WHOI-73-2

THE USE OF FLOWING BIOLOGICAL SYSTEMS IN AQUACULTURE, SEWAGE TREATMENT, POLLUTION ASSAY, AND FOOD-CHAIN STUDIES

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January 1, 1972 - December 31, 1972

TECHNICAL REPORT

Prepared for the National Science Foundation under Grant NSF-RANN GI-32140.

In citing this manuscript in a bibliography, the reference should be followed by the phrase: UNPUBLISHED MANUSCRIPT.

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DSW 032835

STLCOPCB4016797

PCB Uptake and Flushing in Some Marine Systems

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INTRODUCTION

During the first 9 months of work on organic trace contaminants, effort has been concentrated on two areas. First, a functioning analytical laboratory has been established and experimental apparatus was designed, assembled and utilized. Second, an investigation of polychlorinated biphenyl (PCB) pollution in the main sewage-aquaculture food chain and, experimentally, in the oyster link of the chain has begun. In addition, a small comparative study on uptake in open ocean animals (Sargassum community) was carried out. Progress in all phases of this work has been greatly accelerated by the advice and help of Dr. George Harvey and his research group in the Chemistry Department of the Institution.

A 9-week PCB uptake experiment using small oysters was followed by a 7-week flushing period. In order that uptake and flushing curves, as well as final levels, would resemble natural situations, PCBs were added at low levels (0.005-0.05 ppb) similar to those encountered in the marine environment. In that range, three PCB levels were used to provide information on whether a) equilibration of lipid with the surrounding water,

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or b) accumulation from food is the primary mechanism in determining PCB levels in oysters. The complexity of PCB mixtures found in both commercial preparations and environmental samples makes them difficult to quantitate, difficult to distinguish from possible analytical contamination and also causes problems in obtaining any definite information on the behavior of individual isomers with varying arrangement and amounts of chlorine atoms. Therefore a much simpler mixture of purified isomers was employed. Considering the solubility and adsorbtive properties of PCBs and the low levels used, only those materials that would be least likely to add contaminants or pick up the PCBs were used in designing the experimental system. Also, incoming seawater from the busy dock area was filtered to remove as much hydrocarbon as possible, and water in the tanks was analyzed for actual isomer levels.

EXPERIMENTAL DESIGN

Oysters ranging in length from 1-2 cm (from the hatchery of Frank Flower and Son, Bayville, Long Island) were used for the PCB isomer uptake experiment. They were 5 months old at the beginning of the experiment and had been equilibrated for 2 weeks under experimental conditions before addition of PCBs was begun. Four tanks were used, each containing approximately 600 oysters. The first tank served as the control (C), receiving everything that the others did except PCB; the second or low (L) tank received a calculated 0.005 ppb total of PCB isomers in the continuously flowing seawater; the third or medium (M) tank received 0.015 ppb

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and the fourth or high (H) tank received 0.050 ppb. (Although it was difficult to maintain these levels precisely, twice-daily measurements of seawater and PCB flow rates indicate that variation was relatively small, being greatest for the high tank, and the PCB concentration ranges did not overlap.) The mixture of PCB isomers used in this experiment was composed of the following percentages of purified isomers (Analabs, Inc.):

15% 2,3-dichlorobiphenyl

15% 3,4,2'-trichlorobiphenyl

20% 2,5,2',5'-tetrachlorobiphenyl

20% 2,3,2',5'-tetrachlorobiphenyl

15% 2,3,4,2',3'-pentachlorobiphenyl

15% 2,4,5,2',5'-hexachlorobiphenyl

These isomers were chosen among those commercially available for a range of chlorine content from 2-6 per molecule, to include a symmetrical and an asymmetrical isomer of the same chlorine content, and because most have been shown to be major constitutents of the commercial PCBs (Sissons and Welti, 1971 and Webb and McCall, 1972). The isomer mixture was added to the tanks from a more concentrated solution in a 1:1 mixture of re-distilled acetone and distilled water. A modified Mariotte-type bottle was used for PCB addition, with the flow rate regulated by silicon resistance tubing to 0.1 ml/min. The bottles were coated with Dri-Film 87 (G.E.) to make them chemically inert. The acetone concentration in the seawater

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was 50 ppm. The control tank received the solvent but no PCB. During the flushing period, the addition of solvent was discontinued.

Seawater was pumped from beneath the W.H.O.I. dock through PVC tubing, a 100 μ cellulose cartridge filter and a 5 or 10 μ bag filter to a polyethylene head tank. It then passed through a 1 μ cartridge filter, a small pump and finally through a brass filter containing 200 ml of Amberlite XAD-2 (a styrofoam resin from Rohm and Haas Co.) to remove hydrocarbons. The XAD had been prewashed with 100 bed volumes of tap water, 20 bed volumes of 95% ethanol and 100 bed volumes of distilled water. After this filter, the seawater system consisted of only silicon, brass, nylon and glass before reaching the tanks. The flow rate of seawater was 1 liter/min for each tank, giving a retention time of 15-20 minutes. After the uptake phase, the experiment was moved indoors and seawater heated to 18° was used in the flushing phase.

The oysters were fed at a low natural level from a continuous, mixed phytoplankton culture grown out on the dock (and exposed to an unknown variety of materials) on inorganic nutrients. It was pumped into a polyethylene holding tank, where it was metered into each tank by a peristaltic pump at a rate of 11 ml/min through polyethylene tubing. The continuous culture contained an average of about 12,000 μg phytoplankton-carbon/liter. During the course of the uptake experiment, the dominant diatom in the culture changed from Phaeodactylum tricornutum to Chaetoceros spp. and back again.

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The shallow tanks were made of glass and Silicon Seal (Dow), coated with Dri Film-87 and covered with glass lids to reduce airborne contamination. The seawater, PCB solution and phytoplankton culture were swirled in a glass funnel to mix them before entering the tank (see Fig. 5). The tanks were tested with carmine dye particles in the water to obtain as uniform a circulation as possible, and the width (2½ ft) was greater than the length (2 ft) to minimize possible upstream-downstream effects. Oysters were spread over the tank bottom except for edges, corners and under the inflow. The water was approximately 1¼ inches deep.

SAMPLING METHODS

Oysters, their detritus, nutrient-grown phytoplankton, "clean" seawater and isomer-spiked seawater entering and leaving the tanks were sampled at appropriate intervals. All samples were frozen for later analysis.

Oysters from each tank were sampled several times during the first week of both uptake and flushing phases of the experiment, and once a week thereafter. Approximately 20 oysters from a tank were pooled for each sample. After flushing their gut contents for 10 minutes in "clean" seawater, they were opened with a small spatula and both meat and juice were transferred to a vial. The number sampled and the number of those dead (if any) were recorded, and each shell was outlined as an estimat. of size. At attempt was made to include oysters of all sizes and locations

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in the tank. On one occasion large and small oysters and ones located "upstream" versus "downstream" in the tank were sampled.

PCBs in seawater were collected on an XAD column similar to the one described for the incoming seawater line, but smaller in scale, utilizing the method for sampling PCBs in water developed in the Harvey laboratory (Harvey, 1972a). Twelve to twenty liters were sampled on 37 ml of XAD in a 6 inch brass column, with a vacuum pump used to get a flow rate of 5 bed volumes/min. The resin was emptied into a jar and frozen.

The concentrated phytoplankton cultures (and also the sewage) were separated into particulate and filtrate samples. Four liters of plankton or 2 liters of sewage were filtered through prewashed and baked glass fiber filters (0.3 μ pore size, Gelman Type A) in a metal millipore holder, and the filtrate was passed directly through a 37 ml XAD column under an aspirator vacuum.

ANALYTICAL METHODS

Cleaning methods. - Hexane and ether were redistilled in glass, the hexane over elemental sodium. Sodium sulfate and florisil were baked out overnight in a 290°C oven prior to use. Glassware was washed in detergent and tap water, rinsed in acetone and redistilled hexane, and baked out overnight.

Sample preparation. - A 1-2 g sample of pooled small oysters (or 10-20 g of larger ones) was homogenized with hexane and sodium sulfate in a Virtis "23" homogenizer. The extract was further dried on a sodium sulfate

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column and concentrated to 10 ml in a Kuderna-Danish Evaporative Concentrator on a steam bath. A 1 ml aliquot was taken at this point and air-dried to obtain the "hexane-extractable lipid" weight. Lipid was removed from the remainder of the sample on a florisil column, which was eluted with 6% ethyl ether in hexane. The cleaned extract was concentrated to approximately 0.5 ml prior to gas chromatography. Procedural blanks were prepared in the same manner but omitting the sample.

Gas chromatography. - A Micro Tek DSS 2000 gas chromatograph with a ^{63}Ni electron capture detector was used with a 2 mm by 2 m glass column containing 15% QF-1 on Chromasorb Q and 2% OV-17 on Anakrom (mixed 1:3). The solid supports were acid and base washed and HMDS treated. Injection port temperature was 235°C, column temperature 190°C and a detector temperature 290°C. Nitrogen was used as carrier gas at 50 ml/min through the column and 30 ml/min through the purge.

Quantitation. - PCBs and DDT compounds were quantitated by comparing retention times and peak heights with known standards, including p,p-DDE (1,1-dichloro-2,2-bis [p-chlorophenyl] ethylene) p,p-DDD (1, 1-dichloro-2, 2-bis [p-chlorophenyl] ethane), and p,p-DDT (1,1,1-trichloro-2, 2-bis [p-chlorophenyl] ethane), and the PCB mixtures Aroclor 1242 (42% Cl), 1254 (54% Cl) and 1260 (60% Cl). In quantitating Aroclor 1254 in oysters from the control tank, 7-8 peaks were used, but only 2 were clearly visible on chromatograms from oysters dosed with the isomers. Arochlor 1242 was quantitated from 3-6 peaks. When necessary, the result was corrected for the procedural blank. The PCB background (determined from

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the control tank oysters sampled on the same day) was subetracted before quantitating isomers or DDE. The presence of DDD and DDT was confirmed and background levels were revealed by saponification. This was accomplished by shaking the sample with 1 N sodium methoxide in methanol for 45 minutes. Distilled water was added; it was again shaken, and the phases were separated. The saponified hexane extract was dried with sodium sulfate before being gas chromatographed.

PRELIMINARY EXPERIMENTAL RESULTS

The accumulation of individual PCB isomers during the uptake experiment is given in Fig. 1A-C on a wet weight basis for the L, M, and H tanks, respectively. Total isomer accumulation is summarized in Fig. 1D. The "Cl" number refers to the number of chlorines per molecule for each isomer and "sym" and "asym" refer to the symmetrical and asymmetrical isomers with 4 chlorines per molecule. Most early data points are lacking, as those samples have yet to be analyzed. The background PCB level in these oysters was constant throughout the experiment, in the range of 30-100 ppb as Aroclor 1254. There was no systematic difference in background among oysters given different treatments. In some samples Aroclor 1242 (6-50 ppb) and DDE (on the order of 1 ppb) could also be measured. DDD and DDT have not been estimated, as these samples have not yet been saponified. Average concentration factors at equilibrium (considered to be after 6 weeks of uptake) were found not to be dose dependent in the PCB concentration range used here and under these particular environmental conditions. Equilibrium concentration factors were:

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Isomer	Concentration Factor
2 Cl	1,000
3 Cl	7,500
4 sym Cl	8,000
4 asym Cl	12,000
5 Cl	28,000
6 Cl	48,000
Total	17,000

The uptake of all PCB isomers in the low and medium tank oysters had leveled off after six weeks, but in the high tank only the isomer with the lowest degree of chlorination had definitely leveled off after nine weeks. Obviously, the rate and amount accumulated is directly correlated to the degree of chlorination of an isomer. As emphasized in Fig 2B, the amount of each isomer accumulated is also directly proportional to the amount in the water at equilibrium. Fig. 2A illustrated the situation on Day 11, before equilibrium had been reached at higher PCB concentrations in the water and with isomers of higher degrees of chlorination at lower water concentrations. These results disagree with those of Butler (1972), who reported linear uptake of PCB by oysters for a 4 month period with 1 ppb in the water. However, this may not be inconsistent, since, at such a high concentration, equilibrium may not yet have been reached.

Fig. 3A shows the average increase in wet weight of the oysters during the uptake experiment, Fig. 3B the average % lipid on a wet weight basis

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and Fig. 3C the average mortality during the experiment. Averages were used here because no systematic differences in these parameters between oysters given different treatments were found. The oysters grew visibly during at least the first part of the uptake experiment, and wet weight increased for seven weeks before leveling off. Shell deposition as a measure of growth (Duke et al., 1970) was not feasible due to the extreme fragility of new shell material. The lipid percentage first increased and then decreased during the last two weeks of the experiment. The mortality rate was apparently constant during the experiment. It was found that smaller oysters within the size range used had a much higher mortality rate than the larger ones. There is no evidence that the PCBs were related to these factors, although Duke et al.(1970) and Nimmo et al. (1972) found that Aroclor 1254 at approximately 20 times the highest total isomer concentration used in this study reduced the growth of oysters slightly and also caused "generalized tissue responses", including atrophy of digestive diverticula. It is more likely that the changes found in the present study were related to one or both of the following: 1) a gradual decrease in seawater temperature at the end of the summer (beginning after aboug 6 weeks of uptake) from a daily average of about 21°C to 13°C at the end, and 2) copper accumulation in the oysters, caused by leaching from the brass resin filter in the seawater line. After 4 weeks of uptake, most oyster meats were noticeably green. This color increased in intensity until the end of the experiment, although 15-20% of the oysters were still not green at the end. After 4 months in the

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system, copper in the oysters was measured at 1150 ppm on a wet weight basis, compared to 30.8 ppm in a new batch of oysters from the same source.

CHLORINATED HYDROCARBONS IN THE SEWAGE-AQUACULTURE SYSTEM

In order to assess the possible contribution of chlorinated hydrocarbons from domestic secondary sewage treatment effluent, components of the sewage-aquaculture system were sampled during the 1972 summer experimental period. Samples of sewage effluent, phytoplankton grown on that batch of sewage effluent and inorganic nutrient-grown phytoplankton were taken on three occasions. Pooled samples of 3 oysters, 2 clams and 5 mussels each were taken of animals fed sewage-grown phytoplankton and of ones fed only the phytoplankton contained in Woods Hole seawater, after 1 month, 2 months and 3½ months on those diets. Detritus was sampled after 1 month. Finally, the bivalves were sampled after flushing for 1½ months in ambient fall seawater. All samples were frozen for later analysis. Sampling and analytical methods were the same as those described for the PCB uptake experiment.

Preliminary results for the oysters are given in Table 1. Although no conclusions should be attempted on the basis of so few data, it is apparent that some PCB (on the order of 1/10 ppm) is present in all oysters examined, as well as smaller amounts of some DDT-family compounds. The levels are similar in oysters on all diets and they are well below the FDA tolerances of 5 ppm in foodstuffs. The source of

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these chlorinated hydrocarbons in the oysters is yet to be determined, but materials used in the aquaculture system are possibly responsible, in addition to Woods Hole dock seawater. PCB levels in this water on the order of 0.010-0.030 ppb have been measured by George Harvey.

Table 1

Oyster length cm	Diet type	Diet period (months)	PPB Wet Weight Aroclor 1242	Aroclor 1254	DDE	DDD	DDT
2	nutrient-fed algae	2½	53	93	nd*	nd	nd
5	-	0	nd	74	12	9	nd
6	unfiltered seawater	3½	10	34	0.5	1	nd
9	40% sewage-fed algae 60% nutrient-fed algae	3½	23	70	1	2	0.7
9	sewage-fed algae	3½	15	37	0.4	0.7	nd

* nd = none detected

It seems likely that PCB levels in the oysters were not elevated by the diet of domestic sewage effluent-grown phytoplankton for a few months. This would be in agreement with reports indicating that most of the PCB in sewage is found in the sludge, and most of the PCB remaining in the waste water effluent is associated with particulate matter (Holdan,

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1970; Schmidt et al., 1971 and Ahling and Jensen, 1970). It is probable that the affluent from secondary domestic sewage effluent contains comparatively low levels of these industrially disseminated pollutants.

A more comprehensive understanding of chlorinated hydrocarbons in the sewage-aquaculture system will be gained when the remainder of the samples have been analyzed, but a much longer experimental period is needed to be representative of the situation obtained when the bivalves have been fed for most of their lives on sewage-grown phytoplankton. Other factors which should be investigated include flushing patterns when bivalves contaminated to differing degrees are placed in a "clean" environment, the use of other sewage effluent (especially those including industrial wastes), and other possible organic contaminants.

PCB UPTAKE BY SARGASSUM ANIMALS

Both PCB isomer uptake and environmental levels were studied in animals of the Sargassum community of the Sargasso Sea, in order to compare them in open ocean and coastal organisms. Sargassum was swim-collected (on R/V Atlantis II Cruise 71 in the eastern Sargasso Sea) using glass bottles to avoid possible net contamination (Harvey 1972b). On shipboard, the animals associated with the Sargassum were shaken into enamel pans for sorting. The choice of experimental animals was limited to the crabs Portunus sayi and Planes minutus and the shrimp Leander sp. by availability and hardiness. Others, including flatworms, anemones, nudibranchs, gastropods and other shrimp were sampled at several locations

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and frozen for later analysis. PCB addition experiments on shipboard were conducted using the same PCB mixture and tanks as were used for the oyster experiments. The main differences were that the seawater was supplied directly from the ship's system without filtering and no additional phytoplankton was supplied. Analysis of the ship's seawater (by extracting 20 liters with ether-hexane) showed that PCB contamination was low. Analytical methods were the same as previously described. In an initial experiment beginning September 23, 1972, no uptake above background was detected in 1½ days for either crab or the shrimp Latreutes fucorum when exposed to 0.025 ppb of PCB. Therefore, the dose level was raised to 0.1 ppb and the experimental period was extended as long as possible. The second experiment, using animals collected at latitude 30°21'N and longitude 61°20'W, was begun on October 2, 1972. A piece of Sargassum was kept in the tank and changed every 2 days. Portunus and Leander superficially appeared to be healthy for the entire 6 days, although many Planes died during the experiment. The results for the uptake of the isomer with 6 chlorines per molecule are reported in Fig. 4. Uptake of isomers of lower degrees of chlorination was not visible above background levels. Uptake in these Sargassum animals is similar to the oysters in being greatest for the most highly chlorinated isomer. Uptake in Leander appeared to reach equilibrium after approximately 4 days, and it probably has a rapid flushing time. On the other hand, the crabs showed no indication of leveling off and probably flush more slowly. Concentration factors after 4 days of uptake were 800 x for Leander, 900 x

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for Portunus and 2200 x for Planes. Accurate analysis of background levels on shipboard was precluded by contamination in the analytical laboratory that was traced to the air conditioning system. Further analyses have not yet been carried out.

DISCUSSION

It should be emphasized that analyses from all phases of the PCB study are incomplete at this point. Sources of possible variability in the oyster uptake experiment include fluctuations in PCB concentration in the seawater, changes in the environment including water temperature and food concentration and type, nonrandom sampling, wet weight basis for calculations, high background to isomer peak ration in certain cases, and gas chromatographic reproducibility. A crude estimate would be that the values are within 20% of the accurate ones. This results in the greater variation seen in the data for the higher chlorinated isomers and at the higher PCB concentrations. The health of the oysters is questionable near the end of the experiment due to probable copper poisoning, and may have caused the lower lipid percentages as well as possibly affecting PCB levels. The brass filter that released the copper is being replaced with a glass one, if not eliminated entirely.

In spite of these difficulties, it is clear from the results at environmental PCB levels that: 1) the more chlorine in the PCB isomer, the more is accumulated by the oyster, the higher is the uptake rate and the longer is the time before equilibrium is reached. It follows that the

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the total amount of PCB accumulated by the oyster depends on the relative quantities of particular isomers in the mixture. In some organisms, some isomers of low degrees of chlorination are metabolized, but it has not been found that metabolism is roughly proportional to degree of chlorination as seen here. However, the similarity between concentration factors for isomers with 3 and 4 chlorines per molecule may be due to differential metabolism. 2) The amount of PCB accumulated by the oyster at equilibrium is directly proportional to the amount in the water. The concentration factor varied with the degree of chlorination of the isomer, but not with the amount in the water at these levels. This result indicates that for PCB accumulation in the oyster, equilibration with the water is an important factor.

Possible future experiments include studies of the effect of oyster size and environmental conditions on PCB uptake and flushing, uptake patterns from food rather than water, comparison of uptake of DDT compounds and PCBs in the same oysters and metabolism of isomers using ^{14}C-labeled purified isomers.

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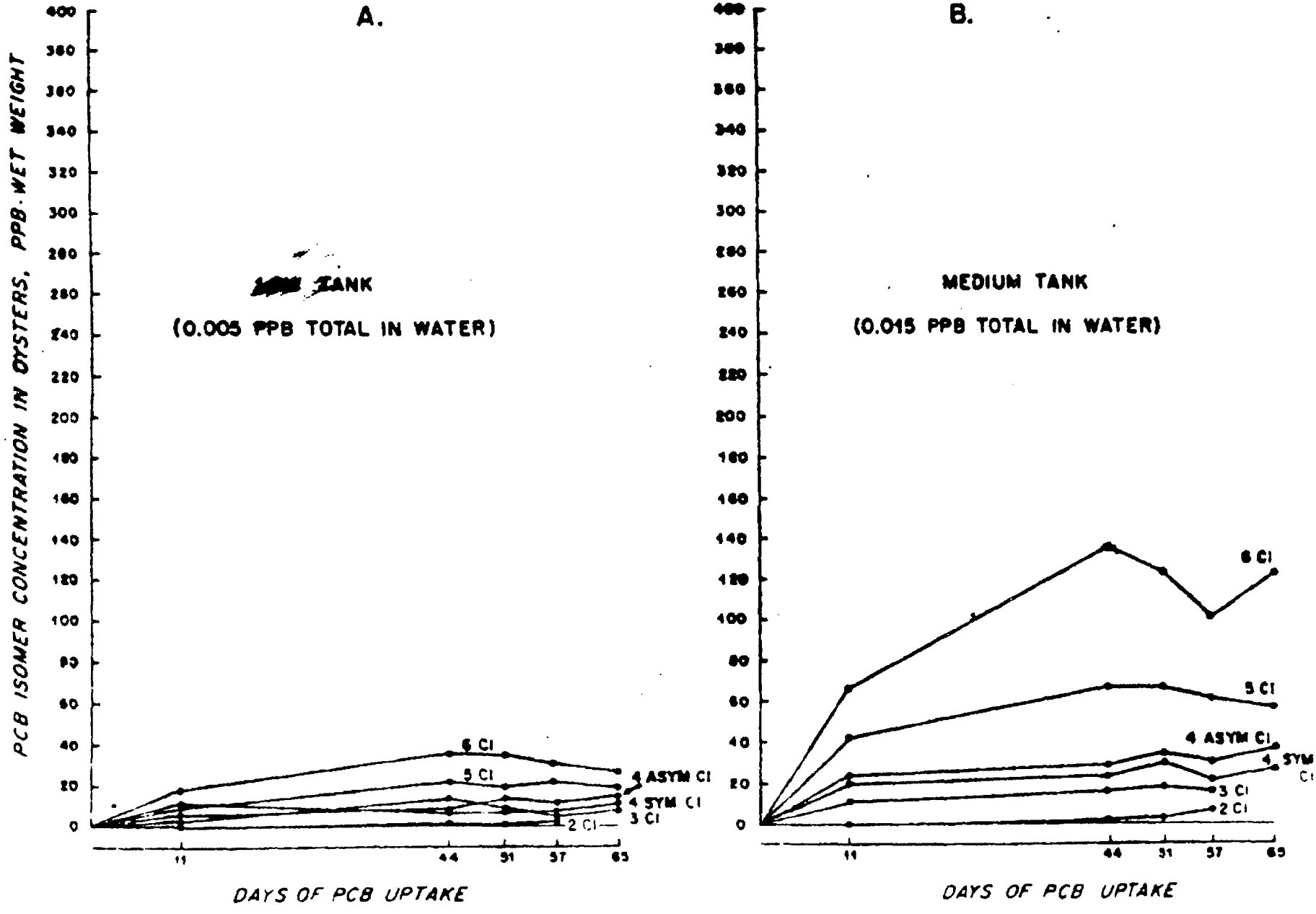

Figure 1 PCB ISOMER ACCUMULATION

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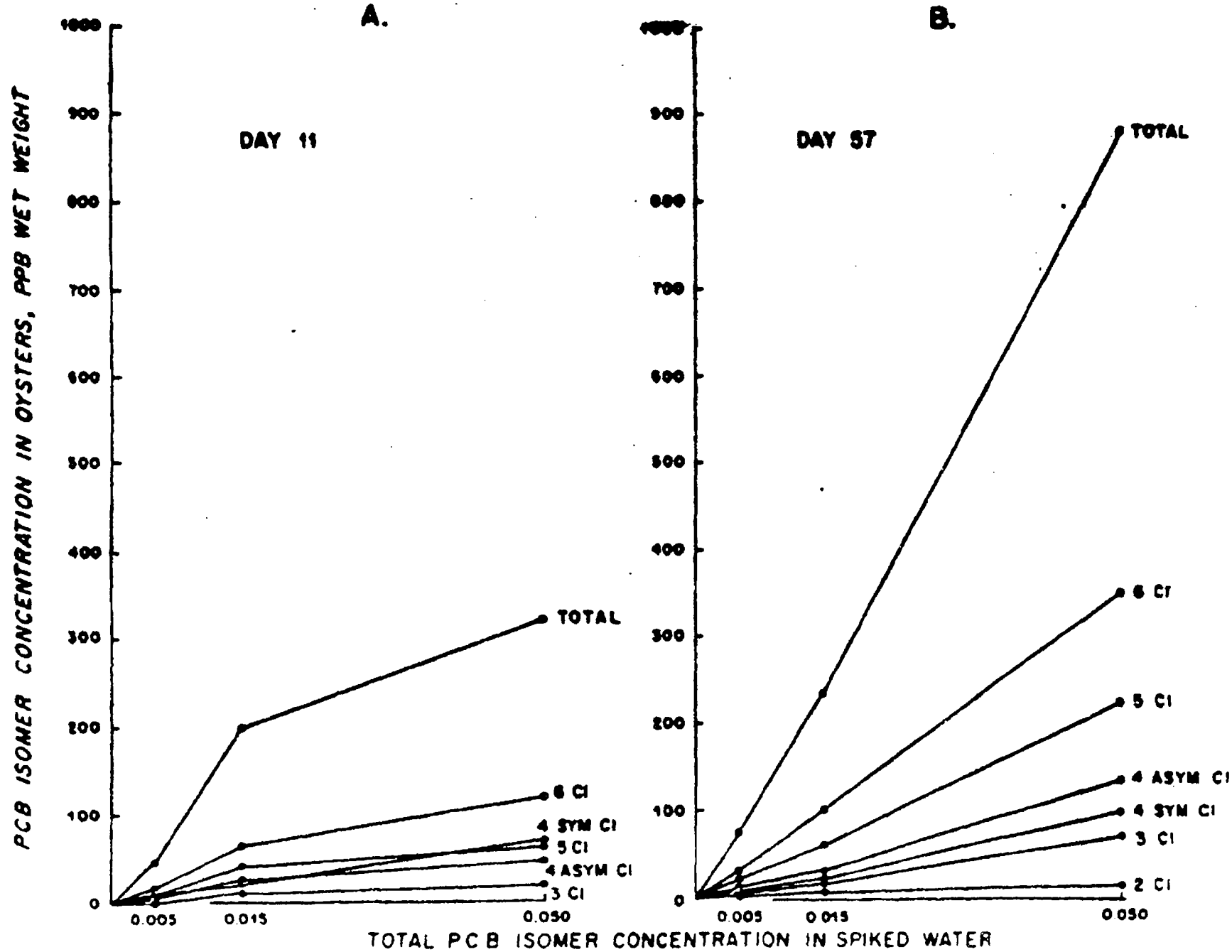

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Figure 2

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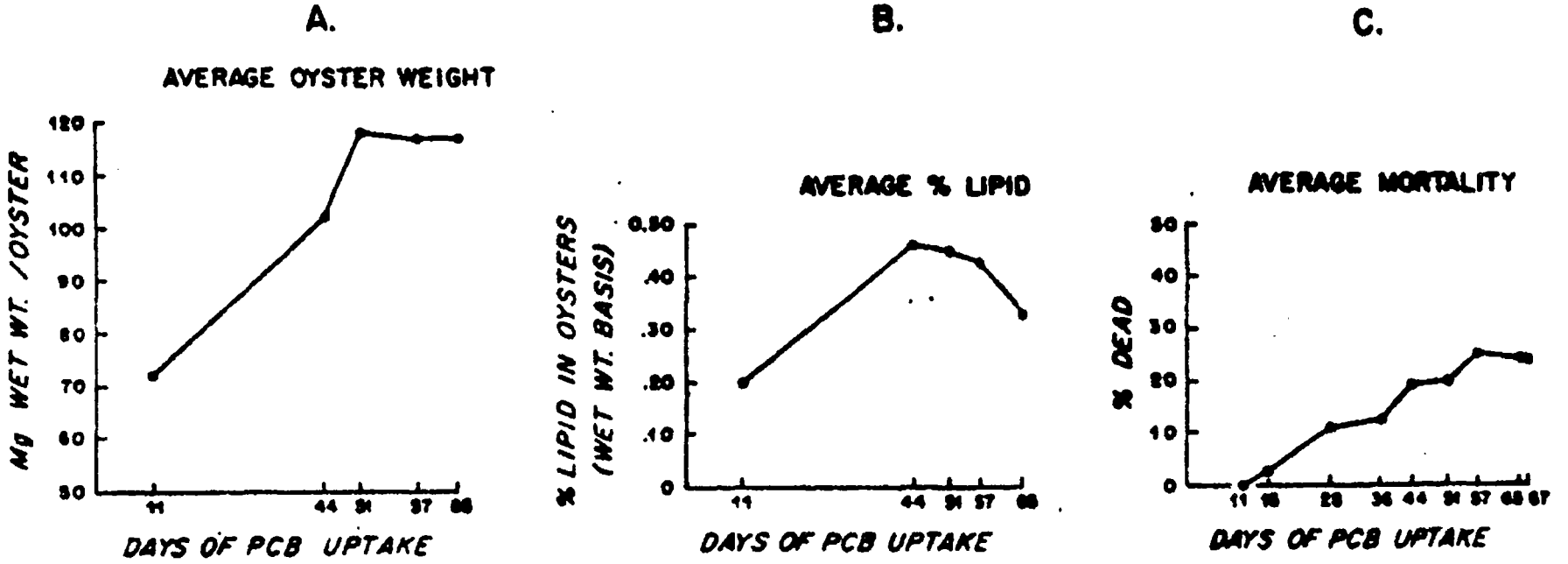

Figure 3: EXPERIMENTAL OYSTER STATISTICS

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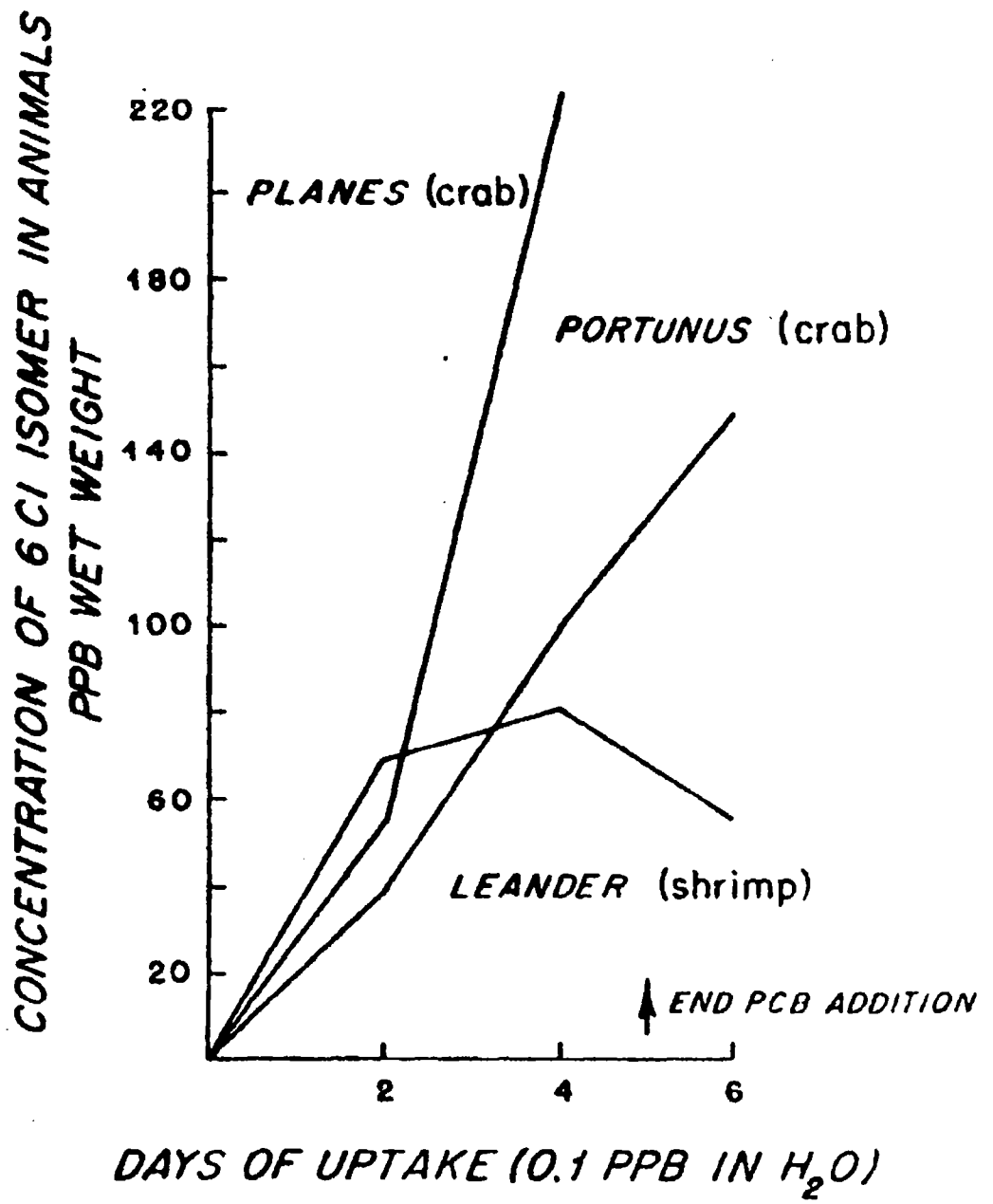

Figure 4:

UPTAKE OF 6 CI PCB ISOMER BY SARGASSUM ANIMALS

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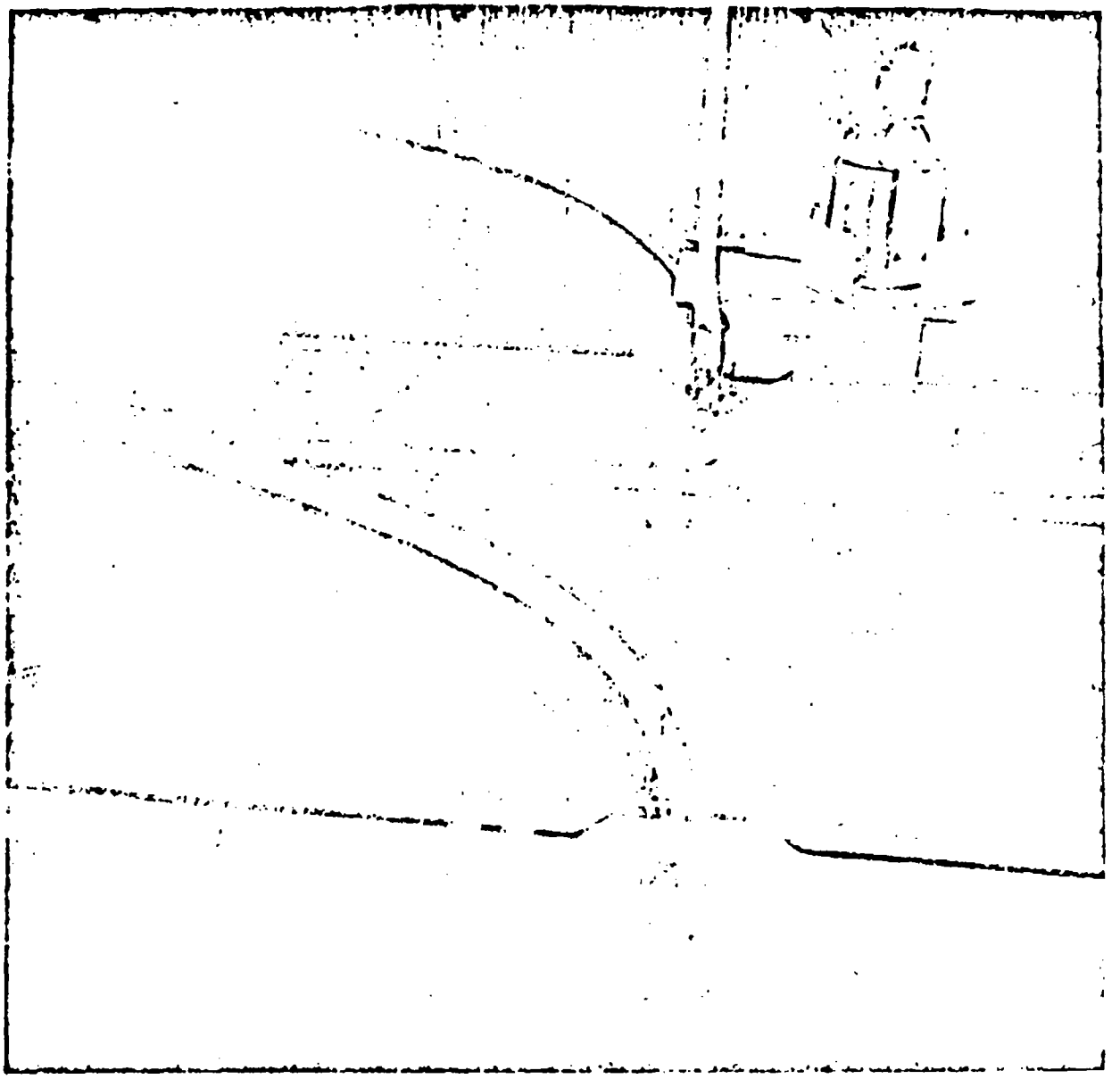

Fig. 5. Flowing system used for PCB uptake experiments.

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