Exhibit 12

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Effects of Aroclor 1242[1] (a Polychlorinated Biphenyl) and DDT on Cultures of an Alga, Protozoan, Daphnid, Ostracod, and Guppy[1]

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The polychlorinated biphenyls (PCB's) are a group of chemicals having physical and chemical properties sufficiently similar to those of DDT to warrant investigation (10). The Monsanto Company, the sole producer of PCB's in the United States, markets them under the trade name of Aroclor. Aroclor 1242 is a mixture of PCB compounds containing approximately 42% chlorine by weight. Diverse in their commercial applications, but most commonly employed as plasticizers, PCB's have increased in usage dramatically since the forties (6). Jensen first reported finding PCB's in pike, other fish, eagle feathers and human hair from Sweden in 1966 (1). Subsequently, scientists from around the world began analyzing wildlife tissues for the presence of PCB's. Chlorinated biphenyls were detected in fish, mussels, and birds from the Rhine River and the coast of the Netherlands (7), in sparrowhawks, kestrels and kittiwakes from Great Britain (4) and peregrine falcons from the United States (5). Cultures of the marine diatom Cylindrotheca closterium have been shown capable of absorbing Aroclor 1242 from sea water containing 0.1 ppm and concentrating it 900 to 1000 times (6). This concentration caused, however, a marked reduction in growth, chlorophyll index, and RNA synthesis of the marine diatom, but no noticeable effect on DNA synthesis.

The aim of the present study was to investigate some of the effects of Aroclor 1242 and DDT on a variety of aquatic organisms. For a more detailed report, please refer to the Master's thesis (9).

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[1] Fisheries contribution No.350

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Methods

Five different organisms were used in the present investigation: the alga Chlamydomonas reinhardtii, the ciliate protozoan Tetrahymena vorax, the cladoceran Daphnia pulex, the ostracod Cypridopsis vidua, and the guppy Poecilia reticulata. Media 63 (12) was the experimental medium in which the effects of the toxicants upon algae, cladocerans, and ostracods were studied. Bioassay media, modified from Fernell and Rosen (3) to contain 1/100 of the organic components and 1/10 of the organic salts, was used for the Tetrahymena studies. Coarsely-filtered pond water was used in the guppy toxicity tests. Since both DDT and PCB have a tendency to adhere to surfaces (2, 14), disposable glassware was used to prevent residues of the chlorinated hydrocarbons from affecting the results of subsequent experiments. The culture vessels were either quart or half-gallon mason jars with half of a disposable plastic Petri plate for a lid or disposable glass test tubes for the protozoan studies. At the start of an experiment all culture vessels and media were autoclaved at 250° C. for 25 minutes to insure sterility (except the guppy experiments in which pond water was used). One hundred percent p,p'-DDT, designated as the "ESA pesticide reference standard" was used in all DDT experiments and the Aroclor 1242 used was obtained directly from Monsanto in St. Louis, Missouri. The test chemical was generally added to the growth media prior to the introduction of the organisms. In order to distribute the test chemical throughout the media, and to ease in making dilutions, acetone was used as a vehicle for DDT, and 95% ethyl alcohol was used for the PCB. A concentration of 0.2 ml solvent/liter media was used, which did not appear to adversely affect the tested organisms as shown by preliminary experiments and usually demonstrated by the solvent controls. Once algal density was determined, a known volume of inoculum was added to each culture so that the initial concentration in the culture vessel would be 0.1×10^6 cells/ml. Adult Daphnia, ostracods, and fish were removed from their stock cultures, randomized, and counted before placing them in the test cultures. Experimental cultures containing algae and/or Daphnia were grown at 28.5° C. ± 1°C. under a bank of fluorescent lights which delivered 900 foot-candles

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(as measured by a GE model 213 lightmeter with a blue filter). Tetrahymena vorax cultures, grown in test tubes, were placed on a roller tube apparatus which rotated the tubes at 1/5 rpm. Protozoan, ostracod, and guppy bioassays were conducted under the conditions of 23.5° C±1°C. and 75 footcandles of light.

During the course of an experiment, population counts of the organisms present in each culture vessel were recorded. The Chlamydomonas cells were killed and

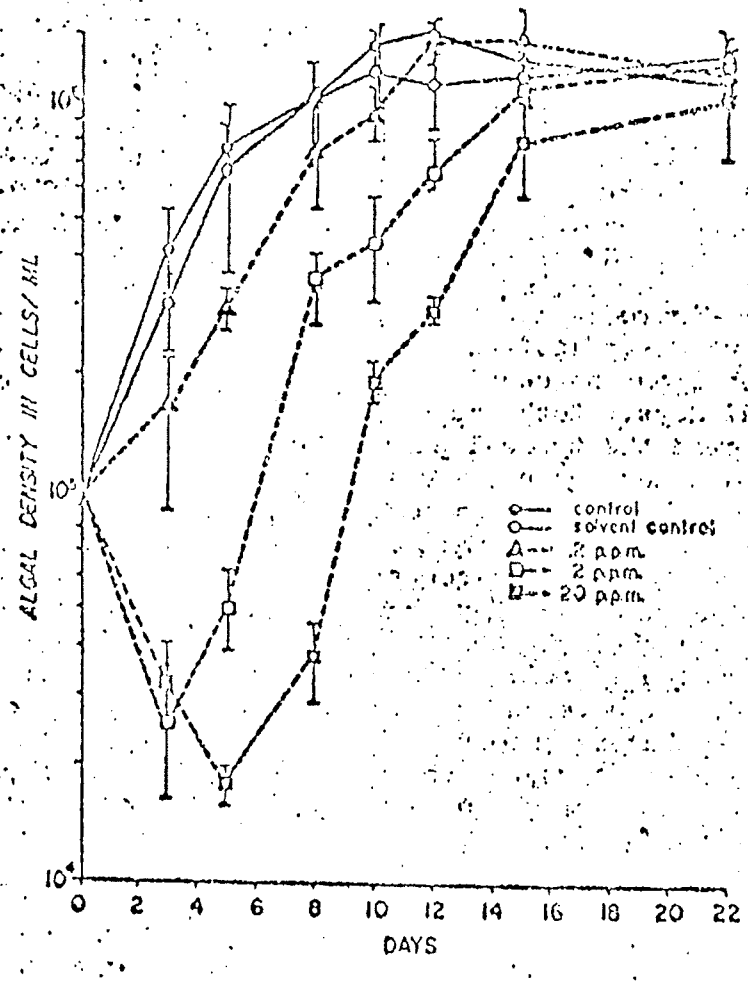

Fig. 1. Effect of the addition of 0.2, 2, and 20 ppm Aroclor 1242 on the growth of Chlamydomonas reinhardtii. Each point represents the mean of two observations whose individual values are indicated as the range.

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stained by adding a drop of I_2-KI solution, then counted using a haemacytometer at 100X. The Tetrahymena were stained in the same manner, then ten 0.01 ml drops of the culture were distributed on a Falcon counting plate and the cells in each counted at 50X. Carbon-14 uptake by algal cells subjected to various concentrations of both DDT and PCB was determined by incubating 5 ml samples with $NaH^{14}CO_3$ and using a standard filtration-planchet method. Gas chromatography analysis was performed by Dr. James Saddler of the Fisheries Research Institute on five algal cultures--a control, a solvent control, and three cultures to which 20, 2, and 0.2 ppm Aroclor had been added. The algal pellets and supernatant samples were extracted with acetonitrile-petroleum ether; purified by elution on a Florisil column; and injected into a gas chromatograph equipped with an electron capture detector.

Observations--Discussion

Figure 1 illustrates the effect of three different doses (0.2, 2, and 20 ppm) of Aroclor 1242-alcohol on the density of Chlamydomonas over a period of 22 days. Both the control and the solvent control followed the typical parabolic growth curve with no initial lag period. There was a period of rapid growth (the log phase) followed by a leveling off of the population size as the culture approached the stationary phase. Aroclor-containing cultures did not follow this pattern, however, but took a much longer time to approach the cell density of the controls. In the Chlamydomonas cultures to which the two highest dosages of Aroclor were added there appeared to be not only a delay before exponential growth began, but an initial drop in the algal density below the original inoculum size. The severity of the response was dose related. After day 5, the growth rate of these inhibited cultures increased so that by day 22 they had cell densities similar to the controls. By day 22, the standing crop of Chlamydomonas in all of the cultures appeared to be about the same. This experiment was repeated several times with similar results, although the degree of inhibition from equal additions of PCB varied somewhat. This variation may be due to heterogeneity of the PCB globular suspensions (14), or to differences in other physical factors from experiment to experiment.

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In order to determine whether ^{14}C uptake by Chlamydomonas cells was also affected by the addition of Aroclor 1242, the experiment was repeated using five different doses of PCB (0.002, 0.02, 0.2, 2, and 20 ppm), incubating $NaH^{14}CO_3$ with samples taken from the cultures at three different points in the growth period. On day 4, the control had the greatest ^{14}C uptake and the PCB containing cultures had uptakes per cell and per ml in inverse relationship to their PCB concentration. By days 11 and 15, the ^{14}C uptake per cell were all markedly reduced and showed no dose-related response. These results were undoubtedly influenced by the varying amounts of nutrients still available. The ^{14}C uptake per ml tended to increase during the duration of the experiment, the values generally in inverse relation to the dose.

A possible reason for the apparent recovery of Chlamydomonas from the initial effects of PCB-containing media is that the PCB was degraded or otherwise made less effective during the course of the experiment. Since an experiment conducted by Risebrough (11) indicated that PCB compounds could be broken down by ultraviolet light, the possibility that the fluorescent lights used in growing the algae cultures were causing degradation of the added PCB was investigated. Mason jars of Media 63 containing 2 ppm Aroclor 1242 were placed under the bank of fluorescent lights for 7, 5, 2 or 0 days prior to their inoculation with Chlamydomonas. All of the PCB-containing cultures exhibited delayed algal growth and there did not appear to be any correlation between degree of inhibition and the length of time the media was under the lights after the PCB was added. This seemed to indicate that neither degradation by light nor adsorption of PCB on the glass were significant factors in the delayed growth and subsequent recovery of the Chlamydomonas cultures. Another possibility is that the PCB may have been metabolized either by the algae or by contaminating bacteria. No difference, however, was noted in the chromatograms between contaminated and axenic cultures, nor in degree of algal growth inhibition, hence bacterial degradation of Aroclor 1242 was probably not a significant factor.

Data from the gas chromatography analysis seem to indicate that the Chlamydomonas was concentrating the PCB, for a much higher concentration was found in the

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algal pellet (which took up about 0.05 ml in volume) than in the one liter of supernatant. For example, in cultures to which 20 ppm (mg/l) PCB had been added, 0.52 mg were detected in the algae, and only 0.04 mg in the supernatant. A large percentage of PCB that was added was not recovered from either the media or the algae. This may have been lost because of adherence to the glass sides of the mason jars, sinking of small PCB globules to the bottom, vaporization, codistillation with water, metabolism by the algae, or loss in the extraction, Florisil clean-up, or injection processes.

The effect of DDT on the growth and ^{14}C uptake of Chlamydomonas cultures was studied. In the tested concentration range of 0.2 ppm to 20 ppm DDT, the algal growth curves did not differ appreciably from those of the controls. While both Wurster (13) and Menzel et al. (8) were able to demonstrate that DDT reduced ^{14}C uptake in various species of marine phytoplankton, in the present study no significant effect of DDT on ^{14}C uptake by Chlamydomonas was noted. Mensel et al. (8) did observe that Dunaliella tertiolecta, a green flagellate, did not appear to be affected by concentrations of DDT as high as 1 ppm. It is thus possible that Chlamydomonas, like Dunaliella, is relatively DDT resistant, and yet may be effected by polychlorinated biphenyls which may restrict ^{14}C uptake by a different mechanism.

The toxicity of PCB and DDT to organisms other than algae was then investigated. The growth curves of the protozoan Tetrahymena vorax grown in the presence of Aroclor 1242 (0.02 to 20 ppm) and in the presence of DDT (0.01 to 100 ppm) seem to indicate that neither chemical has any effect upon the growth rate or population size. Aroclor 1242 appeared to be fairly toxic to Daphnia pulex, however. Young Daphnia died at levels as low as 0.02 ppm. Mortality may be linked with other stresses as well, however, for when the Daphnia were supplied with algal culture, the death rate was cut to one-third. A comparison of the relative toxicity of PCB and DDT to ostracods revealed a greater mortality of Cypridopsis vidua at 20 ppm PCB than at the same concentration of DDT. At 20 ppm there were no survivors out of 20 ostracods in the Aroclor-containing cultures while there were 13 survivors in the DDT cultures. At lower concentrations, however, DDT appeared to have more of an effect than

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the PCB. The guppy Poecilia reticulata had 100% mortality at 20 and 2 ppm, but only 25% mortality at 0.2 ppm. The behavior of the fish just prior to death in PCB-containing cultures was quite different from the normal symptoms of hydrocarbon pesticide poisoning. In some cases behavior included rapid pivoting of the body with the nose pressed close to the bottom of the jar; fungus-like growths were also often associated with dying or recently-dead fish.

To explore the passage of PCB through the food chain, Daphnia in PCB-free media were supplied with algal cells that had been grown in Aroclor-containing media, centrifuged, and resuspended in PCB-free media. All of the three-day old Daphnia fed algae cells grown in media containing 20 ppm were dead by the fourth day, and there were only three survivors from among the thirty fed algae from the culture containing 2 ppm. The mortality rate was higher among control Daphnia that were starved than among those that were given PCB-free algae.

Summary

Aroclor 1242 appeared to temporarily inhibit the growth rate of Chlamydomonas as evidenced by cell counts and ^{14}C uptake, while DDT did not appear to have any effect. A greater concentration of PCB was found in the algal cells than in the media, indicating that concentration and passage of the biphenyl compounds through the food chain may take place. Neither Aroclor 1242 nor DDT in the amounts tested appeared to have any effect on the growth or cell density of Tetrahymena vorax cultures. A summary of the results of the toxicity tests on Daphnia, ostracods, and guppies is presented in Table 1. According to the table, Daphnia pulex were quite sensitive to additions of Aroclor 1242 as low as 0.02 ppm. The toxicity of PCB and DDT to the ostracod Cypridopsis vidua appeared to be approximately the same. Young guppies died in concentrations of 2 ppm Aroclor 1242. Aroclor 1242 appears to have a much lower toxicity to cladocerans and guppies than p,p'-DDT. Although the effect of PCB on algal growth appeared to be temporary, in view of its evident capability to be passed through the food chain and its selective

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TABLE 1

Summary of PCB toxicity studies. +++ = many survivors; ++ = most survivors; + few survivors; 0 = no survivors.

Test Situation	Table #	age	dose of PCB (ppm) 0.0002	0.002	0.02	0.2	2	20	observation day
Daphnia									
fed PCB-containing cells*	2	young	---	---	---	+++	+	0	4
direct addition of PCB-alcohol									
non-fed	3	young	+++	+++	++	0	0	0	4
fed	3	young	+++	+++	++	0	0	0	4
PCB-containing algal cultures	4	adult	---	---	---	---	0	0	3
	5	adult	---	+++	+++	++	0	0	2
	5	adult	---	+++	+++	0	0	0	9
Ostracods									
PCB-containing algal cultures	6	adult	---	+++	+++	+++	++	0	3
Guppies									
direct addition of PCB-alcohol	7	young	---	---	---	---	0	0	7
	8	young	---	+++	+++	++	0	0	1

* Daphnia in PCB-free media were fed washed Chlamydomonas cells grown in PCB suspensions of the indicated doses.

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toxicity to certain zooplankton, it would be unwise to attempt to predict its effect on the ecosystem without further study.

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