CA/
iety for
holds a bachelor's
represented by a
for subscription to
eight scientific
ates, $21 of the
iety publishes the
of microbiologists.
to the contrary,
back issues of the
1 November will
le
Sex
$7.00
B
$30
$18
$21
$15
$26
$18
$9
$25

NOV 24 1978
PCB
PCB

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1978, p. 223-227
0099-2240/78/0035-0223$02.00/0
Copyright © 1978 American Society for Microbiology
Vol. 35, No. 2
Printed in U.S.A.

Effect of Chlorine Substitution on the Biodegradability of Polychlorinated Biphenyls

KENSUKE FURUKAWA,* KENZO TONOMURA, AND AKIRA KAMIBAYASHI
Fermentation Research Institute, Agency of Industrial Science and Technology, Inage, Chiba, Japan

Received for publication 3 August 1977

NOTICE: This material may be protected by copyright

Thirty-one isomers of polychlorinated biphenyl (PCB) were examined for biodegradability by two species of *Alcaligenes* and *Acinetobacter*. The following relationships between chlorine substitution and biodegradability of PCBs were observed. (i) Degradation decreased as chlorine substitution increased. PCB isomers containing more than four chlorines were less susceptible to degradation. (ii) PCBs containing two chlorines on either the *ortho* position of a single ring (i.e., 2,6-) or on both rings (i.e., 2,2'-) showed very poor degradability. (iii) PCBs containing all chlorine atoms on only a single ring were generally degraded faster than when the same number of chlorines were substituted on both rings. (iv) Preferential ring fission of the molecules occurred with nonchlorinated or lesser chlorinated rings. (v) The formation and accumulation of a yellow intermediate was always observed in 4'-chloro-substituted PCBs. (vi) Significant differences between the two organisms with respect to degradability were not observed except for 2,4,6-trichlorobiphenyl.

The widespread distribution of polychlorinated biphenyl (PCB) compounds in the environment is now well documented throughout the world (5-8, 12). The biodegradability of PCBs is of primary importance to minimize their impact on the biota. Chlorinated biphenyls can theoretically represent as many as 210 different compounds containing from 0 to 10 chlorine atoms per biphenyl molecule. Of the possible isomers, 103 are considered most probable (13). Ahmed and Focht (1) studied the degradation of several mono- and dichlorobiphenyls by two species of *Achromobacter* and found that chlorinated benzoic acids formed as a result of oxidative degradation. Murado et al. (9) observed interaction between PCBs and soil fungi. No metabolic changes in PCBs have been observed, but the presence of PCBs in liquid culture depressed the growth of *Aspergillus flavus*. Tucker et al. (12) indicated that commercial PCB mixtures containing predominantly mono- and dichlorobiphenyls readily underwent primary biodegradation by activated-sludge microorganisms. Some studies have been conducted on DDT structure versus biodegradation. Focht and Alexander (2) reported that substituents on the methylene-carbon and *para*-chloro substitution are critical factors governing resistance of DDT and related compounds to aerobic metabolism and decomposition by a *Hydrogenomonas* sp. Pfaender and Alexander (10) reported that pure cultures of bacteria decompose DDT extensively only through a cometabolic process. Francis et al. (3) demonstrated that a *Pseudomonas* sp. rapidly metabolized several nonchlorinated analogs of DDT and several monochloro-substituted biphenyl analogs as the sole carbon source. However, the isolate was unable to metabolize 4,4'-dichlorobiphenyl analogs as the sole carbon source.

In a previous paper (4), we indicated that an *Alcaligenes* sp., strain Y42, isolated from an aquatic sediment sample was capable of cometabolizing a variety of PCB compounds. The primary degradation of various PCB isomers was tentatively observed. However, data were lacking to assess the relationship between chlorine substitution and biodegradability. In this study, we attempted to investigate in detail the effect of chlorine substitution on the biodegradability of PCBs. Thirty-one pure isomers of PCB and two bacterial species of *Alcaligenes* and *Acinetobacter* were used in the present study.

MATERIALS AND METHODS

Microorganisms and cultivation. *Alcaligenes* sp. strain Y42 and *Acinetobacter* sp. strain P6 were used in all experiments. Taxonomic studies and their microbial properties have been reported separately (K. Furukawa, F. Matsumura, and K. Tonomura, Agric. Biol. Chem., in press). Cultivation was carried out in the following mineral medium supplemented with biphenyl (well ground, 1 g) for *Alcaligenes* Y42 and 4-chlorobiphenyl (1 g) for *Acinetobacter* P6 as the sole carbon source: $(NH_4)_2SO_4$, 1 g; KH_2PO_4, 0.2 g; K_2HPO_4, 1.6 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $CaCl_2 \cdot 2H_2O$, 0.02 g; deionized water, 1 liter (pH 7.5).

MONS 001253

Incubation and gas chromatographic determination of PCBs. The organisms were grown for 3 to 4 days, and the culture fluid was filtered once to remove remaining solid biphenyl or 4-chlorobiphenyl. The cells were then harvested by centrifugation and washed twice in 0.05 M phosphate buffer (pH 7.5). The washed cells were resuspended in 10 ml of phosphate buffer to which 0.5 μmol of pure isomers of PCB (93 μg for mono-, 111 μg for di-, 128 μg for tri-, 146 μg for tetra-, and 163 μg for pentachlorobiphenyl) was added. The final optical density of the cells was adjusted to 1.0 at 660 nm. Four flasks were prepared for each compound. One of them was used as a control to obtain a recovery percentage of extraction: each PCB isomer dissolved in ethanol was added in the cell suspension, and the whole mixture was extracted immediately with 10 ml of ethylacetate after acidification to pH 1 to 2 with concentrated HCl. The extraction recovery of various PCBs was 88 to 96%. The other three flasks were shaken on a rotary shaker for predetermined periods and then extracted in the same manner as the control. The ethylacetate layer was removed, and the amount of parent compound was determined by gas chromatograph on a Hitachi model 163 equipped with a ^{63}Ni electron-capture detector. A glass column (1 m by 3 mm ID) containing silicone DC 11 (at 5% on Uniport B, 60 to 80 mesh) was used with injector and detector temperatures of 190 to 240°C, and the nitrogen gas flow rate was 35 ml/min.

Gas-liquid chromatography-mass spectrometry. Ethylacetate extracts obtained from incubation mixtures were evaporated once under a gentle stream of nitrogen gas and dissolved in a small amount of ethylacetate. Bis trimethylsilyl acetamide was added to form the trimethylsilyl derivatives of certain PCB metabolites such as chlorobenzoic acids. The samples were analyzed using a gas-liquid chromatograph-mass spectrometer (JEOL Ltd., model JMS D300) with a coiled glass column (1 m by 4 mm ID) packed with silicone OV1 (at 2% on 80 to 100 mesh Chromosorb G). Helium was used as a carrier gas at a flow rate of 20 ml/min. The mass spectra were measured at 20-eV ionization potential, 300-μA trap current, and 200°C ion source temperature.

Spectrophotometric measurements. A spectrophotometer (Hitachi model 624) was used for spectrophotometric measurements.

Chemicals. 4-Chlorobiphenyl was purchased from Aldrich Chemical Co. All other pure isomers of PCB (>98% purity) were obtained from Analabs, Inc. Bis-trimethylsilyl acetamide was obtained from Tokyo Chemical Industry Co.

RESULTS

Prior to examining the biodegradability of PCBs by *Alcaligenes* Y42 and *Acinetobacter* P6, the effect of cell concentration upon ring fission was observed by using 2,5,4'-trichlorobiphenyl. As described in our previous paper (4), *Alcaligenes* Y42 was capable of producing a yellow intermediate (λ_{max} = 398 nm) from 2,5,4'-trichlorobiphenyl. This compound was probably a *meta* cleavage product. *Acinetobacter* P6 also produced and accumulated a similar yellow intermediate with this substrate. After incubation for 1 h, absorption of the supernatant fluid at 398 nm was measured at several different cell concentrations of the two organisms (Fig. 1). Subsequent degradation tests for all isomers of PCB were performed at a cell concentration of 1.0 optical density unit (660 nm), as indicated by the arrow in Fig. 1. The cell numbers at the point were 2.0×10^8/ml for *Alcaligenes* Y42 and 4.4×10^8/ml for *Acinetobacter* P6 as counted using a Thoma hemacytometer. The optimum pH values were 7 to 9 for *Alcaligenes* Y42 and 7 to 8 for *Acinetobacter* P6. Phosphate buffer of pH 7.5 was therefore used for all degradation studies.

The biodegradation results of various PCB isomers are summarized in Table 1. Monochlorobiphenyls (group A) were degraded very rapidly by both organisms, and no parent compound was observed after 1 h of incubation, so that the degradation rate was expressed as >50 nmol/ml per h. Dichlorobiphenyls with two chlorines substituted on a single ring (group B) were generally degraded rapidly at over 50 nmol/ml per h in both organisms except for 2,6-dichlorobiphenyl. This compound was extremely refractile and hardly degraded even after 4 h of incubation. Dichlorobiphenyl with one chlorine substituted on each ring (group C) was well degraded, although the degradation rate was slow compared with the dichlorobi-

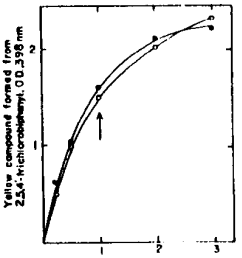
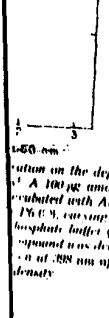


FIG. 1. *Effect of cell concentration on the degradation of 2,5,4'-trichlorobiphenyl. A 100-μg amount of 2,5,4'-trichlorobiphenyl was incubated with Alcaligenes Y42 (●) or Acinetobacter P6 (○), carrying the amount of the cells in 10 ml of phosphate buffer (pH 7.5). The formation of a yellow compound was determined by measuring the absorption at 398 nm after incubation for 1 h. O.D., Optical density.*

MONS 001254

and *Acinetobacter* P6 also
ulated a similar yellow in-
substrate. After incubation
d the supernatant fluid at
d at several different cell
two organisms (Fig. 1).
ion tests for all isomers of
at a cell concentration of
at (660 nm), as indicated
The cell numbers at the
ml for *Alcaligenes* Y42
or *Acinetobacter* P6 as
ma hemacytometer. The
ge 7 to 9 for *Alcaligenes*
cinetobacter P6. Phosphate
erefore used for all deg-

results of various PCB
d in Table 1. Monochlo-
were degraded very rap-
nd no parent compound
of incubation, so that
was expressed as >50
robiphenyls with two
a single ring (group B)
d rapidly at over 50
ganisms except for 2,6-
compound was ex-
dardly degraded even
Dichlorobiphenyl with
on each ring (group C)
ough the degradation
with the dichlorobi-



1-56 nm

ation on the degra-
A 100-μg amount
ubated with Alca-
P6 (0.9, carrying the
hosphate buffer (pH
mpound was deter-
n at 388 nm after
density

TABLE 1. *Biodegradability of a variety of PCB isomers by Alcaligenes Y42 and Acinetobacter P6*

Isomer group	PCB chlorine position[A]	Degradation rate (nmol/ml per h)[B]	
		Alcaligenes Y42[C]	Acinetobacter P6[C]
A	2-	>50	>50
	3-	>50	>50
	4	>50	>50
B	2,3	>50	46.4
	2,4	>50	>50
	2,5-	>50	>50
	2,6-	0	4.1
	3,4-	>50	44.1
	3,5	>50	48.2
C	2,2'-	6.3	14.0
	2,4'-	48.2	49.1
	3,3'	--	18.5
	4,4'-	16.2	25.2
D	2,3,4-	35.1	32.0
	2,3,6-	0	0
	2,4,5-	46.0	32.4
	2,4,6-	3.1	46.0
E	2,5,2'-	1.6	5.1
	2,5,3'	42.1	41.3
	2,5,4'-	21.8	30.4
	2,4,4'	41.3	40.2
	3,4,2'-	15.6	38.6
F	2,3,4,5-	25.8	19.0
	2,3,5,6-	0	0
G	2,3,2'3'-	8.7	7.3
	2,4,2'4'-	0	0
	2,4,3'4'-	0	0
	2,5,2',5'-	0	3.5
	2,6,2',6'-	1.4	0
	3,4,3',4'-	0	0
H	2,4,5,2',5'-	0.6	0

[A] Degradation rate was calculated after 1 h of incubation for mono-, di-, and trichlorobiphenyls and after 2 h of incubation for tetra- and pentachlorobiphenyls.

[B] A 500-nmol amount of each compound dissolved in ethanol (10 mM each) was suspended in 10 ml of phosphate buffer (pH 7.5).

[C] The cell number was 2×10^8/ml in *Alcaligenes* Y42 and 4.4×10^8/ml in *Acinetobacter* P6.

phenyls of group B; 2,2'-dichlorobiphenyl was especially refractile in both organisms. Trichlorobiphenyls with three chlorines substituted on a single ring (group D) showed different extents of degradation in both organisms. The time course of degradation in this group is presented in Fig. 2. 2,3,4- and 2,4,5-trichlorobiphenyls were easily degraded in 1 h, whereas both organisms failed to degrade 2,3,6-trichlorobiphenyl even after 4 h. There was a marked difference in 2,4,6-trichlorobiphenyl metabolism by the two organisms. The compound was mostly metabolized in 1 h by *Acinetobacter* P6; however, it was degraded very slowly by *Alcaligenes* Y42.

Trichlorobiphenyls with two chlorines substituted on one ring and one chlorine substituted on another ring (group E) were generally well degraded (Fig. 3). 2,5,2'-Trichlorobiphenyl was the exception in this group. *Alcaligenes* Y42, especially, degraded this compound very slowly. Difference in biodegradability of tetrachlorobiphenyl with four chlorines substituted on one ring (group F) was striking. 2,3,4,5-Tetrachlorobiphenyl was degraded rapidly at the rate of 25.8 nmol/ml per h in *Alcaligenes* Y42 and at 19.0 nmol/ml per h in *Acinetobacter* P6. In contrast, 2,3,5,6-tetrachlorobiphenyl was never degraded even after 20 h of incubation with either organism. The difference of degradability

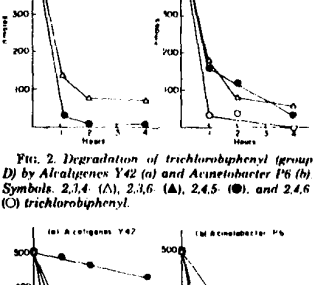


FIG. 2. *Degradation of trichlorobiphenyl (group D) by Alcaligenes Y42 (a) and Acinetobacter P6 (b). Symbols. 2,3,4- (Δ), 2,3,6- (▲), 2,4,5- (●), and 2,4,6- (○) trichlorobiphenyl.*

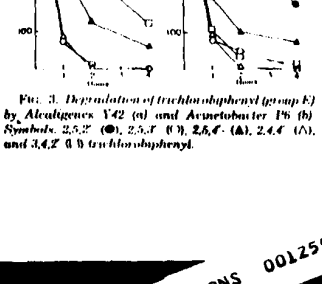


FIG. 3. *Degradation of trichlorobiphenyl (group E) by Alcaligenes Y42 (a) and Acinetobacter P6 (b). Symbols. 2,5,2'- (●), 2,5,3'- (○), 2,5,4'- (▲), 2,4,4'- (Δ), and 3,4,2'- (□) trichlorobiphenyl.*

MONS 001255

between these tetrachloro-compounds appears due to the effects of one chlorine substituted at the 4- or 6-position. Tetrachlorobiphenyls with two chlorines substituted on each ring (group G) generally showed very poor degradability. 2,3,2',3'-Tetrachlorobiphenyl, however, was well degraded at the rate of 8.6 nmol/ml per h with *Alcaligenes* Y42 and 7.2 nmol/ml per h with *Acinetobacter* P6. 2,4,5,2',5'-Pentachlorobiphenyl (group H) was hardly attacked by either organism.

Yellow-colored intermediates were observed from 2,4'-, 4,4'-, 2,5-, 2,5,4'-, 2,4,4'-, 2,4,5-, and 2,3,4,5-chlorobiphenyls metabolism by both organisms, and also from 2,3-, 2,4-, 3,5,-, 3,4,2'-, and 2,3,4-chlorobiphenyls metabolism by *Alcaligenes* Y42. The yellow compounds derived from 2,4'-, 4,4'-, 2,5,4'-, and 2,4,4'-chlorobiphenyls were very stable and accumulated in the reaction mixture for a long period, whereas the yellow compounds from the other chlorinated biphenyls were unstable and the color faded gradually upon further incubation. The formation of chlorinated benzoic acid from each PCB isomer was confirmed easily by isotope peaks of chlorine atoms in their mass spectra. Trimethylsilyl derivatives of monochlorobenzoic acids (M^+, *m/e* 228; M^+-CH_3, *m/e* 213, as a base peak) were detected in the metabolites from 2-, 3-, 4-, 2,2'-, 2,4'-, 3,3'-, and 4,4'-chlorobiphenyls. Dichlorobenzoic acid derivatives (M^+, *m/e* 262; M^+-CH_3, *m/e* 247, as a base peak) were detected in the metabolites from 2,3-, 2,4-, 2,5-, 3,4-, 3,5-, 2,5,2'-, 2,5,3'-, 2,5,4'-, 2,4,4'-, 3,4,2'-, and 2,3,2'3'-chlorobiphenyls. Trichlorobenzoic acid derivatives (M^+, *m/e* 296; M^+-CH_3, *m/e* 281, as a base peak) were detected in the metabolites from 2,3,4- and 2,4,5-trichlorobiphenyls. However, this compound was not observed in the metabolite from 2,4,6-trichlorobiphenyl in spite of complete disappearance of the parent compound in *Acinetobacter* P6. A tetrachlorobenzoic acid derivative (M^+, *m/e* 330; M^+-CH_3, *m/e* 317, as a base peak) was detected in the metabolites from 2,3,4,5-tetrachlorobiphenyl.

DISCUSSION

From the results obtained in this experiment, the relationship between structure of PCBs and their biodegradability can be illustrated by the following points. (i) Degradation decreased as chlorine substitution increased. The resistance of the more highly chlorinated atoms per molecule, especially more than four chlorines, may explain their detection as residues in environmental samples (5, 8, 12). The resistance of tetrasubstituted PCBs may be explained by difficulty in ring hydroxylation due to the two positions of each ring being occupied by two chlorine atoms. (ii) PCBs containing two chlorines in the *ortho* positions of a single ring (i.e., 2,6-) and in each ring (i.e., 2,2'-) showed striking resistance to degradation, as illustrated in studies with 2,6-, 2,2'-, 2,3,6-, 2,5,2'-, and 2,3,5,6-chlorobiphenyls. 2,4,6-Trichlorobiphenyl was the exception in this series of PCBs. This compound was rapidly metabolized by *Acinetobacter* P6, although trichlorobenzoic acid was not detected by gas-liquid chromatography-mass spectrometric analysis, unlike what was observed with 2,3,4- and 2,4,5-trichlorobiphenyls, in which these metabolites could be readily demonstrated. On the other hand, *Alcaligenes* Y42 hardly metabolized this compound. (iii) PCBs containing all chlorine atoms on a single ring were generally degraded faster than those containing the same number on both rings. (iv) Preferential ring fission of molecules occurred with monochlorinated or lesser chlorinated rings; monochlorobenzoic acids were detected from monochlorobiphenyls and group C dichlorobiphenyls; dichlorobenzoic acids were detected from group B dichlorobiphenyls, group E trichlorobiphenyls, and group G tetrachlorobiphenyl; trichlorobenzoic acids were detected from group D trichlorobiphenyls; tetrachlorobenzoic acid was detected from 2,3,4,5-tetrachlorobiphenyl of group F. The formation and stable accumulation of the yellow intermediates was always observed from 2,4'-, 4,4'-, 2,4,4'-, and 2,5,4'-chlorobiphenyls. It should be noted that the compounds of this series have 4'-chlorophenyl in common. (vi) Significant metabolic differences between *Alcaligenes* Y42 and *Acinetobacter* P6 were not observed except for 2,4,6-trichlorobiphenyl. However, the degradation activity per single cell was greater in *Acinetobacter* P6 than in *Alcaligenes* Y42, since the number of cells used for the latter was 4.5 times higher than that of the former.

Tucker et al. (12) demonstrated that commercial PCB mixtures, which contain predominantly mono- and dichlorobiphenyls, readily underwent primary biodegradation by activated-sludge microorganisms, and that as the levels of tri-, tetra-, and pentachlorobiphenyl increased, the biodegradation rates decreased accordingly. These results were generally in good agreement with our data; however, it should be pointed out that 2,6-di- and 2,3,6-trichlorobiphenyls, for example, were hardly attacked in spite of less chlorination. Wendham et al. (14) reported that a lower chlorinated isomer (4-chlorobiphenyl) was highly mutagenic to *Salmonella typhimurium*, whereas higher chlorinated PCBs were only weakly mutagenic. The ecological effect of PCBs has become a matter of

MONS 001256

al by two chlorine
two chlorines in the
ing (i.e., 2,6-) and in
l striking resistance
ed in studies with
and 2,3,5,6-chlorobi-
enyl was the excep-
This compound was
inetobacter P6, al-
d was not detected
y-mass spectromet-
observed with 2,3,4-
in which these me-
monstrated. On the
hardly metabolized
staining all chlorine
generally degraded
the same number
tial ring fission of
monochlorinated or
monochlorobenzoic
monochlorobiphenyls
bis dichlorobenzoic
roup B dichlorobi-
phenyls, and group
hlorobenzoic acids
trichlorobiphenyls;
as detected from
group F. The for-
tion of the yellow
observed from 2,4'-,
biphenyls. It should
of this series have
vi) Significant met-
caligenes Y42 and
observed except for
ever, the degrada-
was greater in *Aci-*
igenes Y42, since
the latter was 4.5
former.
trated that com-
h contain predomi-
phenyls, readily un-
ation by activated-
that as the levels
hlorobiphenyl in-
ates decreased ac-
generally in good
wever, it should be
d 2,3,6-trichlorobi-
ardly attacked in
udham et al. (14)
ed isomer (4-chlo-
genic to *Salmo-*
higher chlorinated
genic. The ecolog-
ome a matter of

increased concern. Knowledge on transformations of PCBs, including microbial degradation, should be accumulated to better interpret their influences on the living organisms. Work is presently under way to reveal the pathway(s) of metabolism of individual PCB isomers by *Alcaligenes* Y42 and *Acinetobacter* P6.

LITERATURE CITED

1. **Ahmed, A., and D. D. Focht.** 1973. Degradation of polychlorinated biphenyls by two species of *Achromobacter*. Can. J. Microbiol. **19**:47-52.
2. **Focht, D. D., and M. Alexander.** 1970. DDT metabolites and analogs: ring fission by *Hydrogenomonas*. Science **170**:91-92.
3. **Francis, A. J., G. I. Ouchi, R. Bramhall, and N. Bohonos.** 1976. Metabolism of DDT analogues by a *Pseudomonas* sp. Appl. Environ. Microbiol. **32**:213-210.
4. **Furukawa, K., and F. Matsumura.** 1976. Microbial metabolism of polychlorinated biphenyls. Studies on the relative degradability of polychlorinated biphenyl components by *Alcaligenes* sp. J. Agric. Food. Chem. **24**:252-256.
5. **Greichus, Y. A., J. J. Worman, M. A. Pearson, and D. J. Call.** 1974. Analyses of polychlorinated biphenyls in bird tissues and Aroclor standards with gas chromatography and mass spectrometry. Bull. Environ. Contam. Toxicol. **11**:113-120.
6. **Hiraizumi, Y., T. Manabe, M. Takahashi, K. Nishida, H. Joh, and H. Nishimura.** 1975. Analysis of PCBs contamination of sediment in the coastal waters of the Seto Inland Sea. La Mer **13**:163-170.
7. **Holden, A. V., and K. Marsden.** 1967. Organochlorine pesticides in seals and porpoises. Nature (London) **216**:1274-1276.
8. **Koeman, J. H., M. C. Ten Noever De Brauw, and R. H. De Vos.** 1969. Chlorinated biphenyls in fish, mussels and birds from the River Rhine and the Netherlands coastal area. Nature (London) **221**:1126-1128.
9. **Murado, M. A., M. C. Tejedor, and G. Baluja.** 1976. Interactions between polychlorinated biphenyls (PCBs) and soil microfungi. Effect of Aroclor-1254 and other PCBs on *Aspergillus flavus* cultures. Bull. Environ. Contam. Toxicol. **15**:768-774.
10. **Pfaender, F., and M. Alexander.** 1972. Extensive microbial degradation of DDT *in vitro* and DDT metabolism by natural communities. J. Agric. Food. Chem. **20**:842-846.
11. **Schmidt, T. T., R. W. Risebrough, and F. Gress.** 1971. Input of polychlorinated biphenyls into California coastal waters from urban sewage outfalls. Bull. Environ. Contam. Toxicol. **6**:235-243.
12. **Tucker, E. S., V. W. Saeger, and O. Hicks.** 1975. Activated sludge primary biodegradation of polychlorinated biphenyls. Bull. Environ. Contam. Toxicol. **14**:705-713.
13. **Widmark, G.** 1968. The OECD study of analysis of PCB. Report from the Institute of Analytical Chemistry, Stockholm.
14. **Wyndham, C., J. Devenish, and S. Safe.** 1976. The *in vitro* metabolism, macromolecular binding and bacterial mutagenicity of 4-chlorobiphenyl, a model PCB substrate. Res. Commun. Chem. Pathol. Pharmacol. **15**:564-570.

MONS 001257