Biodegradation **8:** 153–158, 1997.

Terpene-utilizing isolates and their relevance to enhanced biotransformation of polychlorinated biphenyls in soil

B.S. Hernandez[1], S.-C. Koh[2], M. Chial[1] & D.D. Focht[2,*]
[1] *Departamento de Microbiología, Universidad de Panamá, Republica de Panamá;* [2] *Department of Soil and Environmental Sciences, University of California, Riverside, California, 92521 USA (* author for correspondence)*

Accepted 21 May 1997

Key words: biodegradation, PCB, Aroclor

Abstract

Orange peels, eucalyptus leaves, pine needles and ivy leaves were added separately to soil spiked with Aroclor 1242 (100 $mgkg^{-1}$). Polychorinated biphenyls (PCBs) disappeared after six months in all the amended soils, but not in unamended soils. Although biphenyl was not added to any of the soils, all four amended soils had much higher levels (10^8/g) of biphenyl-utilizing bacteria than the unamended control (10^3/g). Ten random isolates obtained from these soils were identified as coryneform bacteria. Five isolates, that were distinctly different, were studied further with respect to growth on pure terpenes and metabolism of PCBs. The most effective strains were *Cellulomonas* sp. T109 and *R. rhodochrous* T100, which metabolized 83% and 80% of Aroclor 1242, respectively, during a six day period of growth on cymene and limonene, respectively. The *bph*A gene, cloned as a 2.8 Kb *Sa*/I fragment of pAW6194 from *cbpA* (Walia et al. 1990) hybridized to total DNA of all coryneform isolates, and to the well-established PCB degrader *Rhodococcus globerulus*. In contrast, a 5 Kb *Xho*I-*Sma*I fragment of the *bph*A gene (Furukawa & Miyazaki 1986) did not show any homology to the genomic DNA of any of the isolates or to *R. globerulus*, but did hybridize to two other well-known PCB degraders *Pseudomonas* sp. LB400, and *Alcaligenes eutrophus* H850. The data presented herein indicate that terpenes may be natural substrates for biphenyl-degrading bacteria and may enhance substantial transformation of Aroclor 1242.

Introduction

Polychlorinated biphenyls (PCBs) are among the most recalcitrant priority pollutants known. Anaerobic dehalogenation typically occurs with reduction of higher chlorinated congeners to lesser chlorinated ones (Quensen III et al. 1988; Brown et al. 1989; Nies & Vogel 1990). Aerobic metabolism of lesser chlorinated PCBs to chlorobenzoates is fortuitously effected by four sequential steps encoded by the *bphABCD* genes of biphenyl-degrading bacteria (Furukawa 1994). Biphenyl has conventionally been used to isolate and grow bacteria that partially transform PCB congeners (Ahmed & Focht 1973; Furukawa et al. 1978; Bedard et al. 1986, 1987; Kohler et al. 1988) and to enhance biodegradation in soil (Brunner et al. 1985; Focht & Brunner 1985) and sediments (Harkness et al. 1993). However, biphenyl is not a normal constituent of soil, from which biphenyl-degrading bacteria are readily isolated. Thus, the enzymes of the biphenyl/PCB pathway are clearly designed to attack natural substrates having structural similarity to biphenyl. Lignin is not one of those substrates, because it does not enhance biodegradation of Aroclor 1242 in the same soil (Brunner et al. 1985) used in the study presented herein. Moreover, lignin is attacked by non-specific peroxidases and monooxygenases (Kirk 1984), whereas the initial oxidation of biphenyl (Lunt & Evans 1970; Furukawa & Miyazaki 1986; Haddock et al. 1995) involves a specific dioxygenase attack.

Other naturally occurring substrates, similar in structure to biphenyl, may also facilitate biodegradation of PCBs. Donnelly et al. (1994) demonstrated that some plant flavinoids were equal to biphenyl in promoting metabolism of PCBs. The study herein was undertaken to determine if plant residues rich

in terpenes would enhance biodegradation of Aroclor 1242 in soil, and to determine the genetic homology of terpene-utilizing isolates among each other and to well-characterized PCB-degraders.

Materials and methods

Soil incubations

Plant terpenes were considered as likely analogs for the cometabolism of PCBs because the conjugated double-bond structure of the cyclic six-membered ring may be similar to 2,3-site at which dioxygenation occurs with biphenyl. Each treatment, including the control, consisted of 100 g of Altamont soil (pH 6.8) (Brunner et al. 1985) and 10 mg Aroclor 1242. Except for the control, each soil was amended with one of the four plant residues (5 g dry mass): orange peels, ivy leaves, pine needles and eucalyptus leaves. All treatments, including the control, were done in duplicate. Biphenyl was not added to any soils in this investigation. Following a six month aerobic incubation at ambient temperature and 50% soil saturation, 1 g soil was removed for microbial enumeration, and the remainder was extracted by a hexane/acetone (1 : 1, vol/vol) mixture (Brunner et al. 1985) for gas chromatographic analysis.

A separate incubation to determine if plant residues might sequester PCBs was performed. Duplicate treatments containing orange peels and duplicates containing no amendment were added to soil in conjunction with Aroclor 1242 (10 mg100 g^{-1} soil) and autoclaved (121 °C and 15 min). After 6 weeks, soils were extracted, as described above, and analyzed by gas chromatography, as described below.

Gas chromatography

A Hewlett Packard 5890 Series II gas chromatograph equipped with a flame ionization detector and a DB-5 capillary column (30 m $\times$ 0.32 mm ID; film thickness, 0.25 μm; J&W Scientific, Folsom, CA) was used with a helium carrier gas flow of 30 mlmin^{-1} and a 1 : 10 injector split ratio. The temperature program ran from 150 °C to 225 °C using the following ramping conditions: 150 °C for 1 min, 1.5 °C min^{-1} to 165 °C, 3.0 °C min^{-1} to 225 °C. The internal standard 2,3,4,2′,3′,4′ hexachlorobiphenyl (retention time of 34 min) was added prior to extraction.

Microbial enumeration and isolation

Even though biphenyl was not added to any of the soils in this study, numbers of biphenyl-degrading bacteria were analyzed to determine if compounds in plant residues supported growth and maintenance of these bacteria. Soil samples (1.0 g) were taken at the end of the incubation from each of the duplicate flasks. Serial ten-fold dilutions were made in sterile 50 mM phosphate buffer, from which 0.1 ml was inoculated in duplicate plates of mineral salts agar (2% Noble) with biphenyl as the carbon source. Dilutions containing between 100 and 125 colonies were counted following incubation for 10 days at 28 °C. Ten random isolates obtained from plant-amended soils were tested for their ability to use cymene, α-pinene, abietic acid and limonene as a carbon source as these are major terpenoid compounds of the plant residues used in our study (Newall et al. 1996; Ikan 1991). Cultures were grown in 50 ml screw-cap Erlenmeyer flasks containing 20 ml of mineral salts media (Focht 1994). Cymene, pinene and limonene were placed separately (50 ml) into an Eppendorf tube which was fastened with a wire over the neck of the flask. Abietic acid (500 ppm) was added directly to the medium and neutralized as the sodium salt. Growth was noted as positive if turbidity was observed within 20 days.

Molecular biology

The genetic homology of terpene-degrading isolates and other well-known biphenyl-degraders was determined using two different probes of the *bphA* gene, which encodes for the initial dioxygenation of biphenyl (Furukawa & Miyazaki 1986). The *bphA* gene (2.8 kb *SaI I* fragment of pAW6194, also known as *cbpA*) from *Pseudomonas putida* strain OU83 (Walia et al. 1990) was cloned in pU18 and designated as pDF. The *bphA* gene (5 kb *Xho I-SmaI* fragment of pKTF18) originated from *Pseudomonas pseudoalcaligenes* strain KF707 (Taira et al. 1992). Subcloning of gene probes, genomic DNA extractions, and other molecular biological procedures were used (Maniatis et al. 1982). Non-radioactive probes were prepared using the Genius DNA Labeling and Detection Kit (Boehringer Mannheim), and slot blot hybridization was observed by chemiluminescent detection (Lumi-Phos 530) (Koh et al. 1993). Hybridization was conducted at high stringency, allowing over 70% sequence homology.

Table 1. Colony forming units (CFU/g) of biphenyl-degraders with standard deviation

Treatment	Log CFU/g
Control, Aroclor 1242	3.36 ± 0.85
Eucalyptus leaves + Aroclor 1242	7.84 ± 0.84
Orange leaves + Aroclor 1242	8.12 ± 0.07
Ivy leaves + Aroclor 1242	8.12 ± 0.35
Pine needles + Aroclor 1242	7.86 ± 0.56

Metabolism of PCBs

Bacterial strains were grown in 50 ml vials containing 10 ml of mineral salts media (Focht, 1994) and 0.10 mg of Aroclor 1242. A stock solution of Aroclor 1242 in hexane was added to sterile vials and allowed to volatilize before adding the sterile mineral salts solution and inoculum. Limonene or cymene were added as growth substrates by placing 50 μl in a sterile 500 μL Eppendorf tube suspended from a wire inside the vial (Focht 1994). Biphenyl (0.05%) was added directly as a fine powder. Growth on glucose (0.1%) and Aroclor 1242 was used as the comparative control.

After 6 days, 0.5 ml H_2SO_4 (5 M), 5 ml hexane, and 4 ml of Na dodecyl sulfate (12.5%) were added to each vial, which was agitated on a shaking platform for 1 hr. The hexane phase was removed to a test tube, and the remaining aqueous phase was extracted with 5 ml of hexane and shaken for 15 min. The second extract was pooled with the first, and dried over 5 g of anhydrous Na_2SO_4. Hexane extracts were removed, rinsed out of the test tube, and brought up to volume in 10 ml volumetric flasks. GC analyses were performed as described previously.

Results and discussion

Transformation of Aroclor 1242 occurred completely in all the amended soils, but not in the non-amended soil (Figure 1). Decomposition of the lighter mass congeners was evident in the control. Although biphenyl was not added to any of the soils, the numbers of biphenyl-degraders in the amended soils were five orders of magnitude higher than in the non-amended soils (Table 1), which supports the hypothesis that terpenes may be one of the natural substrates for biphenyl-utilizing bacteria.

Ten isolates were obtained on agar plates, containing biphenyl as a carbon source, from plant-amended soils. They exhibited typical coryneform morphology, namely Gram-variable, bent, club-shaped, long rods, and fragmented cocci. The isolates were differentiated as *Rhodococcus*, *Corynebacterium*, and *Cellulomonas* on the basis of cell wall fatty acids. As several isolates were identical, only five, that were distinctly different, were studied further with respect to growth on pure terpenes (Table 2) and metabolism of PCBs (Table 3). Three well-established biphenyl degraders *R. globerulus* P6 (Furukawa et al. 1978; Asturias et al. 1994), *A. eutrophus* H850 (Bedard et al. 1987), and *P. putida* LB400 (Bopp 1986) grew poorly on at least one of the four terpenes tested. Turbidity was not observed in uninoculated controls containing either of the terpenes that were tested.

As there are two genetically different groups of biphenyl-utilizers (Yates & Mondello 1989), the genetic homology of the terpene isolates was pertinent to determine if terpenes are substrates for biphenyl-degraders. The genomic DNA of *P. acidovorans* M3GY (McCullar et al. 1994), a recombinant,

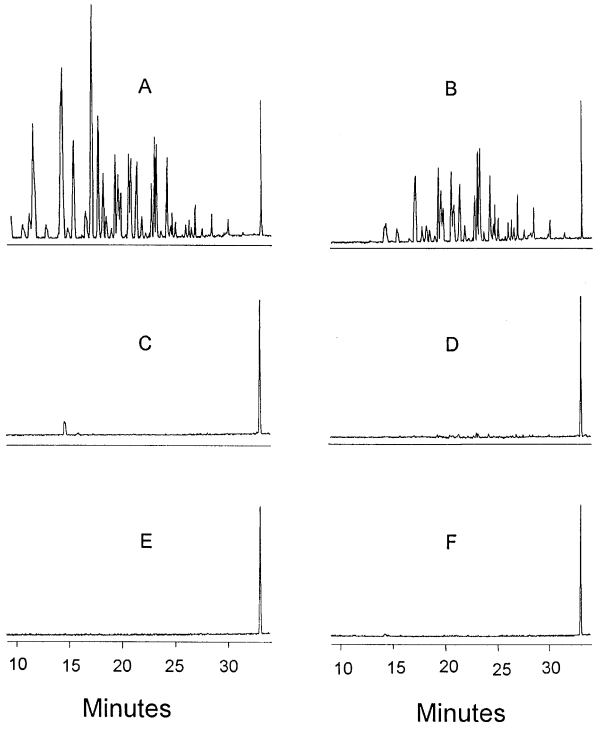

Figure 1. Gas chromatographic analysis of Aroclor 1242 standard (100 ng/μL; (*A*) and soil extracts of terpene-amended and non-amended soils; (*B*) non-amended; (*C*) orange peels; (*D*) ivy leaves; (*E*) pine needles; (*F*) eucalyptus leaves. The peak at 34 min is the internal standard of 2,3,4,2′,3′4′-hexachlorobiphenyl.

Table 2. Growth of biphenyl-utilizing coryneform isolates on terpenes

Strain	cymene	pinene	abietic acid	limonene
R. rhodochrous T100	n/d	+	+	+
R. rhodochrous T101	+	+	+	+
Corynebacterium sp. T104	+	+	+	+
Cellulomonas sp. T109	+	n/d	+	+
R. rhodochrous T110	+	n/d	+	+

n/d : not determined.

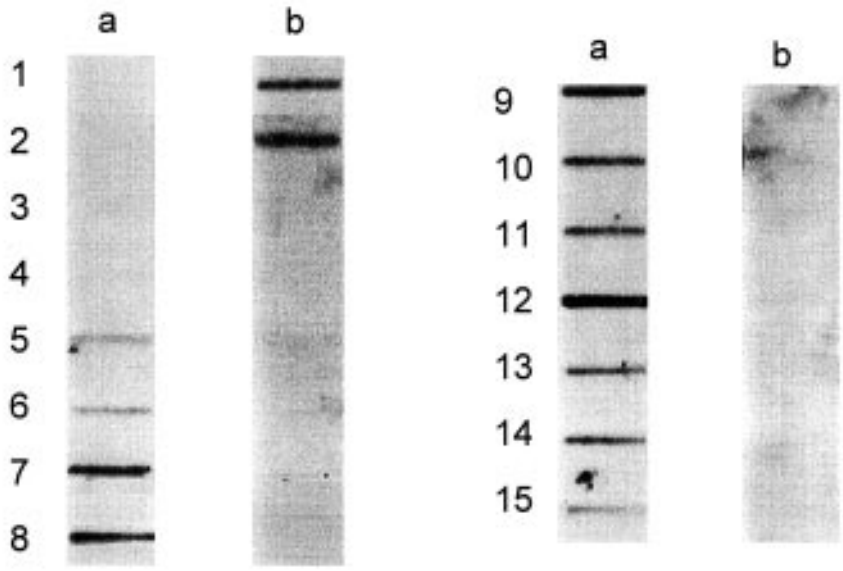

Figure 2. Slot blot hybridization of biphenyl-degrading strains to the *bphA* gene 2.8 kb fragment of pAW6194 (*lane A*) and the *bphA* gene 5 kb *Xho-SmaI* fragment of pKTF18 (*lane B*). Numbers correspond to the following strains: 1. *Pseudomonas* sp. LB400 (Bopp 1986), 2. *Alcaligenes eutrophus* H850 (Bedard et al. 1987), 3. *E. coli*, 4. *Pseudomonas* sp. MB86 (Barton & Crawford 1988), 5. *P. acidovorans* M3GY (McCullar et al. 1994), 6. *Rhodococcus globerulus* P6 (Asturias et al. 1994), 7–15. terpene-utilizing isolates.

Rhodococcus globerulus P6 (Asturias et al. 1994), the parental strain of M3GY, and all terpene-utilizers hybridized to the *bphA* 2.8 kb fragment (Walia et al. 1990), but not to the *bphA* 5 kb fragment (Taira et al. 1992). In contrast, genetic homology to the *bphA* gene 5 kb fragment was observed only in *Pseudomonas* sp. LB400 (Bopp 1986) and *Alcaligenes eutrophus* H850 (Bedard et al. 1987) (Figure 2).

We have focused our attention on the *bphA* gene, based on the rationale that the corresponding gene product may bring about more effective dioxygenolytic removal of *ortho*-chorine substituents, which comprise the most recalcitrant PCB congeners for aerobic attack (Furukawa et al. 1979; Bedard et al. 1987). Bedard questioned the paradigm that *ortho*-chlorines block dioxygenation (Bedard 1990). Indeed, dioxygenation of 2,5,2′,5′ by *Pseudomonas* sp. LB400 caused spontaneous removal of an *ortho*-chlorine to form the corresponding catechol directly in a recent study (Haddock et al. 1995).

Terpene degradation has characteristically been demonstrated to occur by monooxygenase reactions in fungi and bacteria (Trudgill 1994), so that PCB transformation may also have been effected by monooxygenase attack. Nevertheless, all terpene-utilizing isolates grew on biphenyl as a sole carbon source and hybridized to one of the *bphA* genes. That both monooxygenases and dioxygenases may play a role in transformation of PCBs is a subject worthy of future studies.

Table 3. Transformation of Aroclor 1242 by bacterial cultures: % relative to glucose control (%) for each culture

Strain	Substrate	% Transformation
R. globerulus P6	biphenyl	56.6 ± 14.2
R. rhodochrous T100	limonene	79.6 ± 5.6
R. rhodochrous T101	limonene	42.6 ± 3.8
Corynebacterium sp. T104	limonene	52.8 ± 2.8
Corynebacterium sp. T104	cymene	20.6 ± 4.6
Cellulomonas sp. T109	limonene	57.5 ± 4.7
Cellulomonas sp. T109	cymene	82.6 ± 6.3
R. rhodochrous T110	limonene	none
R. rhodochrous T110	cymene	52.7 ± 11.7

No discernible patterns of growth substrate were evident with regard to transformation of PCBs. The two most effective strains were *Cellulomonas* sp. T109, grown on cymene, and *R. rhodochrous* T100, grown on limonene (Table 3). Comparisons between strains able to utilize both terpenes showed greater transformation of PCBs by *Corynebacterium* sp. T104 with limonene, while *Cellulomonas* sp. T109 and *R. rhodochrous* T110 effected greater transformation with cymene. As all of these data are relative to the glucose-grown cells, which are taken as a zero-baseline, the absolute transformation of Aroclor 1242 may be much higher.

The addition of four different plant residues enhanced complete aerobic disappearance of Aroclor 1242 from soil. In contrast, addition of biphenyl has not resulted in complete disappearance of the higher chlorinated congeners when biphenyl is depleted from the soil (Brunner et al. 1985; Focht & Brunner 1985). Two possible scenarios may account for these differences. First, if terpenes are the major substrates which enhance transformation of PCBs, they may be released slowly as other plant residues are metabolized. Second, the 'biphenyl-utilizers' that arose in high numbers ($10^8 g^{-1}$) may be different than those that respond to addition of biphenyl to soil, perhaps in having broader substrate specificity towards PCBs. However, these coryneform isolates grow more slowly on biphenyl, in comparison to typical gram negative biphenyl-utilizers (Bopp 1986; Bedard et al. 1987), so they would not be the dominant PCB-degrading bacteria in soil amended with biphenyl. Although biphenyl has characteristically been the substrate of choice for rapid enrichment of PCB-degrading bacteria, plant residues containing terpenes or other related substrates might be more effective in enhancing biodegradation of PCBs in soil by slower-growing coryneform bacteria.

Finally, the possibility that plant residues may have decreased the amount of extractable PCBs is unlikely as the recovery of Aroclor 1242 from sterilized treatments with orange peels was higher (10.0 ± 0.7 mg), relative to the internal standard, than in soils containing no orange peels (6.2 ± 0.4 mg). This surprising result suggests that orange peels may have adsorbed PCBs in a manner that rendered them more extractable than those adsorbed by soil alone. Thus, plant residues rich in terpenes would have three distinct advantages over biphenyl in bioremediation of PCB in soil, namely as agents which promote bioavailability, are not priority pollutants, and are widespread throughout the world.

Acknowledgements

We thank S. Walia for providing *Pseudomonas putida* strain OU83 and K. Furukawa for providing the *bphA* gene from *Pseudomonas pseudoalcaligenes* strain KF707. This research was supported by the National Science Foundation and the Kearney Foundation of Soil Science.

References

Ahmed M & Focht DD (1973) Degradation of polychlorinated biphenyls by two species of *Achromobacter*. Can. J. Microbiol. 19: 47–52

Asturias JA, Moore E, Yakimov M, Klatte S & Timmis KN (1994) Reclassification of the polychlorinated biphenyl-degraders *Acinetobacter* sp. strain P6 and *Corynebacterium* sp. strain MB1 as *Rhodococcus globerulus*. Syst. Appl. Microbiol. 17: 226–231

Barton MR & Crawford RL (1988) Novel biotransformations of 4-chlorobiphenyl by a *Pseudomonas* sp. Appl. Environ. Microbiol. 54: 594–595

Bedard DL (1990) Bacterial transformation of polychlorinated biphenyls. In: Kamely D, Chakrabarty A & Omenn G (Eds) Biotechnology and Biodegradation. (pp 369–388). Portfolio Publishing Co. and Gulf Publishing Co., Woodlands, TX and Houston

Bedard DL, Haberl ML, May RJ & Brennan MJ (1987) Evidence for novel mechanisms of polychlorinated biphenyl metabolism in *Alcaligenes eutrophus* H850. Appl. Environ. Microbiol. 53: 1103–1112

Bedard DL, Unterman R, Bopp LH, Brennan MJ, Haberl ML & Johnson C (1986) Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Appl. Environ. Microbiol. 51: 761–768

Bedard DL, Wagner RE, Brennan MJ, Haberl ML & Brown JF, Jr (1987) Extensive degradation of Aroclors and environmentally transformed polychlorinated biphenyls by *Alcaligenes eutrophus* H850. Appl. Environ. Microbiol. 53: 1094–1102

Bopp LH (1986) Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. J. Ind. Microbiol. 1: 23–29

Brown JF, Bedard DL, Brennan MJ, Carnahan JC, Feng H & Wagner RE (1989) Polychlorinated biphenyl dechlorination in aquatic sediments. Science 236: 709–712

Brunner W, Sutherland FH & Focht DD (1985) Enhanced biodegradation of polychlorinated biphenyls in soil by analog enrichment and bacterial inoculation. J. Environ. Qual. 14: 324–328

Donnelly PK, Hegde RS & Fletcher JS (1994) Growth of PCB-degrading bacteria on compounds from photosynthetic plant. Chemosphere 28: 981–988

Focht DD (1994) Microbiological procedures for biodegradation research. In: Weaver RW, Angle JS & Bottomley PS (Eds) Methods of Soil Analysis, Part 2. Microbiological and Biochemical Properties. Soil Science Society of America, Madison, WI

Focht DD & Brunner W (1985) Kinetics of biphenyl and polychlorinated biphenyl metabolism in soil. Appl Environ. Microbiol. 50: 1058–1063

Furukawa K (1994) Molecular genetics and evolutionary relationship of PCB-degrading bacteria. Biodegradation 5: 289–300

Furukawa K, Matsumura F & Tonomura K (1978) *Alcaligenes* and *Acinetobacter* strains capable of degrading polychlorinated biphenyls. Agric. Biol. Chem. 42: 543–548

Furukawa K & Miyazaki T (1986) Cloning of a gene cluster encoding biphenyl and chlorobiphenyl degradation in *Pseudomonas pseudoalcaligenes*. J. Bacteriol. 166: 392–398

Furukawa K, Tonomura K & Kamibayashi A (1978) Effect of chlorine substitution of the biodegradability of polychlorinated biphenyls. Appl. Environ. Microbiol. 35: 223–227

Furukawa K, Tonomura K & Kamibayashi A (1979) Effect of chlorine substitution of the bacterial metabolism of various polychlorinated biphenyls. Appl. Environ. Microbiol. 38: 301–310

Haddock JD, Horton JR & Gibson DT (1995) Dihydroxylation and dechlorination of chlorinated biphenyls by purified biphenyl 2,3-dioxygenases from *Pseudomonas* sp. strain LB400. J. Bacteriol. 177: 20–26

Harkness MR, McDermott JB, Abramowicz DA, Salvo JJ, Flanagan WP, Stephens ML, Mondello FJ, May RJ, Lobos JH, Carroll KM, Bracco AA, Fish KM, Warner GL, Wilson PR, Dietrich DK, Lin DT, Morgan CB & Gately WL (1993) *In situ* stimulation of aerobic PCB biodegradation in Hudson River sediments. Science 259: 503–507

Ikan R (1991) Natural products: a laboratory guide. Academic Press, San Diego

Kirk TK (1984) Degradation of lignin. In: Gibson DT (Ed) Microbial Degradation of Organic Compounds. (pp 399–438). Marcel Dekker, New York

Koh SC, Bowman JP & Sayler GS (1993) Soluble methane monooxygenase production and trichloroethylene degradation by a type I methanotroph, *Methylomonas methanica* 68-1. Appl. Environ. Microbiol. 59: 960–967

Kohler H-PE, Kohler-Staub D & Focht DD (1988) Cometabolism of PCBs: enhanced transformation of Aroclor 1254 by growing bacterial cells. Appl. Environ. Microbiol. 54: 1940–1945

Lunt D & Evans WC (1970) The microbial metabolism of biphenyl. Biochem. J. 118: 54

Maniatis T, Firtsch EF & Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

McCullar MV, Brenner V, Adams RH & Focht DD (1994) Construction of a novel polychlorinated biphenyl-degrading bacterium: utilization of 3,4′-dichlorobiphenyl by *Pseudomonas acidovorans* M3GY. Appl. Environ. Microbiol. 60: 3833–3839

Newall CA, Anderson LA & Phillipson JD (1996) Herbal medicines: a guide for health-care professionals. The Pharmaceutical Press, London

Nies L & Vogel TM (1990) Effects of organic substrates on dechlorination of Aroclor 1242 in anaerobic sediments. Appl. Environ. Microbiol. 56: 2612–2617

Quensen JF III, Tiedje JM & Boyd SA (1988) Reductive dechlorination of polychlorinated biphenyls by anaerobic microorganisms from sediments. Science 242: 752–754

Taira K, Hirose J, Hayashida SK & Furukawa K (1992) Analysis of *bph* operon from the polychlorinated biphenyl-degrading strain of *Pseudomonas pseudoalcaligenes* KF707. J. Biol. Chem. 267: 4844–4853

Trudgill PW (1994) Microbial metabolism and transformation of selected monoterpenes. In: Ratledge C (Ed) Biochemistry of Microbial Degradation. (pp 33–62). Kluwer Academic Publishers, Dordrecht

Walia S, Khan A & Rosenthal N (1990) Construction and applications of DNA probes for detection of polychlorinated biphenyl-degrading genotypes in toxic organic-contaminated soil environments. Appl. Environ. Microbiol. 56: 254–259

Yates JR & Mondello FJ (1989) Sequence similarities in the genes encoding polychlorinated biphenyl degradation by *Pseudomonas* strain LB400 and *Alcaligenes eutrophus* H850. J. Bacteriol. 171: 1733–1735