CHAPTER 25

Inhibition of Estuarine Microorganisms by Polychlorinated Biphenyls*

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Over 100 isolates of representative estuarine bacteria and fungi were screened for their ability to grow in the presence of commercial preparations of polychlorinated biphenyls (PCB). Super absorbant sensitivity discs impregnated with up to 0.5 mg of PCB were placed on the surface of freshly inoculated solid media. Twenty-six bacteria, representing both gram-positive and gram-negative strains of varying morphology, showed varying degrees of sensitivity to PCB. In contrast to insensitive isolates, sensitive strains were mainly amylolytic and proteolytic. PCB had negligible effect on the growth of fungi. The sensitivity of select cultures of heterotrophic bacteria to PCB may be of considerable importance to nutrient turnover in estuarine ecosystems.

INTRODUCTION

Polychlorinated biphenyl formulations (PCB's) are chemically and thermally stable and possess high dielectric constants. Because of these properties, PCB's have been important commercially as coolant-insulation fluids in capacitors and transformers, hydraulic fluids, plasticizers, lubricants, and fire retardants. Jensen et al. (1969) were among the first to note the magnification of PCB's in the food chain. Subsequent studies have shown their environmental effects to be similar to those of DDT. The chemistry and persistence of PCB's in the environment and their chronic toxicity for various animals have been reviewed by Peakall and Lincer (1970) and Gustafson (1970). One report (Keil et al. 1972) describes a commercial PCB formulation in concentrations of 0.1 μg/ml which stimulated the growth of *Escherichia coli*. Ahmed and Focht (1973) reported biodegradation of PCB isomers 2 to 5 chlorines by *Achromobacter* pCB. Little information on the interactions of PCB's with heterotrophic microorganisms is available. Our investigation examines the effects of two commercial PCB formulations (Aroclor® 1016 and 1242) on selected estuarine bacteria and fungi.

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MATERIALS AND METHODS

Organisms. Bacterial isolates were obtained from estuarine waters and sediments of Pensacola Bay, near Gulf Breeze, Florida. Yeast isolates were obtained from waters and sediments of Barataria Bay, Louisiana. Biochemical analyses for bacteria were performed by the methods of Colwell and Wiebe (1970) and identifications by means of Breed et al. (1957).

Media. The medium for bacteria contained 1.0 g yeast extract (Difco) and 5.0 g peptone (Difco)/liter of aged artificial seawater (Rila Marine Mix, aged 1 mo in dark) at 20‰ salinity (adjusted with distilled water) and pH 7.4. For solid medium, 20 g agar (Fisher) were added per liter of medium. In growth curve studies, the above medium was diluted to one-half nutrient strength and the desired salinity.

Mycological agar (Difco) prepared with distilled water was used for the growth of fungi. For phosphatase studies, the yeasts were grown on this medium plus 0.02% phenolphthalein diphosphate. The yeasts were also grown in a broth with this formulation.

Test chemicals. Aroclors are commercial PCB formulations containing many isomers. Two Aroclor formulations, 1242 and 1016, containing 42% chlorine, were examined in this study. Concentrations (w/v) of PCB's are based on the Aroclor formulation as received from The Monsanto Company, considered as being 100% PCB.

Sensitivity studies. Bacterial cells for inocula were grown in broth for 18 h at 28 C on a rotary shaker. The culture was diluted 1:1 with sterile 20‰ seawater and 0.1 ml of the dilution was spread on the agar medium. Yeast cultures were grown for 48 h on mycological agar and colonies were suspended in distilled water to produce a cell suspension detectable by sight. The cell suspension was swabbed onto agar and prepared absorbant paper discs were positioned on the surface of the agar.

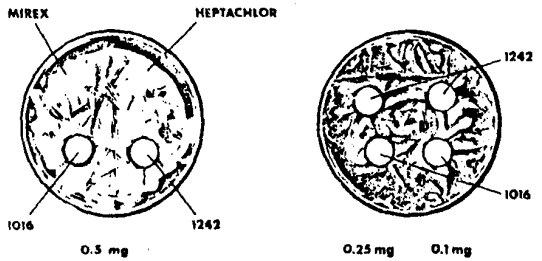

FIG. 1. Growth inhibition of an estuarine bacterium by Aroclor 1242 and 1016 on solid marine medium. Mirex and heptachlor (0.5 mg/disc), chlorinated hydrocarbon insecticides were included for screening purposes only. Bacterial growth appears white on dark medium and the zones of inhibition appear dark surrounding the white disc due to the negative reproduction of the photographic plate. The culture dish served as the negative, placed directly on the photographic paper.

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The absorbant paper discs (Schleicher and Schuell, Inc., No. 7-10-E) were saturated with 0.1 ml of acetone solution containing 1.0, 2.5, and 5.0 mg/ml of PCB formulation. The discs were air-dried for 24 h at room temperature before use. PCB-treated discs and control discs treated only with acetone were placed on the agar plates within 2 h of inoculation. All tests were performed in duplicate and examined for possible inhibition after 24-48 h incubation at 24-28 C. Only isolates sensitive to 0.5 mg of either PCB formulation were tested further for response to PCB's. Sensitivity was defined as a zone of inhibition surrounding the paper disc (Fig. 1).

Phosphatase studies. To test the effects of PCB's on extracellular phosphatases, yeasts were grown in liquid medium and on PCB-impregnated membranes placed on agar plates. After 2-24 h incubation, the membranes were removed and the plates were exposed to vapors of concentrated NH_4OH. Occurrence of reddish zones on the plates demonstrated phosphatase activity.

Growth curve studies. Cells for inocula were grown overnight in liquid medium and inoculated into 50-100 ml of the same medium in a 500-ml Nephelo-culture flask (Bellco, 515-A) to make a final cell concentration of 1%. Cell density was monitored as absorbance using a Bausch & Lomb Spectronic 20 at 660 nm or a Klett-Summerson Photoelectric Colorimeter with a No. 66 red filter. Test chemicals were added in acetone to facilitate dispersion and medium plus acetone cultures were monitored as checks for acetone effects. Salinities were adjusted with distilled water prior to addition of nutrient and the pH was adjusted to 7.4.

Results and Discussion

Of 106 bacterial isolates, growth of 28 was inhibited in varying degrees by the PCB formulations. Sensitive bacteria reacted similarly to both Aroclor formulations (Table 1). The PCB-sensitive bacteria included both gram-positive and gram-negative isolates. Of all strains tested, a slightly greater percentage of the sensitive bacteria were gram-positive (Table 2). These results differ from previous reports of sensitivity of gram-positive bacteria to other chlorinated compounds (Trudgill et al. 1971). Cyclodiene insecticides, shown to inhibit a range of gram-positive bacteria, had no effect on gram-negative bacteria tested (Widdus et al. 1971; Trudgill et al. 1971). Differences in toxicity of PCB's and cyclodiene pesticides to gram-negative bacteria may be related to type of molecule rather than to degree of chlorination.

Biochemical activities of sensitive and nonsensitive bacteria are compared in Table 2. The majority of sensitive strains produced both amylase (75%) and gelatinase (89%), whereas of all strains tested, only 37% were amylase-producers and only 45% were gelatinase-producers. The significance of these results in relation to total nutrient catabolism must await further investigation.

Fig. 2 shows the effect of Aroclor 1242 on the growth of four estuarine bacteria in liquid culture. Two bacteria were completely inhibited for 18 h. Since the PCB's were added in acetone solution, we believe that after volatilization (or degradation) of the acetone, PCB's were adsorbed to the cells and glass, allowing cells with no adsorbed PCB to attain logarithmic growth after 18-20 h incubation (not shown). However, in nature, if the PCB's were adsorbed to the microbial substrate at inhibitory concentrations, no growth would occur.

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TABLE 1. *Inhibition of growth of estuarine bacteria on nutrient seawater medium by PCB's*

GBERL Culture No.	Gram Reaction & Morphology	Genus	Aroclor® 1242 (mg)			Aroclor® 1016 (mg)		
			0.1	0.25	0.5	0.1	0.25	0.5
3	+ ROD	Unknown	++	++	+++	++	++	+++
21	– ROD	Unknown	++	++	+++	++	++	+++
35	– ROD	*Flavobacterium* sp.	++	++	++	+	+++	+++
39	– COCCOID	Unknown	++	+++	+++	++	+++	+++
55	– ROD	Unknown	++	+++	+++	+++	+++	+++
54	+ ROD	*Bacillus* sp.	+++	+++	+++	+	+++	+++
7	+ ROD	*Bacillus* sp.	+	+	+	x	+	++
9	+ ROD	*Bacillus* sp.	+	++	+++	+	++	+++
31	– ROD	Unknown	+	+	+++	+	+	+++
60	+ ROD	*Bacillus* sp.	+	+	+	++	++	++
86	– ROD	*Flavobacterium* sp.	+	+	+	x	+	+
100	– ROD	*Pseudomonas* sp.	+	++	++	+	++	++
8	+ ROD	*Corynebacterium* sp.	x	+	++	x	+	++
11	– ROD	*Achromobacter* sp.	x	+	++	x	+	++
42	+ COCCUS	*Micrococcus* sp.	x	+	++	x	+	++
44	+ COCCUS	*Micrococcus* sp.	x	+	+	–	x	+
93	+ ROD	Unknown	x	+	+	–	x	+
43	+ COCCUS	*Micrococcus* sp.	–	+	++	–	+	++
5	– COCCOID	*Serratia* sp.	–	–	++	–	–	++
13	– ROD	*Achromobacter* sp.	–	–	++	–	–	++
28	– ROD	*Achromobacter* sp.	–	–	++	–	–	++
52	+ ROD	*Corynebacterium* sp.	–	+	+	–	x	+
41	– COCCOID	Unknown	–	–	++	–	–	++
67	– ROD	*Achromobacter* sp.	–	+	+	–	–	+
69	– ROD	Unknown	–	–	++	–	–	++

Degree of sensitivity: +++ (18-20 mm zone), ++ (16-18 mm), + (14-16 mm), x (slightly), – (not sensitive).

TABLE 2. *Biochemical activities of PCB-test bacteria (percent of cultures showing positive reaction)*

Bacteria Tested	Production of					Citrate Utilization	Gram Reaction
	Urease	Amylase	Lipase	Gelatinase	H_2S		
Sensitive cultures	25	75	29	89	4	43	54
Total test cultures	14	37	19	45	5	37	40

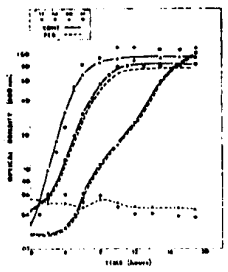

FIG. 2. Growth of estuarine bacteria in liquid marine medium (20‰) containing 10 μg/ml Aroclor 1242 of cultures no. 31 (unknown, gram-negative) and no. 60 (*Bacillus* sp.) were sensitive to PCB's and cultures no. 12 (unknown, gram-negative) and 47 (*Pseudomonas* sp.) were not sensitive. Average data points given for the latter two bacteria, two curves (experimental ——, and control – – –), are not significantly different.

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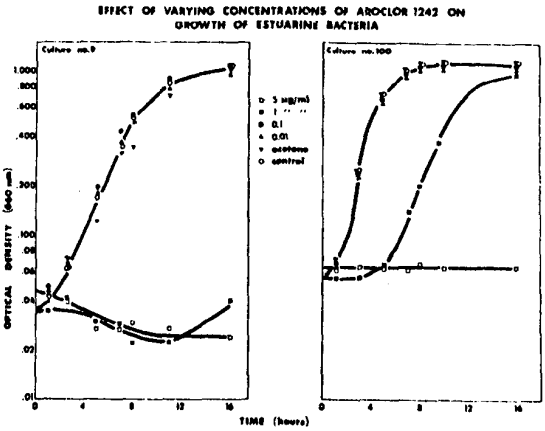

FIG. 3. Dose-response curve showing growth (O.D.) response of estuarine bacterial isolates no. 9 (*Bacillus* sp.) and no. 100 (*Pseudomonas* sp.) to varied concentrations of Aroclor 1242 (0-5 µg/ml). The PCB was added in acetone to facilitate dispersion. Check cultures contained marine broth at 20‰ salinity only and with acetone (0.5 ml/flask).

Due to the insoluble nature of PCB's, the minimum inhibitory level of PCB formulation could not be determined by the paper disc method. The lowest inhibitory concentration of PCB's for two selected sensitive organisms is demonstrated in Fig. 3. Growth of the gram-positive isolate (culture No. 9, *Bacillus* sp.) was inhibited at 1.0 µg/ml, whereas the gram-negative isolate (culture No. 100, *Pseudomonas* sp.) was sensitive at 1.0 µg/ml and completely inhibited at 5.0 µg/ml.

Inhibition of growth of fungi (*Cladosporium* sp., *Cephalosporium* sp., *Saccharomyces* sp., *Candida lipolytica, C. subtropicalis, Pichia spartinae,* and *Kluyveromyces drosophilarum)* by PCB's on paper discs was negligible. Although yeasts failed to grow on membranes completely saturated with PCB's, they grew on membrane areas that were free of PCB's and demonstrated phosphatase activity. In liquid media with PCB's added in petroleum ether (which was evaporated), yeast growth and phosphatase activity were similar to those of controls.

Sensitivity of estuarine bacteria to PCB formulations was greater in liquid than on solid medium. No inhibition of growth of estuarine fungi or phosphatase activity was noted except on PCB-saturated membranes.

Inhibition of bacterial growth by PCB formulations adsorbed onto paper discs is a simple technique which may be used to detect, in the laboratory, the possibility of inhibition in the environment. Although PCB's are almost insoluble in water, they are

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readily adsorbed to solid surfaces (Rizwanul et al. 1974). In nature, if the solid surface to which PCB's are adsorbed is a potential microbial substrate, inhibition of microbial catabolism could occur. Such inhibition could account for unexplained increases in BOD of effluents of sewage treatment facilities that receive industrial wastes containing large amounts of PCB's. The nature of the inhibiting substance in the PCB formulation that acts to interfere with the rate of nutrient turnover is uncertain.

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