Exhibit 9

PREPRINT

(17)

(17)

Growth Responses of Estuarine Bacteria to Mirex, Heptachlor and Polychlorinated Biphenyls[1/]

---

[1]Contribution No., Gulf Breeze Environmental Research Laboratory. Protection Agency, Gulf Breeze, Florida 32561. Associate Laboratory of the National Environmental Research Center, Corvallis, Oregon.

STLCOPCB4021423

MATERIALS and METHODS

Organisms: Eighty-five bacterial isolates from various estuarine environments near Pensacola, Florida were screened for growth responsiveness to heptachlor, mirex and the PCBs, Aroclor® 1016 and 1242. The isolates were maintained and tested in a medium consisting of 1 g/l Difco yeast extract and 5 g/l Difco beef peptone in 20% artificial aged seawater (Rila marine mix), pH 7.4 at 28 C. Fisher agar (2%) was added for solid medium.

Preparation of sensitivity discs: Super absorbant sensitivity discs (Schleicher & Schuell) were saturated with each toxicant dissolved in acetone to give concentrations of 1.0, 2.5 or 5.0 mg/ml. Each saturated disc contained 0.1 ml solution. The discs were allowed to dry thoroughly for 24 hours at room temperature before use.

Screening procedures: The cultures were incubated in nutrient broth tubes for 18h on a rotary shaker at 28 C. One-tenth milliliter of a 1:1 dilution of the broth cultures in sterile 15°/oo seawater was spread on nutrient agar medium and allowed to dry. The toxicant treated, dry paper discs were arranged on the agar surface, four per plate. Discs treated with acetone, but no toxicant, were included as controls. Duplicates of each test were prepared and the cultures were incubated for 24h at 37 C before the results were observed. Isolates sensitive to 0.5 mg of either PCB were further tested at the 0.25 and 0.1 mg concentrations. Sensitivity was defined as a ring of inhibition surrounding the disc (Figure 1).

STLCOPCB4021424

Growth Responses of Estuarine Bacteria to

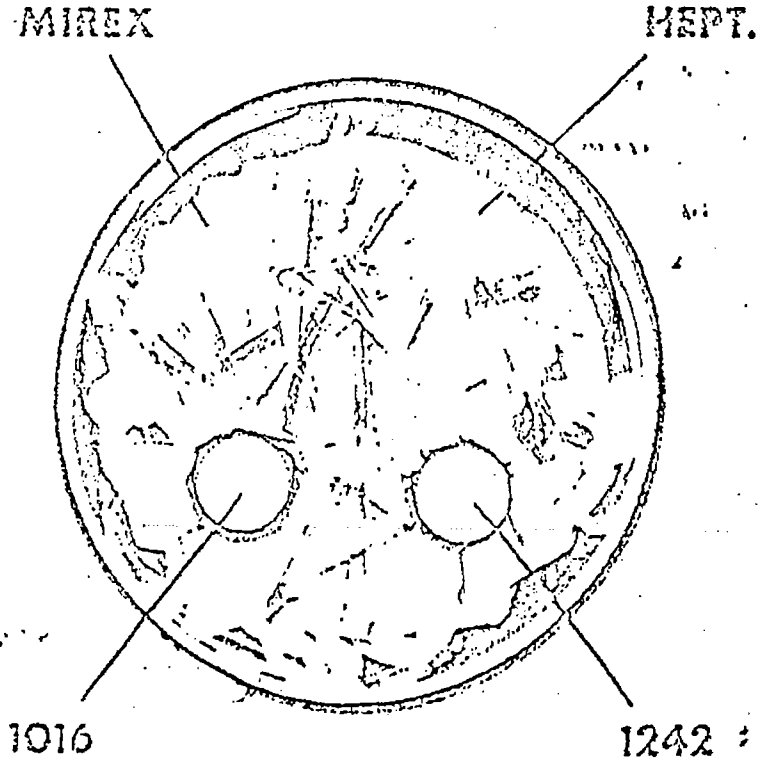
Mirex, Heptachlor and Polychlorinated Biphenyls

Polychlorinated biphenyls (PCBs) have been shown to be stimulating to the in vitro growth of Escherichia coli, an indicator bacterium from human intestinal flora. (Keil, et al., 1972). The lesser chlorinated biphenyls have been shown to be oxidized by a bacterial isolate from sewage, Achromobacter pCB (Ahmed and Focht, 1973). Pesticides and other chemicals in aquatic and estuarine environments have produced various responses in microbial communities (Ware and Roan, 1970). The fate of many of these pollutants depends on their effect upon the total microbial community. Further, responses of individual organisms directly affected, either in a stimulatory or inhibitory fashion, can provide much insight into degradation or persistance of the toxicant in the system. However, the responses of aquatic and estuarine microorganisms to PCBs and organochlorine pesticides has not been established. (Paris and Lewis, 1973; Bourquin, 1973).

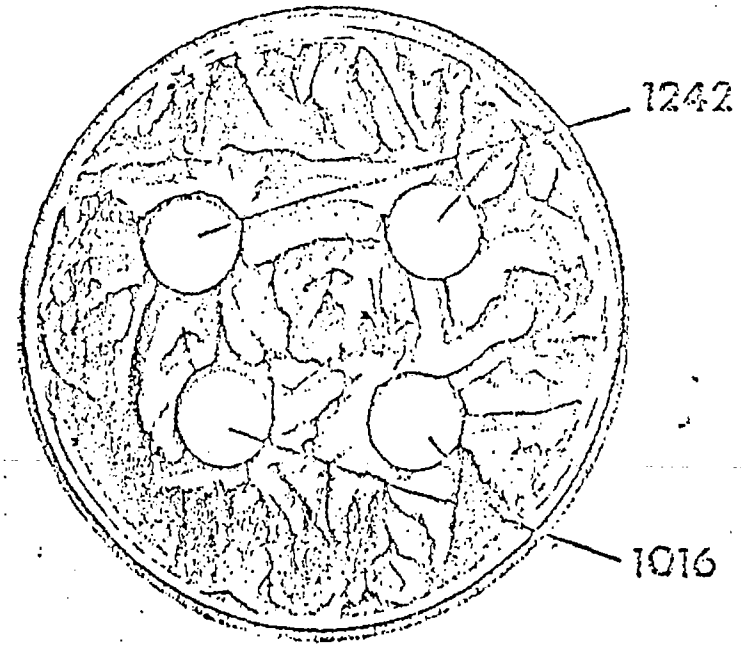
This investigation examines representative estuarine microorganisms responsive to PCBs and organochlorine pesticides using a rapid screeening procedure.

STLCOPCB4021425

GROWTH INHIBITION OF ESTUARINE BACTERIUM BY PCB's



0.5 mg



0.25 mg 0.1 mg

DSM 037602

STLCOPCB4021426

(R) Registered trademark, Monsanto Company, St. Louis, MO. Mention of commercial products or trade names does not constitute endorsement by the Environmental Protection Agency.

STLCOPCB4021427

Table 1

INHIBITION OF GROWTH OF ESTUARINE MICROOARGANISMS BY PCB'S AND PESTICIDES a/

GBERL CULTURE NO.	GRAM REACTION & MORPHOLOGY	AROCLOR^R 1242 (mg) 0.1	0.25	0.5	AROCLOR^R 1016 (mg) 0.1	0.25	0.5	MIREX 0.5 mg	HEPTACHLOR 0.5 mg
3	+ ROD	++	++	+++ b/	++	++	+++	-	-
21	- COCCOID	++	++	+++	++	++	+++	-	-
35	- Coccoid	++	++	++	+	+++	+++	-	-
39	- COCCOID	++	+++	+++	++	+++	++++	-	-
53	- COCCOID	++	+++	+++	+++	+++	+++	-	-
54	+ ROD	++	+++	+++	+	+++	+++	-	-
7	+ ROD (S)	+	+	+	±	+	++	-	-
9	+ ROD	+	++	+++	+	++	+++	-	-
31	+ ROD	+	++	+++	+	++	+++	-	-
60	± ROD	+	++	++	++	++	++	-	-
86	- ROD	+	+	+	±	+	+	-	+
100	- ROD	+	++	++	+	++	++	-	-
8	+ ROD	±	+	++	±	+	++	-	-
11	- ROD	±	+	++	±	+	++	-	-
42	+ COCCUS	±	+	++	±	+	++	±	-
44	+ COCCUS	±	+	+	-	±		nt	nt
93	+ ROD	±	+	+	-	±	+	+	±
43	+ COCCUS	nt	nt c/	++	nt	nt	++	-	-
5	- COCCOID	-	-	++	-	-	++	-	-
13	- ROD	-	-	++	-	-	++	-	-
28	- ROD	-	-	++	-	-	++	-	-
32	+ COCCOID	-	+	+	-	±	+	-	-
41	- COCCOID	-	-	++	-	-	++	-	-
67	- ROD	nt	nt	+	nt	nt	+	±	±
69	- ROD	-	-	++	-	-	++	-	-

a. Growth inhibition on nutrient SW seawater Media, 85 cultures tested

b. (+) Degree of sensitivity, (±) Slightly sensitive, (-) Not sensitive

c. Not tested

DSM 037604

STLCOPCB4021428

RESULTS

Preliminary tests proved techniques customarily employed in anti-biotic sensitivity testing, not applicable in the quantitation of the inhibitory response of mirex, heptachlor, and the PCBs. These toxicants are insoluble in water and the diffusion rates through the agar were variable, yielding non-quantitative zones of inhibition. A relative inhibitory effect, however, could be distinguished between the bacterial strains and toxicant levels tested by using the method described in Table 1.

Of the 85 bacteria tested, 26 demonstrated zones of inhibition to at least one of the test compounds (Table 1). The PCBs were markedly more inhibitory than the organochlorine pesticides. Only 3% of the strains screened showed inhibition at 0.5mg of mirex and heptachlor. This concentration of Aroclor® 1016 and 1242, however, inhibited 31% of the bacterial strains. Further, o.1mg of Aroclor® 1016 inhibited 69% of these strains and Aroclor® 1242 inhibited 62%.

We attempted to correlate PCB-sensitivity response with biochemical activity or cell composition (Table 2). PCB-sensitive bacteria were both Gram positive and negative and no morphological correlation was noted. However, a comparison of biochemical activities of sensitive and non-sensitive bacteria tested showed two distinctly different groupings. Amylase and gelatinase production was shown by 76% and 86% respectively, of the sensitive strains; however, of the total bacterial strains screened, only 33% and 42% of the populations showed these activities. No other biochemical activity differed

STLCOPCB4021429

Table 2.

Comparison of Physiological Activities of PCB-sensitive and Non-sensitive Microorganisms

Bacteria Tested	Production of: Urease	Amylase	Lipase	Gelatinase	H_2S	Nitrate Reduction	Citrate Utilization	Gram Reaction
Sensitive	19*	76	33	86	5	29	48	50
Non-Sensitive	13	33	23	42	6	37	41	

*Percentage of cultures tested showing positive reaction, 100 cultures tested.

DSM 037606

STLCOPCB4021430

distinctly from the non-sensitive bacterial populations. No characteristic gram reaction was shown by the PCB-sensitive bacteria.

DISCUSSION

The sensitivity disc testing procedure commonly used in clinical bacteriology has been successfully applied here as a rapid method for detecting PCB-sensitive estuarine microorganisms in pure culture. Although the procedure is qualitative only, it can serve to eliminate many hours of tedious experimentation involved in the isolation of suitable test organisms for more detailed examinations. This method has been used previously by investigators to study the effects of pesticides on growth of bacteria (Widdus, et al. 1971; Trudgill, et al. 1971). However, no significant response has been shown screening estuarine microbes and chlorinated hydrocarbon pesticides in this study. Conversely, PCB sensitivity in estuarine bacteria was quite dramatic (Figure 1) using this method. Keil et al. (1973) demonstrated no sensitivity to 0.02 mg PCB by E. coli using the same method on non-marine medium. These experiments indicated PCB's stimulate growth of E. coli. No explanation for (Keil et al., 1973) this difference between stimulation in E. coli and inhibiton in (this study) estuarine bacteria has been determined at this time.

The implications of this inhibition, if wide spread in the environment, are obvious. The sensitivity of a large number of heterotrophic bacteria to PCBs may be of considerable importance in an estuarine environment. PCBs have been shown to persist in estuarine sediments in relatively high concentration (9 ppm). Sediment cores taken in close proximity to another showed markedly different concentrations (0.06-9 ppm) indicating the PCBs are pro-

STLCOPCB4021431

bably absorbed onto particulate organic matter during transport and deposition in estuarine sediments (Butler, 1974). The aforementioned concentrations, when considered absorbed onto distinct detrital particles, are not unlike those shown to be inhibitory to hetrotrophic growth in this study. Consequently, PCBs could inhibit the normal turnover of carbon in estuarine sediments by inhibition of specific heterotrophic bacteria attached to organic detritus.

STLCOPCB4021432

REFERENCES

Ahmed, M. and D.D. Focht. Bull. Environ. Contam. Toxicol 10, 70-72 (1973).

Bourquin, A. W., In: The Microbial Degrad. of Oil Pollutants, Eds. Ahearn, D.G. and S.P. Meyers, 237-243 (1973).

Butler, P. A. Personal Communication (1974).

Keil, J. E., S. H. Sandifer, C. D. Graber, and L. E. Priester. Water Research 6: 539-541. 1972

Paris, Doris and David L. Lewis. Residue Reviews 45, 95-124 (1973).

Trudgill, P. W., Widdus, and J. A. Rees, J. of Gen. Microbiol. 69, 1-13 (1971).

Ware, G. W. and C. C. Roan, Residue Reviews 33, 15-45 (1970).

Widdus, R., P. W. Trudgill, and D. C. Turnell, J. of Gen. Microbiol. 69, 22-31 (1971).

STLCOPCB4021433