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Degradation of nonionic surfactants and polychlorinated biphenyls by recombinant field application vectors

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Degradation of polychlorinated biphenyls (PCBs) in the environment is limited by their aqueous solubility and the degradative competence of indigenous populations. Field application vectors (FAVs) have been developed in which surfactants are used to both increase the solubility of the PCBs and support the growth of surfactant-degrading strains engineered for PCB degradation. Surfactant and PCB degradation by two recombinant strains were investigated. *Pseudomonas putida* IPL5 utilizes both alkylethoxylate [polyoxyethylene 10 lauryl ether (POL)] and alkylphenolethoxylate [Igepal CO-720 (IGP)] surfactants as growth substrates, but only degrades the ethoxylate moiety. The resulting degradation products from the alkyl- and alkylphenolethoxylate surfactants were 2-(dodecyloxy)ethanol and nonylphenoldiethoxylates, respectively. *Ralstonia eutropha* B30P4 grows on alkylethoxylate surfactants without the appearance of solvent-extractable degradation products. It also degrades the 2-(dodecyloxy)ethanol produced by strain IPL5 from the alkylethoxylate surfactants. The extent of degradation of the alkylethoxylate surfactant (POL) was greater for strain IPL5 (90%) than for B30P4 (60%) as determined by the cobaltothiocyanate active substances method (CTAS). The recombinant strain B30P4::TnPCB grew on biphenyl. In contrast, the recombinant strain IPL5::TnPCB could not grow on biphenyl, and PCB degradation was inhibited in the presence of biphenyl. The most extensive surfactant and PCB degradation was achieved by the use of both recombinant strains together in the absence of biphenyl. PCB (Aroclor 1242) and surfactant (POL) concentrations were reduced from 25 ppm and 2000 ppm, respectively, to 6.5 ppm and 225 ppm, without the accumulation of surfactant degradation products. Given the inherent complexity of commercial surfactant preparations, the use of recombinant consortia to achieve extensive surfactant and PCB degradation appears to be an environmentally acceptable and effective PCB remediation option.

Keywords: alkylethoxylate; alkylphenolethoxylate; nonylphenoldiethoxylate; *Pseudomonas putida*; *Ralstonia eutropha*; genetically engineered microorganisms

Introduction

Although surfactant degradation and the genetics of polychlorinated biphenyl (PCB) biodegradation may appear to be disparate topics, potential technologies for soil remediation have been proposed which employ soil washing and PCB contaminant biodegradation. Surfactants can be used to increase the solubility of hydrophobic environmental contaminants including PCBs [1,10,38] and have been applied to soil washing or soil flushing technologies for soil remediation [37]. Enhanced water solubility may result in the enhanced bioavailability of contaminants to microorganisms, although contradictory evidence has been reported [20]. Nonionic surfactants may be the most logical possibility for enhancing contaminant bioavailability as they are both effective at solubilizing contaminants and are generally less toxic to microorganisms than anionic or cationic surfactants [35]. Since surfactants may be applied in solutions of 0.5% and higher in soil-flushing technologies [1,38], the surfactant-degradative activities of microbes may have important environmental consequences.

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Considerable research has been conducted on the metabolism and genetics of PCB degradation by naturally occurring microorganisms [2,5,6,11,15,16,18,19,28,36]. In many aerobic PCB-degrading bacteria, PCB degradation is cometabolic, and inducible by biphenyl. Although the genes for biphenyl/PCB degradation have been cloned, the regulation of the biphenyl operon in potential recombinant strains and the compatibility of the biphenyl degradation pathway with existing pathways cannot always be predicted *a priori* [28].

Field application vectors (FAVs), which are genetically engineered microorganisms capable of utilizing a selective carbon source and expressing foreign genes, have been constructed for use in the bioremediation of soils contaminated with PCBs [21–23]. Treatment approaches utilizing FAVs use surfactants to both solubilize PCBs and support the growth of surfactant-degrading bacterial strains bioengineered for PCB degradation. Previous research with PCB-degrading FAVs focused on PCB degradation and did not examine the extent of surfactant degradation or the production of surfactant metabolites [22,23].

Whereas information is available from other studies on surfactant degradation in the environment and the genetics of PCB degradation, successful integration of these disciplines into an effective and environmentally acceptable site remediation technology has not been achieved. The specific objective of the work reported here was to further an

approach to PCB bioremediation using FAVs in which the extent of surfactant and PCB degradation is maximized, while avoiding potential accumulation of surfactant degradation products. Since biphenyl has been shown to enhance PCB degradation in naturally occurring PCB-metabolizing bacteria, the effect of biphenyl addition to the FAV strains on PCB and surfactant degradation was examined.

Materials and methods

Strains and culture conditions

Pseudomonas putida IPL5 and *P. putida* IPL5::TnPCB [22,23] were grown at 25°C on minimal salts medium (PAS [8]) amended with 2000 ppm Igepal CO-720 (IGP) and 10 ppm tetracycline where appropriate. *Ralstonia eutropha* B30P4 and *R. eutropha* B30P4::TnPCB (described below) were typically grown at 25°C on PAS medium amended with 2000 ppm polyethylene glycol (average molecular weight 200) and 10 ppm tetracyline where appropriate.

B30P4 was isolated from a 6-month-old enrichment culture consisting of PAS medium amended with approximately 100 ppm each of polyoxyethylene 10 lauryl ether (POL), Brij 30, Igepal CO-720 (IGP), nonylphenoldiethoxylate, and dodecane inoculated with waste-activated sludge from a sewage treatment plant and soils previously exposed to household detergents or hydrocarbons. Four sequential enrichments were performed using PAS medium containing 2000 ppm POL. Single colonies were isolated from the final enrichment culture on YEPG (yeast extract, 0.2 g L^{-1}; polypeptone, 2 g L^{-1}; glucose, 1 g L^{-1}; NH_4NO_3, 0.2 g L^{-1}) agar plates.

The transposon containing the biphenyl/PCB degradative operon (TnPCB) was transferred to B30P4 by conjugation with *Escherichia coli* S17-1λ pir::TnPCB [22] as previously described. This operon contains the genes (*bph A1A2A3A4BCKHJID*) which are necessary for conversion of biphenyl and chlorobiphenyls to benzoic acid and chlorobenzoic acids [11,15,16]. These genes were cloned from *Ralstonia eutropha* ENV307 (formerly designated as *Pseudomonas* sp ENV307) [23] and the restriction enzyme map of these genes matches the *bph* operon from *Pseudomonas* sp LB400 [11,22,29]. Matings were performed on YEPG agar plates, and recombinants were selected on PAS agar plates amended with 0.2% polyethylene glycol and 10 ppm tetracycline. Recombinants were verified by conversion of 2,3-dihydroxybiphenyl to the yellow *meta*-cleavage product, gene probing (*bphA* and *bphC*), and PCB degradation in resting cell assays [22].

Strain identification

Strains *R. eutropha* B30P4 and *P. putida* IPL5 [22] were identified taxonomically by commercial companies using fatty acid analysis (Microcheck, Northfield, VT, USA) and Biolog analysis (Analytical Services, Essex Junction, VT, USA). Taxonomic identification in this laboratory was performed by partial 16S rDNA sequence analysis.

PCR amplification of a portion of the 16S rDNA was performed using the GeneAmp PCR reagent kit with AmpliTaq DNA Polymerase (Perkin-Elmer, Roche Molecular Systems, Branchburg, NJ, USA) and the 16S rDNA primers 1114f and 1392r [24]. Individual bacterial colonies were transferred into PCR tubes containing buffer, nucleotides, and primers. The samples were heated to 100°C for 5 min to lyse the bacteria and denature the DNA. AmpliTaq DNA polymerase and mineral oil were added to each sample, and PCR amplification was performed for 30 cycles using the following protocol: 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. Final extension to insure full length PCR products was performed at 72°C for 10 min. The amplified DNA was ligated into the TA cloning vector (pCRII) and transformed into competent *E. coli* cells following the manufacturer's protocol (TA Cloning Kit, Invitrogen, San Diego, CA, USA). White colonies were grown in Luria-Bertani (LB) medium containing 50 μg ml^{-1} kanamycin, and plasmids were extracted using an alkaline-lysis procedure as described in the Promega Technical Bulletin .009 (Promega, Madison, WI, USA). The DNA sequence of the 16S rDNA inserts was determined by single primer extensions from the T7 and SP6 promoters on pCRII and the two strands were matched (Retrogen, San Diego, CA, USA). The partial 16S rDNA sequences were deposited in the GenBank Sequence Database at the National Center for Biotechnology Information (NCBI) and received the accession numbers U52348 for *P. putida* IPL5 and U52349 for *R. eutropha* B30P4. These sequences were compared to other 16S rRNA sequences in the Illinois Ribosomal Database [26] using the program Similarity Rank and to all sequences in the nucleotide databases (GenBank, EMBL, DDBJ, PDB) using the Blast N program [3].

Growth substrates and degradation products

The nonionic surfactants Brij 30, POL (alkylethoxylate) and IGP (alkylphenolethoxylate), and compounds similar to the structural moieties present in these surfactants (Table 1), were tested for the ability to support the growth of *P. putida* IPL5 and *R. eutropha* B30P4. Substrate disappearance and accumulation of solvent-extractable degradation products were also examined. All substrates were added as acetone solutions to 15-ml screw cap tubes to achieve a final concentration of 0.2% (wt/vol). After the acetone was evaporated in a 70°C water bath, 5 ml PAS medium was added to the tubes. The tubes were inoculated from frozen culture stocks, and incubated at room temperature on a rotary mixer. PAS medium without substrate addition served as growth controls, and uninoculated PAS medium with substrate addition served as controls for abiotic substrate disappearance.

Analysis of surfactant degradation

Degradation of polyethylene 10 lauryl ether, Igepal CO-720 and Brij 30 was determined by measurement of cobaltothiocyanate active substances (CTAS) [4] in culture supernatants. For CTAS determinations, cultures (1.5 ml) were transferred to microcentrifuge tubes and centrifuged at $10\,842 \times g$ at room temperature. From the supernatant 1 ml was transferred to 15-ml screw cap tubes containing 3 ml cobaltothiocyanate reagent and 8 ml methylene chloride. The tubes were shaken in a horizontal position on a reciprocating shaker for 15 min, and then centrifuged at $760 \times g$ for 5 min. The absorbance of the methylene chloride fraction was determined at 620 nm. The surfactant concen-

Table 1 Chemical formulas and manufacturers of chemicals used in this study

Substrates (surfactant type)	Chemical formula	Manufacturer[a]
Igepal CO-720 (IGP) (nonylphenolethoxylate)	$C_9H_{19}C_6H_4O(CH_2CH_2O)_{11}CH_2CH_2OH$	Aldrich
Igepal CO-210[b] (nonylphenoldiethoxylate)	$C_9H_{19}C_6H_4OCH_2CH_2OCH_2CH_2OH$	Aldrich
Polyoxyethylene 10 lauryl ether (POL) (alkylethoxylate)	$CH_3(CH_2)_{10}CH_2O(CH_2CH_2O)_9CH_2CH_2OH$	Sigma
Brij 30 (alkylethoxylate)	$CH_3(CH_2)_{10}CH_2O(CH_2CH_2O)_3CH_2CH_2OH$	Aldrich
Dodecane	$CH_3(CH_2)_{10}CH_3$	Aldrich
1-Dodecanol	$CH_3(CH_2)_{10}CH_2OH$	Aldrich
1-Nonanol	$CH_3(CH_2)_7CH_2OH$	Aldrich
Polyethylene glycol (avg. mw 200)	$HOCH_2(CH_2OCH_2)_3CH_2OH$	Sigma
2-(dodecyloxy)ethanol	$CH_3(CH_2)_{11}OCH_2CH_2OH$	na[c]

[a]Aldrich Chemical Company, Milwaukee, WI, USA; Sigma Chemical Co, St Louis, MO, USA.
[b]Igepal CO-210 is a mixture of nonylphenolmonoethoxylate and nonylphenoldiethoxylate as determined by GC/MS.
[c]na, not available.

trations were determined by comparison to standard curves derived from 0–2000 ppm of the same surfactants.

Analysis of non-polar surfactant degradation products

Ten-day-old cultures exhibiting visible growth were extracted by addition of 5 ml hexane, 2 ml distilled water, 2 ml ether and 1 g Na_2SO_4. The tubes were shaken on a reciprocating shaker for 1 h, and then centrifuged at $760 \times g$ for 15 min. Two milliliters of supernatant were transferred to another tube containing 0.5 g Na_2SO_4 which was then shaken for 15 min and centrifuged at $190 \times g$ for 15 min. The culture extracts were then transferred to gas chromatography vials. Extraction efficiencies for the growth substrates (and potential degradation products) nonylphenoldiethoxylate, dodecane, dodecanol, and nonanol were 110, 108, 85 and 80%, respectively.

Culture extracts were analyzed using a model GC-9AM gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a split/splitless injector, both of which were kept at 300°C. A DB-5 capillary column (30 m × 0.25 mm i.d., J and W Scientific, Folsom, CA, USA) was used with nitrogen as the carrier gas (1.2 ml min^{-1}) and as the make-up gas (28 ml min^{-1}). Injection of 2 ml was performed using the splitless mode. The column oven temperature was kept at 50°C for 2 min, raised to 80°C at a rate of 10°C min^{-1} and then to 225°C at a rate of 6°C min^{-1}, and then kept at 225°C for 35 min.

Degradation products were further analyzed by comparison with known standard chemicals where possible, and by gas chromatography/mass spectroscopy (GC/MS). The metabolites were identified using a Hewlett-Packard model 5890 gas chromatograph equipped with a BP-5 capillary column (12 m × 0.22 mm i.d.; SGE, Austin, TX, USA). Samples were injected into the gas chromatograph at a column temperature of 40°C and held isothermally for 2 min, and the oven temperature was programmed to increase to 250°C at a rate of 10°C min^{-1} with a helium pressure of 8 psi. Temperatures of the injection port and ionization source were 250°C and 230°C, respectively. Mass spectra analyses were performed on a VG Platform II (Fisons Instruments, Beverly, MA, USA) with electron impact ionization at 70 eV.

PCB and biphenyl analysis

Methods of Lajoie *et al* [22] and Layton *et al* [25] were used for analysis of PCB degradation. PCB extractions were performed by addition of 1 ml of culture to a 25-ml corex centrifuge tube containing 12 ml H_2O, 2 g Na_2SO_4 and 5 ml ether. The tubes were shaken for 1 h on a reciprocating shaker and centrifuged for 15 min at $760 \times g$. From the supernatant 1 ml ether was transferred to a 15-ml screw cap tube containing 4 ml hexane and 1 g silica gel. The extracts were shaken for 15 min, centrifuged at $190 \times g$ for 5 min, and transferred to GC vials. Individual PCB congener concentrations were determined using a model GC-14A gas chromatograph (Shimadzu) equipped with a DB-1 capillary column (30 m × 0.25 mm i.d., J and W Scientific) and an electron capture detector. Injector, detector, and oven temperature programming were the same as that used for detection of surfactant degradation products. PCB concentrations were calculated by normalization using peak 40 (2,3,6,2′,4′,5′-hexachlorobiphenyl/2,4,5,3′,4′-pentachlorobiphenyl) as an internal standard and comparison to Aroclor 1242 standards. Total PCB concentrations were determined from the sum of the concentrations calculated for each of the 40 individual peaks. The average final PCB concentration after 20 days incubation from 15 uninoculated controls initially contaminated with 25 ppm Aroclor 1242 in 0.2% POL was 22.9 ppm. No specific losses of PCB congeners were detected and the extraction efficiency was approximately 92%. A separate experiment was performed to determine the effect of surfactant on PCB extraction in PAS medium containing diatomaceous earth. Final PCB concentrations were 22.3 (± 0.3) and 20.2 (± 1.6) ppm in the presence and absence of 0.2% POL, respectively.

For biphenyl extractions, 1-ml samples were placed into 25-ml corex centrifuge tubes containing 2 g Na_2SO_4 and 12 ml H_2O. The samples were extracted with ether and diluted into hexane as described for PCB extractions. Biphenyl samples were analyzed using a model GC-9AM gas chromatograph (Schimadzu) equipped with a flame ionization detector and a DB-5 capillary column (30 m × 0.25 mm i.d., J and W Scientific). Chromatography conditions were the same as for the surfactant degradation products.

Effect of biphenyl on PCB degradation

The abilities of IPL5 and B30P4 and the recombinant strains B30P4::TnPCB and IPL5::TnPCB to utilize biphenyl or sodium benzoate as growth substrates were determined in PAS medium amended with 0.2% biphenyl or 0.2% sodium benzoate.

B30P4::TnPCB cultures for PCB degradation experiments in resting cell assays were grown on PAS medium amended with 0.2% POL or 0.2% biphenyl. IPL5::TnPCB cultures were grown on PAS medium amended with 0.2% IGP. Cultures were centrifuged, washed with sodium phosphate buffer, and resuspended to an absorbance of 1.0 at 615 nm. Aroclor 1242 was added to resting cells to achieve 10 ppm PCBs, and the cultures were incubated for 48 h, followed by extraction and GC analysis [7].

The effect of biphenyl on surfactant and PCB degradation was determined in growing cell assays. In these experiments B30P4::TnPCB and IPL5::TnPCB were grown in 5 ml PAS medium with 0.2% POL and IGP, respectively, containing 25 ppm Aroclor 1242 and 0.025 g diatomaceous earth with and without the addition of 400 ppm biphenyl. Controls consisted of the same media which remained uninoculated. Experimental and control tubes were prepared in triplicate as described in the following steps. First, 0.025 g diatomaceous earth was added to 15-ml teflon-lined screw cap tubes and autoclaved. Next, PCBs, surfactants and biphenyl were added as acetone solutions. The acetone was evaporated by placing the loosely capped tubes in a water bath at 70°C. Residual acetone was allowed to evaporate by leaving the tubes loosely capped at room temperature overnight. Finally, PAS medium (5 ml) and the inocula (approximately 10^6 cells ml^{-1}) were added. Recovery experiments indicated that no significant losses of PCBs, biphenyl or surfactant were detected as a result of heating using this method. Cultures were incubated for 24 days at room temperature on a rotary wheel after which surfactant, surfactant degradation products, biphenyl and PCB concentrations were determined.

Nonanol was added as an internal standard for subsequent biphenyl analysis to all cultures to a final concentration of 500 ppm. From each, 1 ml of culture was removed for PCB extraction and 1.5 ml was removed for determination of surfactant concentration (CTAS). The remaining culture (2.5 ml) was used for the determination of biphenyl and non-polar surfactant degradation products as described above.

Results

Strain identification

Strain B30P4 was identified taxonomically by two commercial companies. Microcheck identified B30P4 as *Alcaligenes eutrophus* (now *Ralstonia eutropha*) by fatty acid analysis and Analytical Services identified the same strain as a CDC group IV-2 organism using the Biolog substrate utilization method. Since the CDC group 1V-2 strain appears to be closely related to *Ralstonia eutropha* [32], this suggested that B30P4 was a member of the genus *Ralstonia*. The placement of strain B30P4 in the genus *Ralstonia* as *Ralstonia eutropha* was further confirmed by partial sequence analysis of the 16S rDNA. Screening of the B30P4 sequence against 4332 sequences in the Illinois Ribosomal Database using Similarity Rank Analysis (7/2/97, 15 : 05 : 55) yielded a fit of 0.964 to the type strain *Ralstonia eutropha* str 335 (RY Stanier) ATCC 17697 [41]. A Blast N search (5/30/97 14 : 54) of all nucleotide databases also indicated that the B30P4 sequence was 98% similar to the 16S rDNA sequence of the type strain *R. eutropha* str 335 (GenBank accession number M32021) and to four other *Ralstonia eutropha* (*Alcaligenes eutrophus*) 16S rDNA sequences.

Taxonomic identification of IPL5 performed by commercial companies using fatty acid analysis and Biolog analysis placed IPL5 in the genus *Pseudomonas* as *Pseudomonas chloraphis* and *Pseudomonas putida*, respectively. This taxonomic placement into the genus *Pseudomonas* was also confirmed by the 16S rDNA analysis. The Similarity Rank of the 16S rDNA sequence from strain IPL5 (7/2/97; 15 : 01 : 18) with sequences in the Ribosomal Database was 0.940 with *Pseudomonas putida* strain F1. Using a Blast N search (5/30/97 14 : 34) of all nucleotide databases, a 99% similarity was found between the 16S rDNA sequence from strain IPL5 and five *Pseudomonas putida* strains including *P. putida* strain F1 (D87108) and the type strain DSM 291T (Z76667) [30].

Surfactant substrates and degradation products

P. putida IPL5 utilized the alkylphenolethoxylate surfactant IGP and the alkylethoxylate surfactants Brij 30 and POL as growth substrates (Table 2). Degradation of IGP resulted in the accumulation of a grouping of four distinct peaks tentatively identified as nonylphenoldiethoxylates by comparison with the standard Igepal CO-210 using gas chromatography. Three of these products were identified as isomers by GC/MS analysis. Mass spectra analysis indicated that a major metabolite (isomer 1) was 1-ethyl-1-methylnonylphenoldiethoxylate as reported by Maki *et al* [27]. This compound has a retention time of 18.16 min with a molecular ion (m/z) at 308 ($C_{19}H_{32}O_3$) and the base peak at 223. The major fragments were at m/z 279 ($-C_2H_5$), 237 ($-C_5H_{11}$), 223 ($-C_6H_{13}$), 209 ($-C_7H_{15}$), 149, 135, 121, 107, 91, 77, 57, and 55 (Figure 1). Isomer 2 (m/z = 308, retention time 18.29 min) had the same major fragmentation patterns as iomer 1 except it showed molecular ion peaks at m/z 265, 251, 193, and 179. This result indicated a linear nonyl chain present in isomer 2. Another detected compound (retention time 18.46 min) had a very similar fragmentation pattern to isomer 1 with its molecular ion (m/z) at 355. However, the identity of this compound was not determined. These three degradation products were believed to result from the heterogeneity of the initial substrate (IGP).

Extracts of *P. putida* strain IPL5 cultures grown on Brij 30 and POL indicated the appearance of two metabolites. Metabolite I had a retention time of 13.03 min and major fragment ions at m/z 200(0.3), 199(2.7), 168(4.5), 140(5.7), 111(12.1), 97(18.2), 85(29.9), 71(57.4), 63(31.3), 57(100), and 55(64.1). This compound was tentatively identified as 2-(dodecyloxy)ethanol (mw = 230, $C_{14}H_{30}O_2$) by comparison with the standard spectrum obtained from NIST. Metabolite II had a retention of 15.13 min and had a similar major fragmentation pattern to 2-(dodecyloxy)ethanol as

Table 2 Growth of *Pseudomonas putida* IPL5 and *Ralstonia eutropha* B30P4 on surfactants and surfactant moieties

Substrates	*Pseudomonas putida* IPL5			*Ralstonia eutropha* B30P4		
	Growth	Percent degraded (s.d.)[a]	Products detected	Growth	Percent degraded (s.d.)	Products detected
Igepal CO-720	+	94 (2)	nonylphenoldiethoxylate	−	na	na[b]
Polyoxyethylene 10 lauryl ether	+	90 (8)	2-(dodecyloxy)ethanol	+	60 (3)	none
Brij 30	+	75 (16)	2-(dodecyloxy)ethanol	+	81 (16)	none
Igepal CO-210	−	na[b]	na	−	na	na
Dodecane	−	na	na	−	na	na
1-Dodecanol	+	60	none	+	56	none
1-Nonanol	+	>99	none	−	na	na
Polyethylene glycol	+	nd[c]	none	+	nd	none

[a]Degradation of Igepal CO-720, polyoxyethylene 10 lauryl ether, and Brij 30 was determined as a decrease in cobaltothiocyanate active substances (CTAS) in culture supernatants. Degradation of 1-dodecanol and 1-nonanol was determined by gas chromatography of culture extracts.
[b]na, not applicable.
[c]nd, not determined.

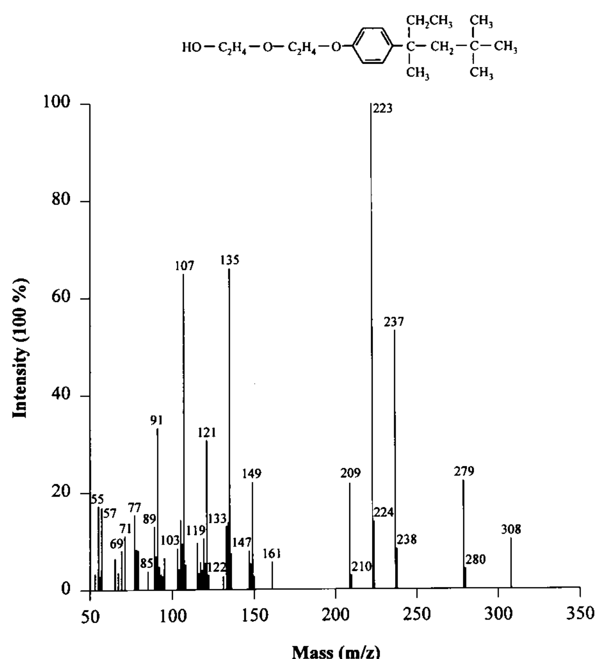

Figure 1 Mass spectrum of 1-ethyl-1-methyl-nonylphenoldiethoxylate formed from the degradation of Igepal CO-720 (IGP) by *Pseudomonas putida* strain IPL5.

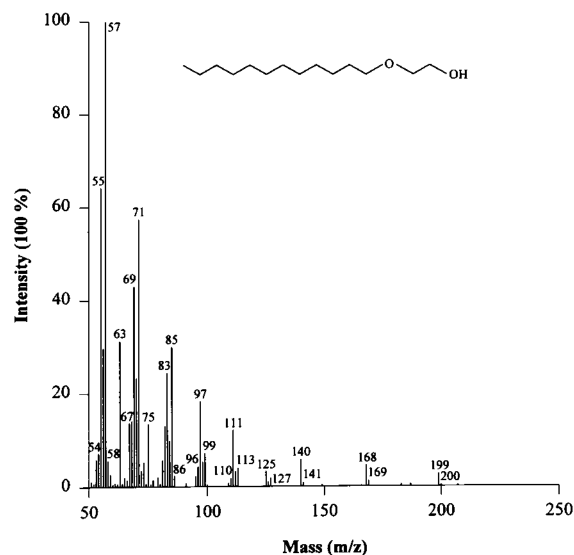

Figure 2 Mass spectrum of 2-(dodecyloxy)ethanol formed from the degradation of polyoxyethylene 10 lauryl ether (POL) by *Pseudomonas putida* strain IPL5.

shown in Figure 2. The structure of this compound was not identified because the molecular weight could not be determined. However, the base peak of the mass spectrum for metabolite II was also at m/z 57 (same as for metabolite I). The molecular ion peaks at m/z 227 and 196 were the major differences between metabolites I and II, and were probably due to the variation of carbon-length in the side chains of the initial substrate (POL).

Dodecanol and nonanol supported the growth of *P. putida* IPL5. After a 10-day incubation, a 60% reduction of dodecanol was observed with no accumulation of detectable metabolites. Disappearance of nonanol was complete (greater than 99% degradation). Polyethylene glycol supported growth, but there was a considerable delay in growth before culture turbidity was evident. Nonylphenoldiethoxylate and dodecane did not support the growth of strain IPL5.

R. eutropha B30P4 utilized the alkylethoxylate surfactants (POL and Brij 30) as growth substrates (Table 2). The amount of POL removed, as measured by CTAS, by strain IPL5 (90%) was greater than that observed by strain B30P4 (60%). However, in contrast to strain IPL5, no degradation products were detected in B30P4 cultures. The alkylphenolethoxylate surfactant (IGP) did not support the growth of strain B30P4. Growth on dodecanol resulted in a 56% reduction of the parent compound with no accumulation of detectable metabolites. Polyethylene glycol readily supported growth without a delay, whereas no growth was observed on dodecane, nonanol or nonylphenoldiethoxylate.

Possible production of surfactant-like products detectable as CTAS by B30P4::TnPCB was evaluated. This strain was

cultured on PAS medium amended with 0.2% sodium benzoate, polyethylene glycol, dodecanol, or a mixture of dodecanol and polyethylene glycol (0.1% each) for 14 days. CTAS measurements indicated that no surfactant-like substances were produced. Cultures of IPL5 grown on sodium benzoate also did not produce surfactant-like products as measured by the CTAS method.

Effect of biphenyl on PCB degradation

The parent strains B30P4 and IPL5 and the recombinant strains B30P4::TnPCB and IPL5::TnPCB grew using sodium benzoate as the sole source of carbon and energy. Neither of the parent strains grew on biphenyl. B30P4::TnPCB grew on biphenyl, but IPL5::TnPCB did not grow on biphenyl. Attempts to grow IPL5::TnPCB on IGP in the presence of 0.2% biphenyl were also not successful suggesting that biphenyl or its metabolites are inhibitory to this strain.

The patterns of congener degradation in resting cell assays using strain B30P4::TnPCB grown on either biphenyl or POL and IPL5::TnPCB grown on IGP were similar (Table 3). Although the extent of PCB degradation appears somewhat higher when B30P4::TnPCB is grown on biphenyl, it is not clear if this difference is significant.

The effects of biphenyl (400 ppm) on PCB degradation by B30P4::TnPCB and IPL5::TnPCB grown on 0.2% POL and IGP, respectively, in growing cell assays are indicated in Table 4. The extent of surfactant degradation (disappearance of CTAS) was similar to that observed in the absence of PCBs (94% in Table 2 *vs* 93% in Table 4). The surfactant degradation product nonylphenoldiethoxylate was observed in IPL5 cultures grown on IGP. No surfactant degradation products were observed in B30P4::TnPCB cultures. Biphenyl (400 ppm) was completely degraded in cultures containing either strain. A slight increase in PCB degradation was observed in biphenyl-amended B30P4::TnPCB cultures. In biphenyl-amended IPL5::TnPCB cultures, PCB degradation was significantly repressed although minimal effect on surfactant degradation was evident.

Potential PCB, CTAS, and biphenyl disappearance due to adsorption to cells was evaluated in experiments using killed cells. B30P4::TnPCB and IPL5::TnPCB grown on PAS medium amended with 0.2% (wt/vol) POL were centrifuged and washed with PAS medium, resuspended in PAS medium, and killed by heating at 70°C for 1 h. The cells were then added separately to 5 ml PAS medium containing 0.2% POL, 25 ppm Aroclor 1242 and 400 ppm biphenyl, and incubated at room temperature on a rotary incubator for 2 days. Comparison of extracts from the killed cultures and uninoculated controls indicated that less than 3% of the surfactant (POL) was lost through adsorption to the killed cells. No losses of PCBs or biphenyl were observed. In addition, losses in the uninoculated controls attributable to volatilization were less than 10% for biphenyl and 2% for PCBs over a 10-day incubation period.

PCB and surfactant degradation in mixed cultures

Experiments comparing individual pure cultures (B30P4::TnPCB and IPL5::TnPCB) and mixtures of the two strains indicated favorable synergistic effects for surfactant degradation resulting from the combination of strains. Cultures containing both strains exhibited more extensive POL degradation than cultures containing B30P4::TnPCB alone, without the accumulation of the surfactant degradation product 2-(dodecyloxy)ethanol typically observed in IPL5::TnPCB cultures grown on POL (Table 5). This indicates that B30P4::TnPCB can degrade 2-(dodecyloxy)ethanol as depicted in Figure 3.

Degradation of PCBs in treatments using B30P4::TnPCB or B30P4::TnPCB and IPL5::TnPCB was greater than PCB degradation in the treatment using IPL5::TnPCB alone. The pattern of PCB congeners degraded by B30P4::TnPCB and IPL5::TnPCB (Figure 4) was similar to that for B30P4::TnPCB as seen in Table 3. No further benefit was derived from biphenyl addition in terms of surfactant or PCB degradation (data not presented).

Discussion

In treatment strategies involving FAVs the surfactant should both solubilize the PCBs and provide the carbon and energy source for growth of the recombinant strains and PCB degradation. Surfactant degradation is important in several respects. First, the extent of surfactant degradation will determine the efficiency of substrate utilization for growth and contaminant degradation. Second, the presence of residual surfactant in the environment or the persistance of solubilized PCBs due to incomplete surfactant degradation is undesirable. Third, the production of surfactant degradation metabolites may be problematic.

The identification of bioengineered strains designed for field release is of importance in the EPA approval process. B30P4 and IPL5 were identified taxonomically using fatty acid analysis, Biolog analysis, and partial 16S rDNA analysis. These methods agreed on the placement of these strains in the genus *Ralstonia* for B30P4 and *Pseudomonas* for IPL5. The high degree of sequence similarity (98% and 99%) of the 16S rDNA from B30P4 and IPL5 with the type strains of *Ralstonia eutropha* and *Pseudomonas putida* further confirms the taxonomic identification of *Ralstonia eutropha* for B30P4 and *Pseudomonas putida* for IPL5.

PCB degradation was previously demonstrated in surfactant-amended soils from an industrial site treated with a specific FAV consisting of *Pseudomonas putida* IPL5::TnPCB and the alkylphenolethoxylate surfactant Igepal CO-720 [22]. This and other studies [27] demonstrated the production of nonylphenoldioethoxylates from alkylphenolethoxylate surfactants by some *Pseudomonas* strains. Alkylphenols have been observed in anaerobic sludge at sewage treatment plants employing anaerobic digestion [12,13]. Surfactants are partially degraded to alkylphenols in the aeration basin, but are resistant to further degradation under anaerobic conditions. However, sludge treatment by extended aeration appears to ultimately result in the degradation of these compounds. Nonylphenols and nonylphenolethoxylates in treated effluents are known to be toxic to aquatic organisms [9,31], and are suspected as potential environmental estrogens in humans [14,34,40]. Alkylethoxylate surfactants are more readily degradable than the alkylphenolethoxylate surfactants, and degradation products are much less likely to accumulate [35,39]. Results using indi-

Table 3 Biodegradation of Aroclor 1242 by *Ralstonia eutropha* B30P4::TnPCB and *Pseudomonas putida* IPL5::TnPCB

Peak No.	Congener[a]	Percent degradation in resting cell assays[c]			
		Starting concentration[b]	B30P4::TnPCB Biphenyl[d]	B30P4::TnPCB POL[e]	IPL5::TnPCB IGP[f]
2	2,2′/2,6	0.27	90	80	>97
3	2,4/2,5	0.045	>98	>98	>98
4	2,3′	0.045	>98	>98	>98
5	2,3/2,4′	0.12	>99	>99	>99
6	2,6,2′	0.58	24	20	20
7	2,5,2′	0.09	>99	>99	>99
8	2,4,2′/4,4′	0.43	94	85	95
9	2,3,6/2,6,3′	0.07	70	45	80
10	2,3,2′/2,6,4′	0.48	65	65	60
11	2,5,3′	0.11	>99	75	>99
12	2,4,3′	0.7	>98	55	>99
13	2,5,4	0.7	>99	>99	>99
14	2,4,4′	0.65	30	20	30
15	2′3,4/2,5,2′,6′	0.33	>98	85	95
16	2,3,4′/2,4,2′6′	0.09	55	35	75
17	2,3,6,2′	0.05	50	35	50
18	2,3,2′,6′	0.05	55	50	54
19	2,5,2′,5′	0.31	95	90	95
20	2,4,2′,5′	0.26	85	55	80
21	2,4,2′,4′	0.11	0	20	0
22	2,4,5,2′	0.14	>99	65	>99
23	2,3,2′,5′	0.34	90	65	>99
24	3,4,4′/2,3,2′,4′	0.37	45	35	50
25	2,3,4,2′/2,3,6,4′/2,6,3′,4′	0.3	30	25	25
26	2,3,2′,3′	0.09	95	79	>99
27	2,4,5,4′	0.19	0	0	0
28	2,5,3′,4′	0.39	75	40	65
29	2,4,3′,4′/2,3,6,2′,5′	0.48	25	15	15
30	2,3,6,2′,4′	0.13	30	0	20
31	2,3,3′,4′/2,3,4,4′	0.29	0	0	0
32	2,3,6,2′3′/2,3,5,2′,5′	0.10	50	0	25
33	2,3,5,2′,4′/2,4,5,2′,5′	0.11	70	25	25
34	2,4,5,2′,4′	0.1	15	0	0
35	2,4,5,2′,3′/2,3,5,6,2′,6′	0.1	25	0	0
36	2,3,4,2′,5′	0.1	35	0	0
37	2,3,4,2′,4′	0.07	25	0	0
38	2,3,6,3′,4′/3,4,3′,4′	0.11	0	0	0
39	2,3,4,2′,3′	0.07	0	0	0
40	2,3,6,2′,4′5′/2,4,5,3′,4′	0.07	0	0	0
41	2,3,4,3′4′/2,3,4,2′3′,6	0.1	0	0	0
Total percent degraded[g]			68%	56%	65%

[a]Identification of individual congeners based on comparison with Bedard *et al* [7].
[b]Starting concentration in ppm of each peak area in 10 ppm Aroclor 1242.
[c]% Degraded of individual congeners by live bacteria compared to killed bacteria after 2-day incubation with 10 ppm Aroclor 1242 in resting cell assays. Levels of apparent degradation less than 15% are considered nonsignificant and are reported as 0% for clarity [7].
[d]B30P4::TnPCB was grown in PAS medium [8] with 2000 ppm biphenyl.
[e]B30P4::TnPCB was grown in PAS medium with 2000 ppm polyoxyethylene 10 lauryl ether.
[f]IPL5::TnPCB was grown in PAS medium with 2000 ppm Igepal CO-270.
[g]Total % degraded was calculated from the percent degradation for each peak and the weight percent values for each peak of Aroclor 1242 as determined by Bedard *et al* [7].

vidual growth substrates, CTAS measurements, and GC/MS analysis suggest that polyoxyethylene 10 lauryl ether can be completely degraded by the IPL5 and B30P4 consortium. However, it is possible that some non-detectable products may be present in the aqueous phase. Alkylethoxylate surfactants consist of an aliphatic portion and a polyethylene glycol portion. Aliphatic compounds should be solvent extractable, whereas polyethylene glycol products may be present in the aqueous fraction. Complete degradation of the alkylethoxylate is inferred from the fact that the bacteria utilize polyethylene glycol as a growth substrate and that no aliphatic products are detected.

Strains *Ralstonia eutropha* B30P4::TnPCB and *Pseudomonas putida* IPL5::TnPCB each have some distinct advan-

Table 4 The effect of biphenyl on surfactant and PCB degradation by *Pseudomonas putida* IPL5::TnPCB and *Ralstonia eutropha* B30P4::TnPCB

Strain	Treatment[a]		Percent degraded (s.d.)[d]		
	Surfactant[b]	Biphenyl[c]	Surfactant	Biphenyl	PCBs
B30P4::TnPCB	POL	−	60 (0.56)	na[e]	64 (15)
B30P4::TnPCB	POL	+	57 (6.6)	>99	71 (6.0)
IPL5::TnPCB	IGP	−	93 (16)[f]	na	53 (4.3)
IPL5::TnPCB	IGP	+	86 (18)[f]	>99	32 (4.8)

[a]All treatments were performed in triplicate and contained a starting concentration of 25 ppm Aroclor 1242.
[b]The starting concentrations of surfactants were 2000 ppm. Surfactant abbreviations: POL, polyoxyethylene 10 lauryl ether; IGP, Igepal CO-720.
[c]The starting biphenyl concentration was 400 ppm.
[d]Percent degraded was calculated from ppm values for each treatment and corresponding uninoculated controls. s.d. = standard deviation of percent values.
[e]Not applicable.
[f]Surfactant metabolites (nonylphenoldiethoxylates) were detected by GC.

Table 5 Surfactant and PCB degradation by *Pseudomonas putida* IPL5::TnPCB and *Ralstonia eutropha* B30P4::TnPCB alone and in mixed culture

Strain	Percent degraded (s.d.)[a]	
	POL[b]	PCB[c]
B30P4::TnPCB	61 (5.7)	65 (11)
IPL5::TnPCB	88 (4.1)[d]	28 (5.6)
B30P4::TnPCB and IPL5::TnPCB	89 (0.8)	73 (25)

[a]Percent degraded was calculated from ppm values for each treatment and corresponding controls. All treatments were performed in triplicate. s.d. = standard deviation of percent values.
[b]The starting concentration of the surfactant polyoxyethylene 10 lauryl ether (POL) was 2000 ppm.
[c]The starting PCB (Aroclor 1242) concentration was 25 ppm.
[d]The surfactant metabolite 2-(dodecyloxy)ethanol was detected by GC.

tages and disadvantages for use in surfactant-based treatment of PCBs. Commercial surfactant preparations often contain a complex mixture of individual components [33]. The more extensive decrease in CTAS by IPL5 appears to indicate that this strain can degrade a wider array of these surfactant components than B30P4. This is further suggested by the observation that IPL5 can use both the alkylethoxylate and alkylphenolethoxylate surfactants as growth substrates. However, this strain can only degrade the ethoxylate moiety of these surfactants resulting in the accumulation of potentially undesirable products. Although strain B30P4 grows slower than IPL5 and dose not degrade as many of the individual surfactant compounds in POL, no solvent-extractable degradation products were detected. B30P4 also degrades 2-(dodecyloxy)ethanol that results from IPL5 degradation of the alkylethoxylate surfactant. Attempts to achieve more complete surfactant degradation with B30P4 by using higher purity surfactants did not result

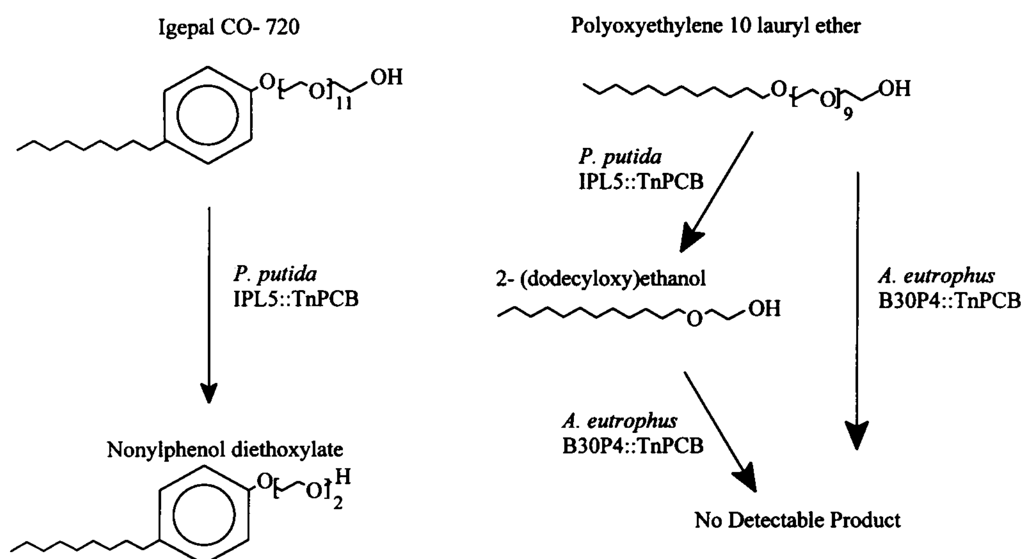

Figure 3 Schematic diagram depicting the surfactant substrate ranges of *Pseudomonas putida* IPL5 and *Ralstonia eutropha* B30P4 (*A. eutrophus*), and the resulting degradation products.

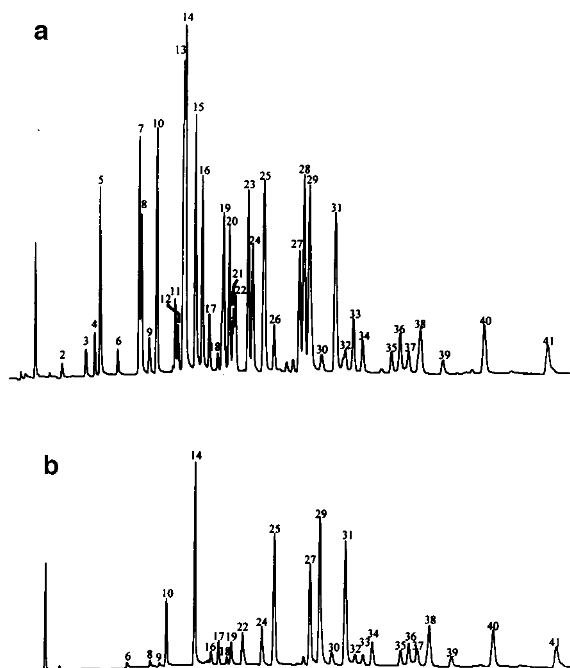

Figure 4 Degradation of individual PCB congeners (Aroclor 1242) after 20 days by the *Pseudomonas putida* IPL5::TnPCB and *Ralstonia eutropha* B30P4::TnPCB consortium grown on polyoxyethylene 10 lauryl ether (POL). Panel (a) is the uninoculated control and panel (b) is the inoculated experimental treatment. The peak numbers correspond to the congeners identified in Table 3. Peak 40 serves as a non-degraded internal standard in both the experimental and control treatments and has approximately the same relative peak area in both treatments.

in a considerable improvement in CTAS removal (data not presented). The most extensive surfactant degradation without the accumulation of degradation products was observed by using a combination of both strains. As surfactant degradation by consortia has been observed for other surfactants [17], and is undoubtedly important in the environment, the use of a combination of recombinant strains for contaminant degradation may be the natural choice.

The observed accumulation of nonylphenoldiethoxylates, and the presence of degradation-resistant branched alkyl chains and an aryl nucleus in the IGP surfactant, indicate that surfactant degradation by strain IPL5 occurs via ethyoxylate shortening. The lack of hydrophobic degradation products in B30P4 cultures grown on alkylethoxylate surfactants, and the ability of this strain to grow readily on aliphatic alcohols and polyethylene glycol, suggests that B30P4 degrades both the alkyl and ethoxylate groups. Whether degradation occurs via central ether scission, α/β-oxidation of the alkyl group, or ethoxylate shortening cannot be established from the available data [39]. Addition of the biphenyl degradative operon to strain IPL5 did not confer the ability to grow on biphenyl. When the recombinant strain (IPL5::TnPCB) is grown on benzoate a transient brown metabolite is observed that is not seen in benzoate-grown cultures of the parent strain (IPL5). This metabolite is not observed in surfactant-grown cultures of either strain. This suggests that interference between the indigenous benzoate pathway and the biphenyl operon is occurring.

Previous results indicated that PCB degradation by IPL5::TnPCB may be limited by gene expression [22]. Attempts to increase PCB degradation via biphenyl induction were not effective. Growth of IPL5::TnPCB was severely inhibited at high biphenyl concentrations (2000 ppm). In growing cell assays, biphenyl amendment (400 ppm) resulted in a considerable decrease in PCB degradation, although the added biphenyl was degraded. These results indicate that the biphenyl genes or enzymes are not completely functional in IPL5. Addition of biphenyl does not induce PCB degradation, but apparently competes with the PCBs for the degradative enzyme. Alternatively, metabolites resulting from interference by the indigenous benzoate degradative pathway may inhibit PCB degradation.

Addition of the biphenyl operon to B30P4 confers the ability to use biphenyl as a sole source of carbon and energy. Amendment of resting and growing cell assays with biphenyl appears to result in some increase in PCB degradation, suggesting induction of the biphenyl operon, although the effect is relatively small. These results indicate that the biphenyl operon is more compatible with B30P4 than with IPL5. This is perhaps not unexpected as both the original source of the PCB genes (ENV 307; [23]) and B30P4 have been identified as *R. eutropha*.

Experiments with the alkylethoxylate and alkylphenolethoxylate surfactants, biphenyl, and the two recombinant strains indicate that the most effective combination appears to be the use of the surfactant POL in combination with both strains in the absence of biphenyl. This approach achieves the highest observed surfactant and PCB degradation without the accumulation of surfactant degradation products. Problems with potential human toxicity concerns and possible competitive inhibition of PCB degradation associated with biphenyl amendment are also avoided.

The results of studies on surfactant degradation in the context of PCB degradation suggest possible alternatives for future improvements in FAV design. Whereas the alkylethoxylate surfactants are more desirable than the alkylphenolethoxylate surfactants in terms of potential toxicity associated with degradation products, the alklethoxylate surfactants may be too easily degraded by indigenous populations for some potential environmental applications. The growth of indigenous surfactant-degrading strains may result in competition with added surfactant/PCB-degrading strains. In this respect, the less degradable surfactants may be superior. A suitable compromise may involve the use of alkylethoxylate surfactants with longer ethoxylate chains to decrease biodegradability or the use of linear alkylphenolethoxylates to increase biodegradability over the branched alkylphenolethoxylates [35]. The most appropriate design is likely to depend to some extent on the application. Further efforts to increase PCB gene expression in the absence of normal inducers may increase the rate of PCB degradation. The use of alternative promoters or increased gene copy number are obvious alternatives.

Issues of surfactant degradation pertain not only to FAVs, in which surfactant degradation is intentional, but also to other soil washing or surfactant-based treatment technologies where surfactant degradation by indigenous

strains may occur. Degradation of more readily degradable alkylethoxylate surfactants may result in oxygen depletion, contaminant desolubilization or pore clogging in the subsurface. Partial degradation of the more recalcitrant alkylphenolethoxylate surfactants may result in accumulation of degradation products or persistence of residual surfactants and solubilized contaminants. The fate of the surfactants in all treatment schemes in which they are employed should be considered to avoid potential difficulties caused by the degradative activities of indigenous microbial populations.

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