultures without and with the culture filtrate. Thus, even in the presence of the surfactant-containing filtrate, *P. paucimobilis* did not degrade the sorbed substrate appreciably.

Because extracellular surfactant production by the consortium was not detected, the possible role of synthetic surfactants was tested. The surfactants were added to the cultures at two concentrations, and mineralization of 400 μg biphenyl sorbed to 0.2 g beads and 400 μg biphenyl in 20 ml solution was measured. The test bacteria were *P. paucimobilis*, a pleomorphic non-spore-forming, gram-positive rod (classified as strain CDC group A-5 subgroup B) and strains Ly, My and Mw, all of which were isolated from consortium SBP-1. These bacteria mineralized biphenyl in solution but not sorbed biphenyl.

Table 1 The effect of surfactants on the extent of mineralization of biphenyl in solution by two bacteria. *ND* not done

Surfactant	Surfactant conc. (μg/ml)	Biphenyl mineralized (%)	
		P. paucimobilis	Pleomorphic rod
Triton X-100	0	6.9 ± 4.8	11.5 ± 0.3
	20	10.6 ± 0.2	10.2 ± 0.3
	2000	3.3 ± 0.3	3.7 ± 0.4
Tween 80	0	10.9 ± 1.1	12.0 ± 0.4
	20	3.5 ± 0.1	5.5 ± 5.4
	2000	8.2 ± 4.7	2.8 ± 0.7
Enordet LXS-814	0	5.4 ± 1.2	10.9 ± 1.1
	20	5.4 ± 4.6	8.2 ± 4.7
	2000	ND	3.5 ± 0.1
Neodol 25-3S	0	14.2 ± 0.2	15.8 ± 0.7
	20	10.1 ± 0.5	13.9 ± 2.3
	2000	8.0 ± 0.6	6.0 ± 4.9
Taurocholic acid	0	10.8 ± 0.1	7.8 ± 3.0
	20	10.8 ± 1.5	6.4 ± 1.8
	2000	9.8 ± 0.6	11.2 ± 2.2
Dimethyldioctadecyl-ammonium bromide	0	5.0 ± 4.0	8.8 ± 2.6
	20	11.4 ± 2.0	8.7 ± 2.3
	2000	11.2 ± 4.0	6.1 ± 4.1
Benzethonium chloride	0	11.6 ± 3.1	12.2 ± 2.4
	20	12.2 ± 0.4	10.1 ± 0.5
	2000	3.1 ± 1.0	4.1 ± 0.3
Palmityl sulfobetaine	0	ND	9.3 ± 8.0
	20	ND	12.8 ± 3.3
	2000	ND	1.5 ± 0.0

Triton X-100 was used to determine the concentration of the surfactants in solution in the presence of beads. In 20 ml solution initially containing 20 μg Triton X-100/ml and 0.2 g beads, surfactant was not detected at 24 h by measuring the absorbance at 270 nm, but 3.5 μg/ml remained in solution if the initial concentration was 2.0 mg/ml. If the other surfactants behaved similarly, all would be sorbed at the lower concentration, but some would be present in solution at the higher conentration. The surfactants tested were Triton X-100, Tween 80, Tergitol 15-S-3 and Enordet LXS-814, which are nonionic; taurocholic acid, Neodol 25-3S and surfactant produced by *B. licheniformis* JF-2, which are anionic; dimethyl dioctadecylammonium bromide and benzethonium chloride, which are cationic; and palmityl sulfobetaine, which is zwitterionic.

The effects of the surfactants on the metabolism of biphenyl in solution by *P. paucimobilis* and the pleomorphic rod were different (Table 1). Some surfactants were toxic to one or both organisms only at 2000 μg/ml, and others were inhibitory even at 20 μg/ml. Some did not depress this activity even at the

Table 2 The effect of Tergitol and *B. licheniformis* culture filtrate on the extent of mineralization of biphenyl in solution by three bacteria. *BL B. licheniformis* culture filtrate

Isolate	Surfactant tested	Biphenyl mineralized (%)	
		No surfactant	Surfactant
Ly	Tergitol	21.7 ± 1.0	16.0 ± 1.4
	BL	18.7 ± 0.8	17.6 ± 0.7
My	Tergitol	20.8 ± 1.3	20.6 ± 1.3
	BL	18.1 ± 1.0	10.0 ± 5.1
Mw	Tergitol	12.6 ± 7.4	21.6 ± 0.5
	BL	17.5 ± 0.4	6.5 ± 1.6

higher level, and a few were stimulatory. However, addition of the surfactants resulted in either no or less than 1% mineralization of the sorbed compound in 429 h. Because the inocula of each organism varied somewhat, the percentages of biphenyl mineralized in the absence of the surfactants were not identical.

In tests with strains Ly, My and Mw, the incubation times were 237 h and 304 h when the surfactant was Tergitol 15-S-3 and the *B. licheniformis* culture filtrate respectively, and the concentration of Tergitol 15-S-3 was 50 μg/ml. Neither Tergitol 15-S-3 nor the culture filtrate appreciably increased the rate of mineralization of biphenyl in solution (Table 2). The values represent duplicate determinations. The large standard deviation precludes a conclusion on whether Tergitol increased the activity of strain Mw, and a statistical analysis failed to reveal an effect. The three isolates did not mineralize sorbed biphenyl in the presence of the surfactants.

To determine the activity of cells attached to the beads, the mineralization of sorbed biphenyl was determined after removal of the aqueous phase. Four 300-h-old consortium cultures in biometer flasks were pooled, and the beads were suspended in 5 ml sterile 6 mM phosphate buffer (pH 6.8) and repeatedly passed through a pipette tip to remove readily detachable cells. The detached cells were added to the aqueous portion of the pooled culture, which was centrifuged for 30 min at 10 000 *g*. The pellet was suspended in 5 ml phosphate buffer, and the cell density was determined by viable counts. The cells were then added to biometer flasks containing 0.2 g beads, 400 μg sorbed nonradiolabeled biphenyl, 100 000 dpm radiolabeled biphenyl and 20 ml inorganic salts solution to a density of 9×10^5 cfu/ml. The flasks were incubated for 50 h at 30°C on a rotary shaker operating at 112 rpm, and the liquid then was removed by gentle vacuum suction. The colonized beads were aseptically washed six times with phosphate buffer, fresh salts solution was added, and the flasks were again incubated on a shaker at 30°C. In flasks with unwashed beads, the liquid phase was simply removed, and a fresh salts solution was added. Mineralization of the sorbed biphenyl occurred in cultures

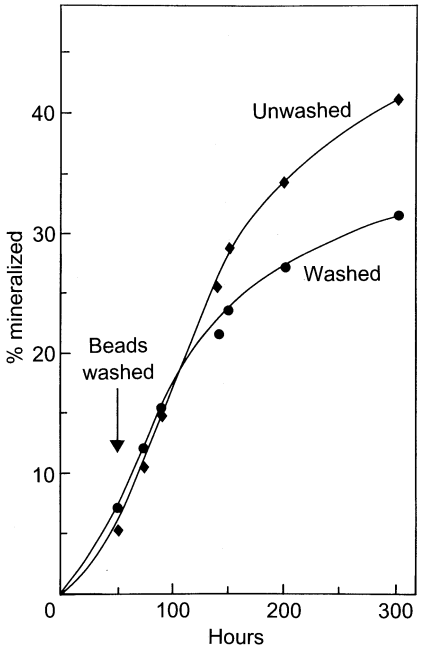

Fig. 4 Mineralization of 400 μg biphenyl sorbed to beads that were washed or not washed

containing either unwashed or washed beads (Fig. 4). The values for the washed and the unwashed beads from the time of washing at 50 h until 150 h were not significantly different. The rate of mineralization of biphenyl in the cultures with unwashed beads was significantly higher ($P = 0.05$) thereafter. At 300 h, 41.2% sorbed biphenyl was mineralized in the cultures with unwashed beads compared to 31.7% with washed beads.

A system was designed to wash continuously the beads taken from an active consortium. The beads and spent culture medium from two biometer flasks containing the consortium were aseptically poured into bags of sterile bolting cloth. The beads were washed continuously with a sterile inorganic salts solution supplied through a reservoir. The solution contained 250 μg cycloheximide/ml to avoid clogging by fungi, which were present in the consortium. In the first 44.5 h, before the beads were transferred to the columns, an average of 24.8% of the biphenyl had been mineralized by duplicate cultures of the consortium. The beads and culture medium from two biometer flasks containing the consortium were transferred aseptically to the columns at 44.5 h. In the next 63.5 h, biphenyl was not further mineralized (Fig. 5); in this period, 254 exchanges of solution occurred in the reservoir containing the beads and presumably the cells originally in the culture medium had been washed out, leaving only attached cells. At some time after 128.5 h, the sorbed biphenyl was slowly mineralized.

Isolates Ly, Mw and My were grown for 5 days at 30°C on agar containing 7 μg biphenyl, 0.1% yeast

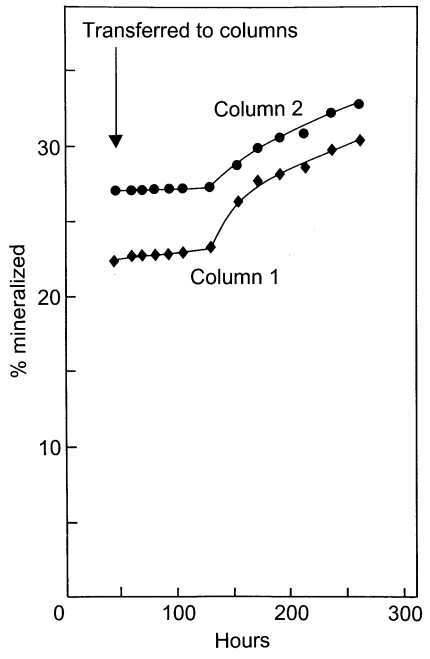

Fig. 5 Mineralization by consortium of 400 μg sorbed biphenyl in duplicate continuous columns

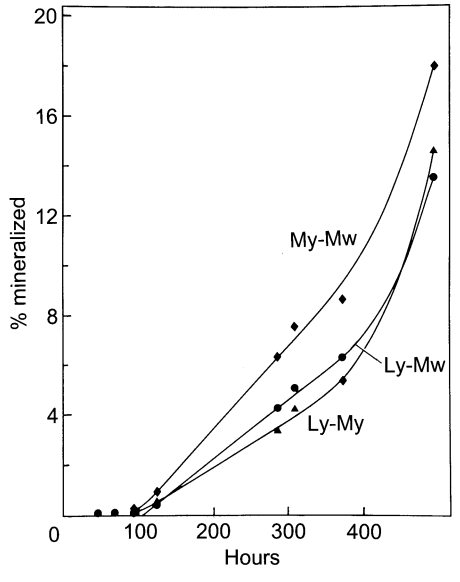

Fig. 6 Mineralization of sorbed biphenyl by two-membered cultures My-Mw, Ly-My, and Ly-Mw

extract and 0.05% casamino acids/ml, and colonies of each isolate were suspended in 5 ml inorganic salts solution. Duplicate biometer flasks were inoculated with combinations containing two of these three cultures (Ly plus My, Ly plus Mw and My plus Mw) to a final density of 4×10^5 cfu/ml of each isolate. Each biometer flask contained 0.2 g beads, 120 μg sorbed biphenyl and 20 ml inorganic salts solution supplemented with 20 μg each of yeast extract and casamino acids/ml. Prior tests had shown that the three isolates in pure culture did not metabolize sorbed biphenyl. At 383 h, little mineralization of the sorbed biphenyl was evident, 2.1% being mineralized by the Ly-My combination and less than 1.0% by Ly-Mw and My-Mw combinations.

Portions (1.0 ml) of these two-membered cultures were transferred to biometer flasks containing 0.2 g beads, 1600 μg sorbed biphenyl and 20 ml inorganic salts solution. None of the two-membered cultures showed appreciable activity in the first 124 h (Fig. 6). Thereafter, appreciable biodegradation occurred, and 18%, 15% and 14% of the substrate was mineralized in 493 h by two-membered cultures My-Mw, Ly-My, and Ly-Mw respectively.

Discussion

The biodegradation of sorbed biphenyl by the consortium was faster than the rate at which much of the substrate was desorbed. This suggests that the mechanism by which the microorganisms acquire the substrate is not dependent on the rate of spontaneous desorption. However, because a more rapid desorption was noted in the first hour (although the value for that rate is somewhat equivocal because of the small number of points), such data do not exclude the possibility of spontaneous desorption being required for microbial acquisition of the substrate. A stronger argument comes from observations that the consortium and two-membered cultures but not individual species of bacteria utilized the bound substrate; if the consortium and the two-membered cultures relied on spontaneous desorption from the beads, then the pure cultures should also have been active. Differences in substrate affinities or thresholds between the mixtures and the pure cultures seem most unlikely because such arguments would require that one species affects the affinity or the threshold of the second species.

A second mechanism of substrate acquisition entails the production of surfactants. According to this argument, one species in the mixtures is producing a surfactant that allows another species to grow on and degrade the compound. If one species produced the surfactant and also could metabolize the substrate, it should have been possible to obtain such a pure culture from microbial mixtures provided with sorbed biphenyl as the sole carbon source, but no such isolate was obtained. The two isolates functioned together but not apart, and both utilized soluble biphenyl. The lack of stimulation of biodegradation of sorbed biphenyl by the microbial and synthetic surfactants also argues against a role for such excretions.

A third mechanism assumes that cells acquire the substrate by physical contact with the substrate. Indeed, this is the mechanism by which an *Arthrobacter*

sp. acquires a hydrophobic substrate present in a nonaqueous liquid (Efroymson and Alexander 1991). Such utilization may involve lipids at the bacterial surface, the hydrophobic molecule on the solids dissolving first in these lipids before entering the cytoplasm. The results obtained here with washed beads are consistent with such a mechanism. However, it is difficult to envision why cells of two but not of one species should be able to accomplish the degradation by this mechanism or how bacteria can make physical contact with a substrate retained in micropores as small as those in SM-7 beads (average micropore diameter of 9 nm). It is noteworthy that Harms and Zehnder (1995) proposed that the rate of utilization by *Sphingomonas* sp. of 3-dichlorodibenzofuran sorbed to porous Teflon was influenced by the tendency of the bacterium to adhere to the sorbent.

The data reported herein do not exclude other mechanisms of utilization. Thus, Steen et al. (1980) reported that sorption to suspended sediments rendered chlorpropham and di-*n*-butyl phthalate unavailable for biodegradation, and the models of Ogram et al. (1985) suggested that the rate of desorption limited the degradation of 2,4-dichlorophenoxyacetic acid sorbed by soil.

The micropores present in SM-7 beads distinguish them from synthetic sorbents used in other studies of the utilization of sorbed substrates, such as glass cylinders (Kefford et al. 1982) or glass slides (Thomas and Alexander 1987). The fact that biphenyl can sorb within the micropores makes these beads more realistic simulants of soils and sediments, in which binding of hydrophobic molecules may occur in such small pores. Sorption to surfaces like glass does not involve such a binding.

The surfactants were chosen to include the classes of surface-active agents that are commonly produced by microorganisms, as most of the biosurfactants that have been described are anionic or nonionic (Reiser et al. 1989). Moreover, surfactants in these charge classes also commonly are the least toxic to bacteria (Glassman 1948). Both synthetic and microbial surfactants have been used to desorb organic contaminants from natural surfaces. For example, Triton X-100 desorbs polychlorinated biphenyls from contaminated sand (Viney and Bewley 1990). Aronstein et al. (1991) showed that the biodegradation of phenanthrene sorbed to soil was enhanced by nonionic ethoxylates without appreciable surfactant-induced desorption. Moreover, Berg et al. (1990) reported that *Pseudomonas aeruginosa* produced an emulsifier capable of desorbing hexachlorobiphenyl from soil. The present findings do not exclude the possibility that acquisition of biphenyl may require a microbial surfactant because biosurfactants have some specificity for the molecule used as a carbon source of the producing microorganism. For example, Ito and Inoue (1982) showed that 43 synthetic nonionic surfactants did not replace the extracellular sophorose lipid produced by *Torulopsis bombicola* in stimulating growth on water-insoluble alkanes, and the consortium may contain an organism producing a surfactant that allows it to use sorbed biphenyl.

Although this investigation does not fully determine the mechanism of acquisition of the sorbed substrate, it does show that the rate of mass transfer from sorbent to water, at least as determined in the absence of microbial activity, is not necessarily the rate-limiting step in the biodegradation of sorbed substrates. Because of the ubiquity and quantity of sorbed hydrophobic pollutants and of the widespread use of environmental-fate models of contaminants that do not consider mechanisms of microbial utilization other than those governed by spontaneous desorption, it is important to explore further the mechanisms by which microorganisms acquire substrates that are retained by particulate matter in soils, sediments and aquifers.

Acknowledgements This research was supported by a training grant (5-T32-ES07052) and a research grant (ES05950) from the National Institute of Environmental Health Sciences.

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