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The degradation of biphenyl and chlorobiphenyls by mixed bacterial cultures

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1. SUMMARY

Pseudomonas sp. HV3 grows on naphthalene but not on biphenyl, as the sole source of carbon. When the cells of *Pseudomonas* sp. HV3 grown on naphthalene were shaken with biphenyl as the carbon source in a mineral salt solution, a yellow metabolite identified as the *meta*-cleavage product of biphenyl was excreted. The degradation of biphenyl stopped here, but was completed if either 2-methyl-4-chlorophenoxy acetic acid (MCPA)-degrading mixed culture or a *Nocardia* strain was added to the growth solution. Neither of these uses naphthalene or biphenyl as growth substrate. The mixed culture of *Pseudomonas* sp. HV3 and *Nocardia* sp. also degrades the commercial polychlorinated biphenyl (PCB) mixture Aroclor 1221. A yellow metabolite was likewise produced in the degradation, and sometimes two different peaks of the yellow metabolite were observed. The gas chromatography-mass spectrometry (GC-MS) analyses showed that 40–87% of Aroclor 1221 was degraded during an incubation time of 6–21 days. Chlorobenzoic acids were found as metabolites.

2. INTRODUCTION

PCBs have been manufactured commercially since 1929, with current production estimated at about two million tonnes. Although the use of PCBs is prohibited in many countries, their release into the environment continues and they are regarded as global contaminants. PCBs are produced by the direct chlorination of biphenyl and a large number of congeners are formed in the process. The complexity of the mixtures, which are found in low concentration in almost all environmental samples, places heavy demands on analytical and waste-handling procedures. Bacterial degradation is one of the major routes in the environmental breakdown of PCBs. A number of reports have shown that different bacteria isolated from PCB-contaminated sites are capable of degrading both single congeners and commercial mixtures of PCBs [1–6].

In this work we report the degradation of bi-

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phenyl by mixed bacterial cultures and the degradation of the commercial PCB mixture Aroclor 1221 by a co-culture consisting of a *Pseudomonas* sp. HV3 strain and a *Nocardia* sp. strain. These strains were not originally isolated as biphenyl- or chlorobiphenyl-degrading strains.

3. MATERIALS AND METHODS

3.1. Organisms

Pseudomonas sp. HV3, *Nocardia* sp. and the MCPA-degrading mixed culture 260 were isolated from field soil, obtained from the Agricultural Research Centre, Vantaa, Finland [7,8]. *Pseudomonas* sp. HV3 was isolated as a phenoxy herbicide MCPA- and dichlorobiphenyl (2,4-D)-degrading bacterium. Apart from growing on herbicides, it can grow on benzoic, *meta*- and *para*-toluic, 3-chloro- and 4-chlorobenzoic, salicylic, and 3-methyl- and 4-methylsalicylic acids and on naphthalene. *Pseudomonas* sp. HV3 possesses the enzymes for both catechol *ortho* and catechol *meta* cleavage of the aromatic ring [9].

Nocardia sp., first described by Trojanowski et al. [10], was isolated by Kilpi as a vanillic acid-degrading bacterium. Nocardia sp. grows well on many *meta* and *para* methoxy-substituted aromatic acids, e.g., vanillic, isovanillic, *meta*- and *para*-anis, veratrum, and ferulic acids and also on benzoic and *meta*-, *para*- and *ortho*-hydroxybenzoic acids (S. Kilpi, unpublished data).

The mixed culture 260 is derived from the mixed culture M1 [8]. This culture can grow on MCPA, 2,4-D and common aromatic compounds such as benzoate, *meta*-, *para*- and *ortho*-hydroxybenzoates and vanillate.

3.2. Chemicals

Aroclor 1221 (Monsanto Chemical Corp.) was obtained from OY Nokia AB. Other chemicals were purchased from commercial manufacturers.

The composition of Aroclor 1221 according to Albro and coworkers is presented in Table 1 [14].

Table 1

The composition of Aroclor 1221 (batch No. AM-25) [14] rounded to whole numbers by us

Congener	Mol%
Biphenyl (BP)	16
2-ChloroBP	32
3-ChloroBP	3
4-ChloroBP	19
2,2′-DichloroBP	5
2,4-DichloroBP	3
2,3′-DichloroBP	3
2,4′-DichloroBP	10
3,4-DichloroBP	1
3,4-DichloroBP	1
4,4′-DichloroBP	4
Total	97%

In addition to the congeners listed in the table, three congeners with 2, eight with 3, one with 4 and two with 5 chlorine substituents were detected, all of them having a mol% < 1.

Many tri- to pentachlorinated congeners were detected in our work as well, but these were disregarded since the mol% of the above-mentioned 11 congeners (giving 10 peaks in the chromatogram) totalled 97%. Small differences in the different batch numbers of Aroclor 1221 have been reported as in other commercial mixtures. Although the mixture used was of a different batch number than the one analyzed by Albro et al. [14], the composition they report was considered to be a reasonable basis for the calculation of total Aroclor 1221 breakdown.

3.3. Media and culture

The mineral salt solution described by Horvath and Alexander was used as growth medium [11]. The carbon source was added separately to the sterilized medium, naphthalene and biphenyl as crystals, and commercial PCB mixture (Aroclor 1221, chlorine content 21%) as a liquid 10 μl/50 ml or 4 μl/10 ml mineral salt solution, giving PCB concentrations of 237 and 474 μg/ml, respectively.

The cells were grown in a rotary shaker at room

temperature (25°C). Growth was monitored by measuring the turbidity of the growth solution with a Klett–Summerson colorimeter (filter 54) and by following the appearance and disappearance of the yellow metabolite (the degradation product of the *meta*-cleavage pathway) by measuring the absorption spectra in neutral, alkaline and acidic pH from 480 to 240 nm with a Varian Cary 219 spectrophotometer. In some experiments the cells were first cultivated on biphenyl and then their ability to degrade Aroclor 1221 was studied. The cells were grown in the mineral salts solution + biphenyl crystals for 2–3 days, after which the cell suspension was filtered through glass wool to remove remaining solid biphenyl, centrifuged at 4°C, washed once with the mineral salt solution and resuspended into a 10-ml volume of mineral salt solution.

Samples from different cultures were plated on minimal agar (minimal salt solution + 2% Bacto agar) to which Aroclor 1221 or biphenyl was added as the sole source of carbon. Aroclor and biphenyl were sprayed onto the uninoculated plates as diethyl ether solution (0.05%) and the plates were allowed to stand overnight at room temperature before inoculation [12]. Peptone yeast extract agar [13] was used as a rich medium.

3.4. Chemical analyses of Aroclor 1221 degradation

3.4.1. Sample preparation

After growth, the samples (the entire 50 ml incubation mixture) were acidified (pH 1–2) with concentrated HCl and extracted four times with 10 ml ethyl acetate [2]. The combined ethyl acetate fractions were evaporated in a rotary evaporator and the residue was suspended in 1.5 ml of ethyl acetate. The control was mineral salt solution + Aroclor 1221 + cells, which was shaken about 1 h at room temperature and then acidified with concentrated HCl.

3.4.2. Thin-layer chromatography

The plates (DC Plasticfolia Kieselgel F254 and DC Alufolia 60 F254) were activated for 2 h at 105°C and cooled in an desiccator. Toluene–dioxane–acetic acid or benzene–dioxane–acetic acid (90 : 20 : 4), or *n*-hexane–ethanol–acetic acid (5 : 2 : 1 or 10 : 2 : 1) was used as solvent, the running time was 1–2 h. Aroclor 1221, Aroclor 1242, biphenyl and monochloro- and dichlorobenzoic acids as 20 mM acetone solutions were used as references. The plates were investigated under UV light (254 nm).

3.4.3. Gas chromatography–mass spectrometry

A GC/MS system consisting of a Hewlett–Packard 5790 gas chromatograph, a Hewlett–Packard 5970A quadrupole mass selective detector and a Hewlett–Packard 9825B data station was employed in the analyses.

The ethyl acetate solution from section 3.4.1 was used for the determination of individual congeners as such. The column was 15 m, 0.20 mm i.d. FS capillary with a 5% phenylmethyl-silicone cross-linked stationary phase of 0.33-μm film thickness (Hewlett–Packard). Helium was the carrier gas (1 ml/min). Temperature program: 70°C (1 min), 70°–100°C progr. 42°C/min, 100–275°C progr. 8°C/min. Splitless injection, injector temperature 275°C. The following mass fragments were used for detecting Aroclor 1221 congeners: m/e 154 for biphenyl, m/e 188 and 152 for mono-, m/e 222 and 152 for di-, m/e 256 and 186 for tri-, m/e 292 for tetra-, and m/e 326 for pentachlorinated biphenyls.

For determination of the chlorobenzoic acid concentrations the ethyl acetate solution from section 3.4.1 was evaporated to dryness in nitrogen flow, the residue was dissolved in dry diethyl ether and the chlorobenzoic acids were methylated with diazomethane. The ether was again evaporated with nitrogen and the residue dissolved in heptane. The column was a 25 m, 0.20 mm i.d. FS capillary column with a BP20 stationary phase (similar to Carbowax 20 M) of 0.25 μm film thickness (Scientific Glass Engineering). Carrier gas, see above. Temperature program: 85°C (0.5 min), 85°–235°C progr. 20°C/min. Splitless injection, injector temperature 235°C. The following mass fragments were used for detecting the chlorobenzoic acids as methyl esters: m/e 139, m/e 111 and m/e 141 for mono-, and m/e 173, m/e 175 and m/e 145 for dichlorobenzoic acids.

All samples were run in duplicate.

4. RESULTS

4.1. Biphenyl-degrading mixed cultures

Pseudomonas sp. HV3 grows on naphthalene as the sole source of carbon, but cannot grow on biphenyl. When cells of *Pseudomonas* sp. HV3 grown on naphthalene were inoculated into a mineral salt solution containing biphenyl as the sole carbon source, a yellow metabolite was observed in the growth solution. This metabolite gave absorption spectra typical for the degradation product of biphenyl, with absorption maxima at 430 nm in neutral and alkaline pH and at 330 nm in acidic solution [15].

When *Pseudomonas* sp. HV3 was grown alone the degradation of biphenyl stopped with the appearance of the yellow metabolite. If the incubation was continued, the solution became red, perhaps due to polymerization reactions. The degradation of biphenyl was completed when *Pseudomonas* sp. HV3 was cultivated together with the phenoxy herbicide-degrading mixed culture 260 or with the *Nocardia* strain.

The *Nocardia* strain has the enzymes of the β-oxoadipate pathway, both the protocatechuate and the catechol branch, and the enzymes of the gentisate pathway (S. Kilpi, unpublished data). Neither culture 260 nor the *Nocardia* strain are able to grow on naphthalene or on biphenyl and they do not show any activity for the *meta*-cleavage pathway of *ortho*-diphenols.

Biphenyl was degraded rapidly by both mixed cultures. The turbidity of the growth solutions increased in a few days and the yellow metabolite disappeared. Biphenyl was degraded at 28°C as well as at room temperature (Fig. 1).

4.2. Aroclor 1221-degrading mixed culture

The ability of the two biphenyl-degrading mixed cultures to degrade commercial PCBs was studied with Aroclor 1221, Aroclor 1242 and Clophen A30. The growth experiments were done at 28°C and at room temperature in a rotary shaker. No growth occurred in the cultures where Aroclor 1242 or Clophen A30 was used as the carbon source. Only with the coculture *Pseudomonas* sp. HV3 + *Nocardia* sp. (*Ps. HV3* + *Noc*) and with Aroclor 1221 as the carbon source could growth be shown. The growth was slow at first but accelerated after continuous reinoculation into fresh medium. A yellow metabolite eventually appeared in the growth solution with an absorption maximum at 398 nm in neutral and alkaline pH and at 330 nm in acidic pH. Furukawa and Matsumura [2] found absorption maxima for PCB degradation products between 398 and 415 nm in neutral and alkaline pH, and Liu [16] found an absorption maximum at 395 nm for Aroclor 1221 degradation products in neutral and alkaline pH.

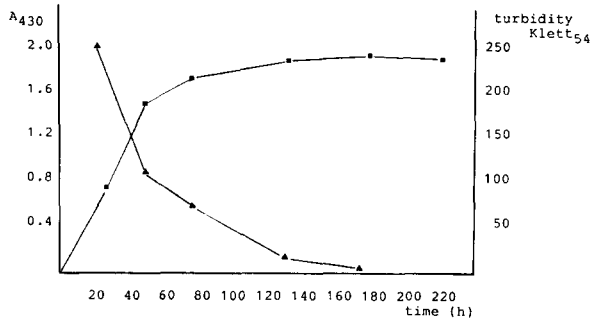

Fig. 1. Degradation of biphenyl by the mixed culture *Ps.HV3* ± *Noc*. The cells were grown at room temperature in mineral salts solution, with biphenyl crystals as the sole carbon source. ■, Growth of bacteria; ▲, disappearance of the yellow metabolite A_{430nm} (pH 12). The absorbance was measured from the supernatant of the growth solution, which was diluted 1 : 5.

It was interesting to note the appearance of a yellow metabolite at two different times during some incubations of the co-culture *Ps.HV3* + *Noc* with Aroclor 1221. The first peak appeared after 4–7 days of incubation and the second after 9–12 days. Aroclor 1221 consists mainly of mono- and dichlorobiphenyls (approx. 81%) (Table 1) and the two peaks may have been due to these two types of chlorobiphenyl congeners degrading at different times (Fig. 2).

Samples from cultures of different age were plated on agar containing Aroclor 1221 or biphenyl. According to Sylvestre [12], bacteria able to grow on biphenyl or chlorobiphenyl agar will form a clear zone around the colony, providing a useful way of screening for chlorobiphenyl- or biphenyl-degrading bacteria in natural sources.

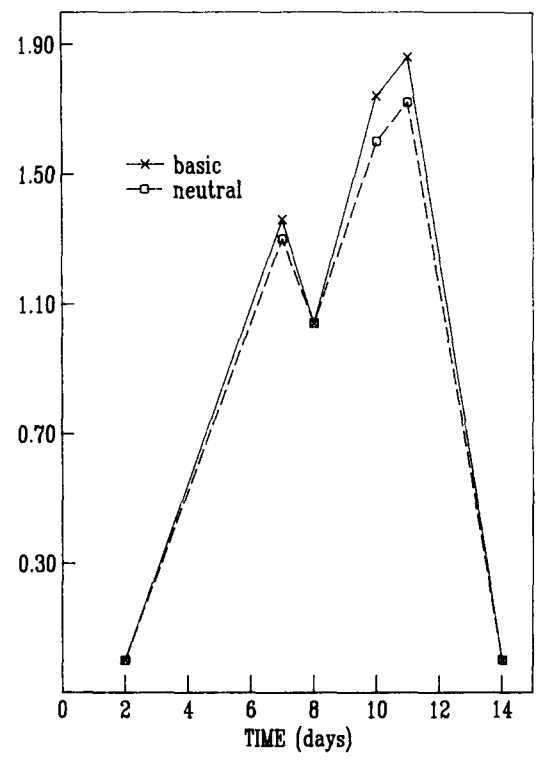

Fig. 2. Appearance and disappearance of the yellow metabolite (A_{396nm}) in the degradation of Aroclor 1221 by the mixed culture *Ps.HV3* + *Noc*. The absorbance was measured from the growth solution at 480–260 nm.

When this method was used with our co-culture no clear zones formed around the colonies, but the surfaces of the plates turned yellow and only later did small colonies appear on the plates. The yellow colour first appeared on the plates of smallest dilution. The greatest dilutions did not cause a change in colour. The cell numbers were 10^6–10^7 cell/ml.

Different colonies were picked up from the agar plates and their ability to grow in Aroclor + mineral salt solution was tested, however, single colonies with the ability to grow on Aroclor + mineral salts solution were not found. When bacteria (not single colonies) from a yellow plate were inoculated into an Aroclor 1221 mineral salt solution, the culture began to grow.

4.3. Aroclor 1221 degradation

Some cultures were extracted as described in MATERIALS AND METHODS and the extraction products were analyzed by thin-layer chromatography (TLC). The results showed that Aroclor 1221 had clearly diminished in the test samples compared with the control sample. Some metabolites could be seen but only in small amounts.

Samples from two different Aroclor 1221 degradation experiments were subjected to GC/MS analysis. In the experiments AI and AII the cells were grown in a mineral salt solution containing 237 mg/kg of Aroclor 1221 for 21 days. Cell density was approx. 10^7 cell/ml. In the experiments BI and BII the cells were first cultivated in biphenyl + mineral salt solution for 3 days, centrifuged and diluted to a cell density of 10^8 cell/ml and then incubated in mineral salt medium with Aroclor 1221 (474 mg/kg) for 6–8 days.

The results (Fig. 3, A and B) showed a clear connection between the degradability and chlorine content. Biphenyl was degraded most rapidly and among the chlorinated congeners the degradation rate was highest for 2-chlorobiphenyl. 3-Chloro- and 4-chlorobiphenyl were degraded at a slightly lower rate, but with no significant difference in degradation rate between them.

The primary degradation was greater in experiments AI and AII, where the incubation time was longer and Aroclor 1221 concentration smaller than in experiments BI and BII. The total de-

Table 2

Chlorobenzoic acids (μg/ml) found as metabolites in the degradation of Aroclor 1221 by the mixed culture *Ps.HV3* + *Noc*

	AI	AII	BI	BII
2-Chlorobenzoic acid	17	8	8.6	0.8
3-Chlorobenzoic acid	0.7	0.3	0.6	0.1
4-Chlorobenzoic acid				
2,4-Dichlorobenzoic acid	0.2	0.1	0.1	0.03
2,5-Dichlorobenzoic acid	0.1	0.02	0.2	0.1
2,6-Dichlorobenzoic acid	ND	ND	ND	ND
3,4-Dichlorobenzoic acid	0.5	0.3	0.1	0.04
3,5-Dichlorobenzoic acid	ND	ND	ND	ND

ND, not detected.

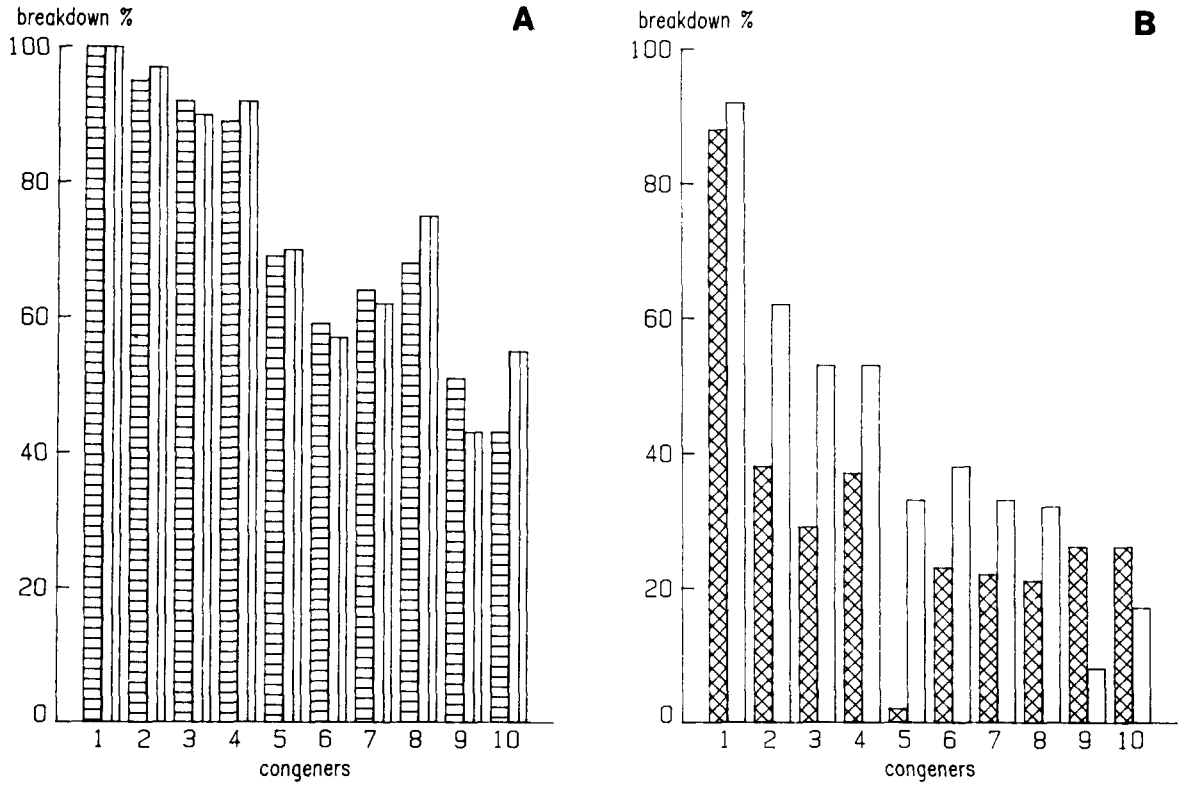

Fig. 3. Degradation of Aroclor 1221 by the mixed culture *Ps.HV3* + *Noc.* Horizontally striped bars and vertically striped bars, AI and AII cells, respectively, grown on Aroclor 1221 at 25 °C for 21 days. Crosshatched and open bars, BI and BII cells, respectively, grown on biphenyl and then on Aroclor 1221; BI—6 days, BII—8 days (see the text). Aroclor 1221 congeners are: 1, biphenyl (BP); 2, 2-chloroBP; 3, 3-chloroBP; 4, 4-chloroBP; 5, 2,2′-dichloroBP; 6, 2,4-dichloroBP; 7, 2,3′-dichloroBP; 8, 2,4′-dichloroBP; 9, 3,4- and 3,4′-dichloroBP; 10, 4,4′-dichloroBP.

gradation in experiments AI and AII was circa 87% and in experiments BI and BII only 40%.

The cultures grown on Aroclor 1221 were also analyzed for chlorobenzoic acids, which are known to be metabolites in the degradation of PCBs [4,17,18], small amounts were found (Table 2), corresponding in composition to the original chlorobiphenyl congeners of Aroclor 1221 (Table 2; Fig. 3).

5. DISCUSSION

Commercial PCB mixtures and their individual congeners are degraded by certain bacterial mixed cultures and pure cultures isolated from natural sources [1–6]. The chlorobenzoate pathway, which has been extensively studied by Furukawa and co-workers [4,17,18] seems to be a major metabolic route in this degradation.

Bacterial metabolism of PCB is considered to proceed via a dioxygenase-catalyzed reaction in the same way as with other aromatic compounds, e.g., benzene, benzoic acid and naphthalene [19–21]. The initial step in the degradation of PCB is presumably similar to the degradation of naphthalene by *Pseudomonas* [21–23] and biphenyl by a *Beijerinckia* strain [15]: two atoms of molecular oxygen are incorporated at the 2,3 position of the less chlorinated ring of PCB congeners to form *cis*-dihydrodiol compounds (Fig. 4). The dihydrodiols are then dehydrogenated to yield 2,3-dihydroxy compounds. The *meta* cleavage should occur between the C1 and C2 atoms of the dihy-

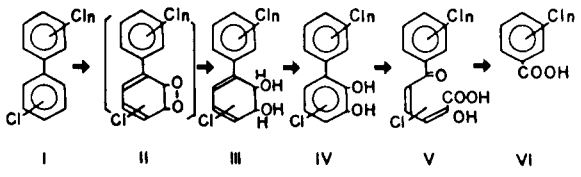

Fig. 4. Proposed major pathways for metabolism of PCB by *Alcaligenes* sp.Y42 and *Acinetobacter* sp.P6. n = 1 to 4 [18].

droxy compound. The yellow *meta*-cleavage metabolites of many PCB congeners are usually rapidly converted to chlorobenzoic acids [4]. The degradation of Aroclor 1221 by our co-culture *Ps.HV3* + *Noc* likewise seems to proceed via the chlorobenzoate pathway; for the characteristic yellow metabolite was observed and chlorobenzoic acids were found as metabolites. Since the *Nocardia* strain cannot grow on chloroaromatic compounds and has no *meta*-fission enzymes, it seems probable that *Pseudomonas* sp. HV3 is a more active component in our co-culture *Ps.HV3* ± *Noc*, at least at the start of the degradation. The *Pseudomonas* strain can grow on naphthalene and the cells grown on naphthalene produce a yellow metabolite (the ring-fission product) from biphenyl. The enzymes of *Pseudomonas* sp.HV3 that have been studied, the catechol dioxygenases [9] and the enzymes oxygenating benzoates and salicylates (not shown), have displayed a low substrate specificity. Thus it may be that the enzyme system active in the degradation of naphthalene also acts in the degradation of biphenyl and chlorobiphenyls, which are structural analogues of naphthalene.

The role of the *Nocardia* strain in this degradation is not yet clear. Perhaps it effects the degradation of the yellow compound to the chlorobenzoic acids, because the degradation of Aroclor 1221 by *Pseudomonas* sp. HV3 alone stopped at this stage.

The Aroclor degradation was studied in two different experiments. In experiments AI and AII the bacteria were grown directly in Aroclor 1221, whereas in experiments BI and BII they were first grown on biphenyl to increase the number of cells, and then incubated with Aroclor 1221. The cell density, Aroclor concentration and incubation times were also different in the two sets of experiments so that they cannot directly be compared. At least the degradation of PCBs could in all cases be shown. The degradation of Aroclor 1221 by this co-culture is noteworthy in that neither of the constituent strains had been isolated as biphenyl or chlorobiphenyl-degrading strains. Nevertheless, these two bacteria together possess the necessary enzyme capacities for this kind of degradation. We are now continuing the experiment by attempting to adapt the co-culture to more highly chlorinated Aroclor mixtures.

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