Exhibit 27

Effects of Aroclor® 1254 on Laboratory-Reared Embryos and Fry of Sheepshead Minnows (*Cyprinodon variegatus*)[1]

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ABSTRACT

Artificially fertilized sheepshead minnow (Cyprinodon variegatus) eggs were exposed to logarithmic concentrations of Aroclor 1254 (10.0 to 0.1 µg/ℓ) in seawater averaging 30 C and 24 °/oo in an intermittent-flow bioassay. Fertilization was not affected but significantly fewer embryos developed in the 10.0 µg/ℓ and fewer fry survived in concentrations greater than 0.1 µg/ℓ. Fry were more susceptible to Aroclor 1254 than were juveniles or adults.

Sheepshead minnow eggs were artificially fertilized and maintained at temperatures from 15 to 35 C and in salinities from 0 to 35 °/oo to determine efficient culture conditions. Fertilization was not affected by the temperature and salinity ranges chosen, but hatching success was greatest (χ^2; α=0.01) at a temperature range of 24 to 35 C and a salinity range of 15 to 30 o/oo.

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Polychlorinated biphenyls (PCB's) occur in estuaries in many states (Butler, 1973), and the occurrence of one, Aroclor 1254, in nearby Escambia Bay and its acute toxicity to estuarine animals has been documented (Duke et al., 1970). Hansen et al. (1971) found 5 µg/l of Aroclor 1254 toxic to the juvenile estuarine fishes, pinfish (Lagodon rhomboides) and spot (Leiostomus xanthurus), in 14 to 45 day bioassays. We are unaware of data on the effects of this PCB on embryonic and larval stages of estuarine fishes.

The sheepshead minnow (Cyprinodon variegatus) is found in brackish waters from Cape Cod, Massachusetts to Brownsville, Texas (Hildebrand, 1917). It is important in estuarine food chains as a voracious omnivore and as food for predators such as croakers (Micropogon undulatus) and spotted seatrout (Cynoscion nebulosus) (Darnell, 1958). The size, hardiness, high fecundity, and generation time of the sheepshead minnow makes it a nearly ideal laboratory test fish.

In this paper, we determined water temperatures and salinities suitable for the culture of embryos and fry of the sheepshead minnow and observed the effect of Aroclor 1254 in water on the various life stages of this fish.

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METHODS AND MATERIALS

Temperature and Salinity Studies

Nearly identical methods were used in the temperature and salinity experiments to fertilize eggs. Adult fish were acclimated for one week in salt water averaging 25 C and 20 o/oo. The following week, female fish were given three intraperitoneal injections of 50 I.U. human chorionic gonatrophic hormone to induce egg maturation. Over 70% of the fish produced viable eggs. Eggs were manually stripped and deposited in 40 ml of filtered seawater. Only large, round, clear eggs were selected for fertilization. Testes from 7-10 acclimated males were excised, macerated in 20 ml of filtered seawater and mixed with eggs in a 100 ml beaker, allowing 30 minutes for fertilization. Seawater conditions used for fertilization in the temperature test was 25 C and 20 o/oo salinity while the salinity test conditions were 30 C and the test salinity. Each experiment lasted as long as was required for embryos to hatch plus an additional two weeks to determine survival of fry.

The effect of temperature on hatching was investigated by withdrawing 25 eggs from the 100 ml beaker with a wide-bore pipette and placing them in two, 1 dishes of 25 C, 20 °/oo seawater. Each of the dishes were partially submerged in a bath at 15, 20, 25, 30, or 40 C in one temperature test and 22, 24, 26 or 28 C in another. One hour later, when temperatures in the dishes equalled the bath temperature, fertilization was confirmed microscopically. The criterion for fertilization was cleavage. Dishes were checked daily during the 3-week static tests and dead or non-fertile eggs and larvae were removed. The criteria for embryo mortality was

an opaque, white, fungal growth and for the larvae a white coloration of the trunk musculature. Temperatures were monitored continuously and were within 1 C of the desired temperatures. Salinity alterations due to evaporation in both the salinity and temperature tests were compensated for daily by addition of distilled water.

Fertilization procedures for the salinity study were identical to those of the temperature study except that the number of eggs in each salinity varied from 35-75, depending on egg availability. Filtered seawater from Santa Rosa Sound, Florida was diluted to give salinities of 0, 5, 10, 15, 20, 25, 30 and 35 °/oo. The 30 and 35 °/oo concentrations were attained by adding Rila® salts to the water from the Sound. Salinities, checked daily with a TS® refractometer, were within 2 °/oo of the desired level.

Aroclor 1254 Exposure

An Aroclor 1254, intermittent-flow bioassay was accomplished using suitable culture conditions determined in the temperature and salinity tests. Eggs were fertilized in control water and placed in Petri dishes, four dishes (20 eggs per dish) in each PCB concentration and control. Fertility was confirmed after one hour. Temperatures in both tests averaged 29 C (range 27-31 C) and salinity averaged 24 °/oo (range 16-32 o/oo). The salinity fluctuated with that of Santa Rosa Sound. The toxicant dosing system used in this test was a modification of the apparatus of Brungs and Mount (1970). In our system the toxicant and carrier were injected into the delivery tube leading to each exposure tank (Figure 1). Our apparatus

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allowed us to retain the advantages of the Mount and Brungs (1967) dosing apparatus and to select any concentration of toxicant while maintaining the same concentration of carrier in each toxicant concentration.

Seawater used in this bioassay was pumped from Santa Rosa Round into a constant head box in the laboratory. An oscillating pump (1A), regulated by a rheostat, pumped water from the head box through a 20 μ -pore polypropylene filter and into the compartments in the dosing apparatus. After all compartments were filled, the self-starting siphon in the last compartment emptied into a receiving bucket (1D). The weight of the bucket being filled operated two micro-switches. One switch (1a) shut off the oscillating pump and the other (1e) activated a solenoid (1E) that raised the lever of the injection apparatus (1F). This rising lever was connected to gears that forced the barrels of six 50-ml syringes (1G) equal distances. Mixing bottles (1B) received injections from a control syringe containing the carrier (polyethylene glycol 200). Stock solutions of the carrier and Aroclor 1254 were mixed to give concentrations 0.1, 0.32, 1.0, 3.2 and 10.0 μg/ℓ of Aroclor per liter of water. The water then flowed to the distribution boxes (Figure 2) containing one large and four small compartments, each with a standpipe or siphon. Each compartment filled completely during each cycle and the siphon delivered water to the aquarium in which adults and juvenile fish were held. Water remaining in the four small compartments flowed slowly out through submerged holes in the standpipes into each of four containers holding eggs or fry. Each container received 100 ml of water per cycle. The number of cycles per day ranged from 100 to 140 and were sufficient to replace water in each egg container at least three times each day.

Toxic effects of polyethylene glycol 200 in this test (7.4 mg/ℓ) were not anticipated because (1) 1% v/v polyethylene glycol was not lethal to juvenile sheepshead minnows in 96 hour tests and (2) in earlier tests at this laboratory, toxicity of 5 µg/ℓ of Aroclor 1254 to pinfish was not changed by an increasing solvent concentration one hundred times (0.1 to 10 mg/ℓ) (Hansen, unpublished data).

Chemical Methods

Aroclor 1254 concentrations in test and control water were determined weekly by gas chromatography. Methods were the same as those of Nimmo et al. (1971), except that an OV-101 column was used and all peak heights were averaged for PCB quantification. Measured PCB concentrations in test water were typically 35% to 60% of nominal concentrations; control water contained no detectable PCB (<.03 µg/ℓ). Recovery efficiency of Aroclor 1254 was greater than 60%. Measured concentrations were not corrected for percentage recovery.

Dissolved oxygen was determined weekly using the modified Winkler method (Strickland and Parsons, 1968). Concentrations seemed adequate and above 50% saturation.

Statistical Methods

The Chi-square (χ^2) test was used in the statistical analysis of the data. In temperature and salinity studies the maximum positive response was compared with all other responses to determine the most efficient culture conditions. Data in the two temperature experiments were analyzed separately.

RESULTS AND DISCUSSION

Temperature Study

Temperature determined the number of days required for hatching (time-to-hatch) and affected the survival of embryos and fry of the sheepshead minnow (Table 1). Sheepshead minnow eggs hatched at water temperatures from 22-40 C but none hatched at lower temperatures. Hatching success was greatest at temperatures ranging from 24-35 C. Fry survival did not differ in the range of 22 to 35 C. The longest time-to-hatch was 15 days at 22 C, and decreased rapidly, leveling off at about 4 days at temperatures ranging from 30 to 40 C.

Salinity Study

Salinity affected survival of embryos and fry but did not affect time-to-hatch (Table 2). Survival to hatching was greater at salinities from 15 to 30 o/oo and fry survival did not differ in that range. Under natural conditions, adult and juvenile sheepshead minnows occur in a wide salinity range (Simpson, 1856). Our data show that embryos and fry also can survive in a wide salinity range.

Aroclor 1254 Study

Aroclor 1254 affected the survival of both embryos and fry of the sheepshead minnow, but had no effect on time-to-hatch. Embryos developed and hatched at all PCB concentrations, but hatching success at 10 µg/l was significantly less than that of control eggs. After the eggs hatched, survival of fry was significantly less than control fry at all PCB concentrations except 0.1 µg/l. Mortality increased with an increasing concentration of PCB. Three-week LC50 in exposed embryos and fry was estimated at 0.93 µg/l (S.E. = 0.17 µg/l). Fry did not die immediately

after hatching but were killed over most of the two-week period of post hatch exposure. Many of the dying fish developed fin rot as previously described in other PCB exposed fishes (Hansen et al., 1971).

Aroclor 1254 was more toxic to fry of sheepshead minnows that it was to juveniles, adults or to the fertilization of eggs (Table 4). In three week exposures to the same concentrations of this PCB, mortality of the juveniles was significantly greater (twenty-four percent) in the 10µg/ℓ concentration. Adult fish were not killed in a three-week exposure but became lethargic and exhibited fin rot. The concentration of PCB in the fry compared to that in the water (concentration factor) was nearly identical with that of adult fish. Concentration factors in fry ranged from 1.6 to 3.2×10^4 and those for adults ranged from 1.1 to 3.2×10^4. A twenty-four hour static test in 10.9µg/ℓ PCB showed no inhibition of fertilization of sheepshead minnow eggs. The greater sensitivity of early life stages of this fish to Aroclor 1254 stresses the need for bioassays designed to assess the effects of such chemical pollutants on these stages.

ACKNOWLEDGEMENTS

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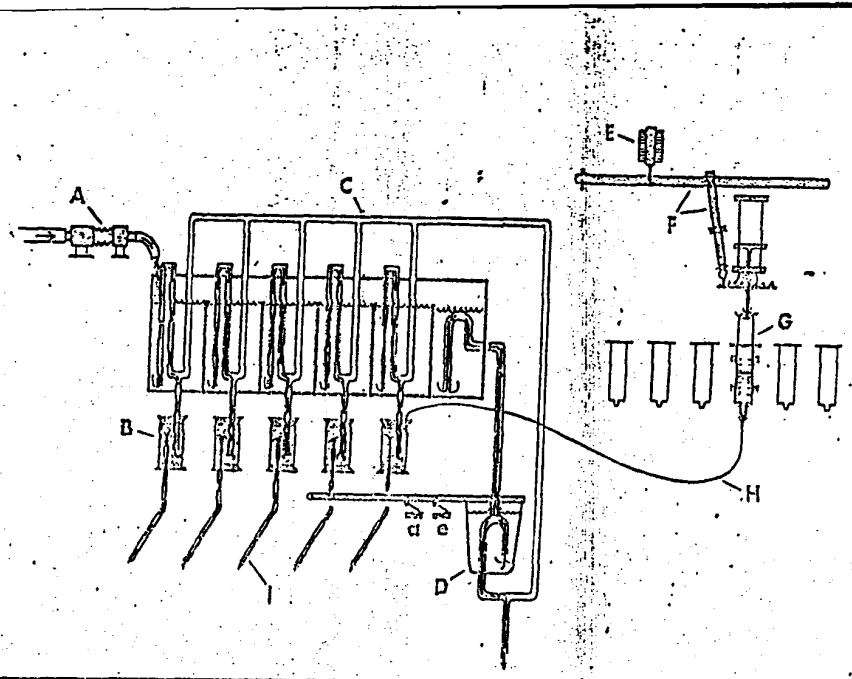


FIGURE 1. Dosing apparatus: A-oscillating pump; a-pump micro-switch; B-mixing bottles; C-vacuum lines; D-receiving bucket; E-solenoid; e-solenoid micro-switch; F-injector lever apparatus; G-50cc. syringe; H-toxicant delivery tubing; I-water delivery tube.

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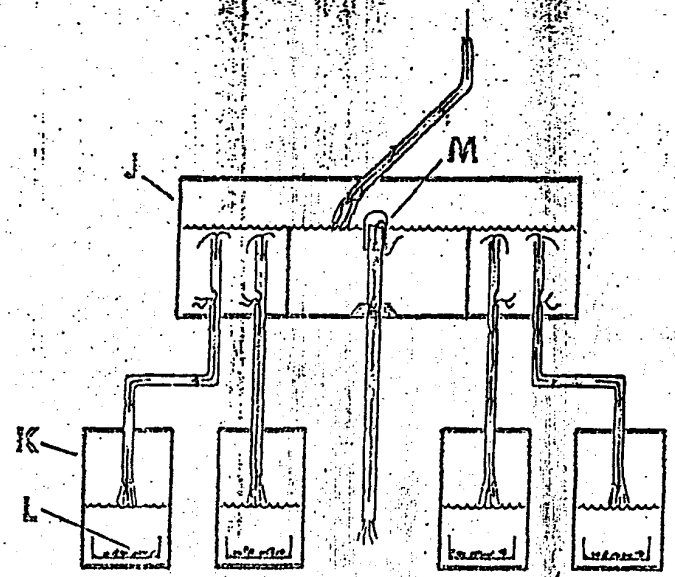


FIGURE 2. Distribution apparatus: J-distribution box; K-egg/larvae trays; L-Petri dishes; M-siphon.

Table 1.—Effect of temperature on fertility, time-to-hatch, hatch success and survival of fry for two weeks following hatching of sheepshead minnows (Cyprinodon variegatus). Temperatures varied ± 0.5 C; salinity averaged 20 o/oo, ± 2 o/oo.

Temperature (°C)	Eggs		Hatching		Fry Survival %
	Number	Fertile %	Days #	Survival %	
15	50	92	No hatch	0**	0**
20	50	98	No hatch	0**	0**
22	50	100	15.0	28.0**	100
24	50	96	9.0	50.0	100
25	50	98	7.0	83.7	80
26	25*	92	7.0	78.0	100
28	50	90	6.0	55.5	92
30	50	94	4.5	82.9	90
35	50	94	4.0	70.2	100
40	50	94	4.0	8.5**	50

* Loss of 25 eggs due to spillage.

** Significantly less than the greatest fertility or survival (χ^2;α = 0.01).

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Table 2.—Effect of salinity on fertility, time-to-hatch, hatch success and survival of fry for two weeks following hatching of sheepshead minnow (Cyprinodon variegatus). Temperature 30 ± 2 C; salinity varied ± 1 o/oo.

Salinity o/oo	Eggs		Hatching		Fry Survival %
	Number	Fertile %	Days #	Survival %	
0	60	35	No hatch	0*	0
5	35	46	7.0	12*	50
10	75	35	7.0	35*	89
15	35	51	5.5	67	92
20	75	44	6.0	67	100
25	35	54	5.5	53	90
30	75	49	5.5	73	93
35	35	37	6.0	30*	50

* Significantly less than the greatest fertility or survival (χ^2 α = 0.01).

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Table 3.--Effect of Aroclor 1254® on fertility, time-to-hatch, hatch success and survival of fry for two weeks following hatching of sheepshead minnow (Cyprinodon variegatus). Temperature averaged 30 C (range 27-31 C); salinity averaged 24 o/oo (range 16-32 o/oo).

Concentration (µg/ℓ)		Eggs		Hatching		Survival
Nominal	Measured	Number	Fertile %	Days #	Survival %	%
Control	<0.03 µg/ℓ	160	86	7.0	79	89
0.1	0.06	160	86	6.5	69	95
0.32	0.16	160	86	7.0	73	62*
1.0	0.36	160	91	7.0	82	63*
3.2	1.04	160	85	7.0	75	40*
10.0	3.48	160	91	6.5	57*	8*

* Significantly different from control fish (χ^2 α = 0.01).

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Table 4.--Relative susceptibility of various life stages of sheepshead minnows (Cyprinodon variegatus) to Arocior 1254® in a flow-through system. Criteria are infertility of eggs and death of embryos, larvae, juveniles, and adults.

Life Stage	Exposure (days)	Concentration (µg/l)	
		Minimum Affecting	Maximum Not affecting
Egg fertilization*	0.4	----	10.0
Embryos	7	10.0	3.2
Larvae	21	0.32	0.1
Juveniles	21	10.0	3.2
Adults	21	----	10.0

* 24-hour static test.

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