EFFECTS OF COPPER ON EARLY LIFE HISTORY STAGES OF NORTHERN ANCHOVY, *ENGRAULIS MORDAX*

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ABSTRACT

The sensitivity to copper of embryonic and larval stages of the Northern anchovy, *Engraulis mordax*, was determined using a flow-through bioassay system. Northern anchovy embryos were exposed continuously from 8 to 10 hours after fertilization until hatching, and the larvae were exposed within 12 hours after hatching until yolk-sac absorption. During the testing both total copper concentrations and the percent copper in labile forms were determined. From the cumulative mortality versus measured copper exposure data, a series of median lethal concentrations (LC_{50}) were determined. These LC_{50} values were used to construct comparative toxicity curves.

The northern anchovy life stage most sensitive to copper was the embryonic stage. For northern anchovy embryos the 12-hour LC_{50} was 200 μg Cu/l, and the estimated incipient lethal concentrations (ILC_{50}) was 190 μg Cu/l; a sensitive period of embryonic development was noted prior to closure of the blastopore. The 12 hours, 24 hours, and ILC_{50} for northern anchovy larvae were 460, 400, and 370 μg Cu/l.

Copper is one of the wastes commonly discharged into coastal waters that has been shown to be toxic to marine fishes (Becker and Thatcher 1973; Lewis and Whitfield[3]). Increased copper concentrations in coastal marine waters have resulted from the release of municipal wastewater (Schafer[4]), power plant effluents (Young et al.[5]), and marine antifouling paints (Young and Alexander[6]). In polluted waters, concentrations have been recorded as high as 16,800 μg Cu/l in municipal waste effluents (Schafer footnote 4) and 1,800 μg Cu/l during start up of a power plant (Martin et al. 1977).

One important factor in the toxic effect of copper on marine fishes is the life history stage when the exposure occurs. Few studies have examined the comparative sensitivities of the major life stages of marine fishes: embryo, larva, and adult. The spot, *Leiostomus xanthurus*, was found to be more sensitive to copper in the embryonic stage than in the larval stage (Engel et al. 1976). The incipient lethal concentration (ILC_{50}—that concentration that kills 50% of a population during an exposure sufficiently long that acute lethal action has ceased [Sprague 1969]) for Pacific herring, *Clupea harengus pallasi*, embryos exposed to copper was found to be approximately 30 times lower than the ILC_{50} for Pacific herring larvae (Rice and Harrison 1978) and 7 times lower than the ILC_{50} for Pacific herring adults (Harrison and Rice[7]). Natural mortalities that occur during the early life stages have been suggested to be a major factor in reducing the size of a given year class of fish (May 1974; Cushing 1975; Vaughan and Saila 1976). Pollutants that have an impact on the survival of fish embryos or larvae might further reduce the size of a given year class of fish.

In addition to the life stage, the chemical form of copper to which fishes are exposed may play an important role in the toxic response (Lee 1973; Neff and Anderson 1977; Chapman and McCrady[8]). Copper in seawater can exist in many

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forms. We will use the terminology proposed by Batley and Florence (1976). According to these authors, labile copper, as defined by experimental conditions, includes ionic, as well as some dissociable, complexed forms; bound copper is that fraction of the total copper which is not labile and includes soluble copper-organic complexes, copper bound to high molecular weight organic materials, and copper occluded in or adsorbed onto highly dispersed colloids. Although current copper emission standards are defined in terms of the total copper concentration in the water (Anonymous 1972, 1976) complexation of copper has been shown to reduce its toxicity to marine organisms (Lewis et al. 1972, 1973; Davey et al. 1973; Sunda and Guillard 1976; Harrison et al.[9]). Ionic copper has been suggested as the form most toxic to freshwater fishes (Pagenkopf et al. 1974). During our testing of the early life stages of the northern anchovy, we determined both total copper concentrations and the percent copper in the labile forms.

Northern anchovy, *Engraulis mordax*, is a pelagic, filter-feeding fish that spawns in upwelling waters along the Pacific coast of the United States and Mexico (Ahlstrom 1960). During recent years, the northern anchovy catch has been the third largest commercial catch on the Pacific coast (McAllister 1976; Pinkas 1977). Having conducted earlier tests on the sensitivity to copper of Pacific herring during its early life stages (Rice and Harrison 1978), we set two objectives for the present study: to conduct similar tests on the northern anchovy and to compare the sensitivities of these two species of fish during their early life histories. We continuously exposed northern anchovy embryos and larvae to copper over a range of concentrations and then constructed comparative toxicity curves.

METHODS

Northern anchovy embryos were collected in San Francisco Bay, Calif., between the Tiburon Peninsula and Angel Island. Collections and tests were carried out over a period of 2 yr. Collections were made with a 0.5 m, 505 μm mesh, nylon plankton net, towed for 2 min just below the surface of the water. Collections from each tow were placed into a plastic bag half full of seawater; the bag was inflated with air and then held in an insulated ice chest containing seawater from the collection site. Water temperature at the collection site was between 17° and 18.5° C; upon arrival at Lawrence Livermore Laboratory the temperature of the water in the ice chest was always <19° C.

The water for the bioassay system was obtained from the University of California Marine Station at Bodega Bay, Calif. This water is pumped from the ocean off the open coast in an area that receives little anthropogenic input. The water contains low levels of trace metals, dissolved organics, and particulate material. The collected seawater was stored in a 40,000 l underground tank and passed through a filter with 1.0 μm openings prior to experimental use.

Two embryo tests were conducted during 1976 (test I: 7 July 1976; test II: 23 July 1976) and one embryo test during 1977 (test III: 27 June 1977). All larval tests were conducted during 1977 (test I: 6 June 1977; test II: 13 June 1977).

All transfers of embryos or larvae were carried out with a large-bore, polished glass pipette. During embryo tests, healthy embryos estimated to be 8-10 h old (stages IV-V, Ahlstrom 1943) were placed either directly into exposure chambers containing seawater with known concentrations of copper or allowed to hatch in control seawater. Larvae used during larvae tests hatched within the 12-h period preceding the test; hatched larvae were placed directly into exposure chambers containing seawater with known concentrations of copper. Approximately 50 embryos or 30 larvae were used in each exposure chamber.

Anchovy embryos and larvae were exposed to copper in 500 ml clear glass, flow-through, exposure chambers (Figure 1) which, in turn, were immersed in a water bath (mean temperature: 16.8°±1.0° C). Seawater containing a known concentration of copper, as copper chloride, was pumped into each chamber from a 19 l plastic jug at a rate of 5 ml/min. About 5 h were required to replace 95% of the water in the exposure chamber; the mixture of seawater and copper in the 19 l plastic jugs was prepared daily.

The height of the water in the exposure chambers was maintained by a constant-level, outflow siphon. The mouth of the siphon, located at the base of the exposure chamber, was covered with nylon netting (265 μm pore size) to prevent the loss

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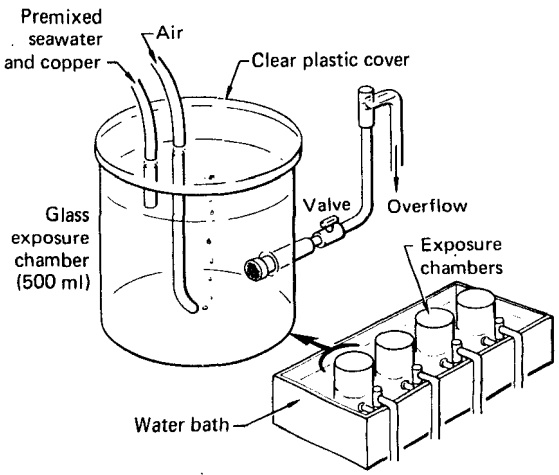

FIGURE 1.—Diagram of the exposure chamber and flow-through bioassay system used to expose northern anchovy embryos and larvae to copper.

of organisms from the chamber. The bottom outlet from each exposure chamber was fitted with a valve that could be closed to allow removal of the chamber from the water bath. This was important because during each observation the exposure chambers were removed and illuminated from the side. In this manner both live and dead organisms could be examined. Observations were made every 2-4 h during the copper exposures. During embryo exposures, a gentle stream of bubbles was delivered to the bottom of the exposure chamber; during larval exposures, no aeration was used. Overhead illumination was provided by the fluorescent lighting in the laboratory and followed the regular ambient photoperiod. The number of embryos or larvae exposed during each test is given in Tables 1 and 2.

Exposures continued until 1) all animals died, or 2) in the case of the embryos, until hatching was complete, or 3) in the case of the larvae, until the

TABLE 1.—Samples mortality data used to calculate LC_{50} values for northern anchovy embryos exposed to different copper concentrations. Chi-square tests used the null hypothesis that the relationship between dose and response followed the logit model.

Exposure time (h)	Control[1]	Measured exposure concentration (μg Cu/l ± SD) 92 ±8	116 ±14	171 ±31	177 ±30	190 ±12	197 ±41	242 ±46	272 ±22	285 ±19	531 ±66	564 ±39	589 ±28	[2]LC_{50} (μg Cu/l)	Chi-square value[3]	df
	Proportion dead[4]															
2	0.00	0.04	0.02	0.00	0.02	0.03	0.16	0.21	0.03	0.27	0.77	0.93	0.85	409±22	29.14**	11
4	.00	.04	.03	.01	.14	.24	.32	.44	.29	.61	.96	.93	.94	292±16	38.82***	11
6	.00	.14	.03	.08	.14	.47	.44	.63	.51	.86	.98	.96	.98	235±10	40.67***	11
8	.00	.21	.09	.12	.25	.59	.49	.72	.64	.88	.98	1.00	1.00	213± 8	29.24**	11
12	.00	.25	.09	.15	.27	.62	.62	.76	.75	.95	1.00			199± 8	40.06***	9
18	.00	.27	.09	.17	.31	.67	.65	.80	.78	.98	1.00			193± 7	40.65***	9
25	.00	.27	.09	.17	.38	.67	.69	.84	.85	.98	1.00			186± 6	40.49***	9
32	.00	.27	.09	.17	.39	.67	.70	.84	.88	.98	1.00			185± 9	41.88***	9
No. organisms exposed	260	28	90	94	124	34	161	112	66	96	126	29	104			

[1]Labile copper = 1.3 (SD = 0.1) μg Cu/l.
[2]±95% confidence limit.
[3]**$P \leq 0.01$; ***$P \leq 0.001$.
[4]Corrected for control mortality.

TABLE 2.—Samples mortality data used to calculate LC_{50} values for northern anchovy larvae exposed to different copper concentrations. Chi-square tests used the null hypothesis that the relationship between dose and response followed the logit model.

Exposure time (h)	Control[1]	Measured exposure concentration (μg Cu/l ± SD) 277±6	289±29	427±7	531±42	[2]724	[3]LC_{50} (μg Cu/l)	Chi-square value[4]	df
	Proportion dead[5]								
4	0.00	0.14	0.02	0.42	0.49	0.76	523±58	7.20	4
8	.00	.15	.18	.49	.53	.84	485±54	4.02	4
12	.00	.16	.16	.50	.65	.87	457±46	2.61	4
20	.00	.07	.30	.59	.79	1.00	412±34	7.35	4
26	.00	.06	.30	.71	.85	1.00	391±31	7.61	4
32	.00	.08	.41	.75	.87	1.00	375±31	11.15*	4
40	.00	.11	.41	.73	.87	1.00	374±32	8.86	4
46	.00	.01	.51	.79	.87	1.00	372±30	27.28***	4
No. organisms exposed	74	71	33	61	60	26			

[1]Labile copper = 1.3 (SD = 0.1) μg Cu/l.
[2]Single measurement.
[3]±95% confidence limit.
[4]*$P \leq 0.05$; ***$P \leq 0.001$.
[5]Corrected for control mortality.

yolk sac was absorbed. The criterion for embryo mortality was the appearance of opacity of the embryo, and the criterion for larval mortality was failure to respond to a prod with a polished glass rod. Cumulative mortality with time, percentage hatching, and the stage of development at mortality were taken to be indices of the toxic effect of copper.

Total copper concentrations were measured every other day during all tests. Labile copper concentrations were measured every other day during embryonic test III and larval tests I and II. Total copper in samples containing >200 μg Cu/l was determined by direct aspiration of seawater into the flame of a Model 303 Perkin Elmer[10] atomic absorption spectrophotometer (AAS) with a deuterium background corrector; total copper in samples containing <200 and >10 μg Cu/l was determined by direct injection of a sample aliquot into an HGA 2100 model graphite furnace after 1:1 dilution of the sample with ultrapure 2 N HNO_3. Labile copper, defined operationally as that fraction passing through a 0.45 μm filter and retained by NH_4-Chelex resin, was determined by the method of Riley and Taylor (1968). Eluants from the columns were analyzed directly in the flame or in the graphite furnace of the AAS.

The mean total copper concentrations measured during each test are given in Tables 1 and 2. The percentage of the total copper in the labile form for all concentrations averaged 96% (SD = ±2.60). The mean pH of the exposure seawater for all tests was 8.06 (SD = ±0.05).

The primary measure of toxicity for this study was the copper concentration resulting in 50% mortality over a given time (median lethal concentration, LC_{50}). This toxicity measure was determined by performing weighted least squares estimates and maximum likelihood estimates for the parameters α and β in the logit model:

$$P(x) = \frac{e^{\alpha + \beta x}}{1 + e^{\alpha + \beta x}}.$$

The linear transform of the logistic function is $\text{logit}\,P = \ln P(x)/1-P(x) = \alpha + \beta x$; thus if $\text{logit}\,P$ is plotted against x, the points should fall on a straight line with α as the intercept and β as the slope (Berkson 1953). The weighted least squares estimates for α and β were found first and then used as the initial estimates for the maximum likelihood estimates (Koshiver and Moore 1979).

In our calculation of LC_{50}, $P(x)$ is the proportion responding at dose x. Our method followed that outlined by the American Public Health Association (1976) except that the logit analysis was used in place of a probit analysis. For each observation time, an estimated LC_{50} value was determined. The series of LC_{50} values obtained were used to construct a toxicity curve that was used to estimate the incipient lethal concentration (lethal threshold concentration, ILC_{50}; Sprague 1969).

RESULTS

Northern anchovy embryos continuously exposed to copper showed high mortality during the first 8-10 h of exposure (Table 1). After 10 h, the mortality rate was relatively constant until hatching (Figures 2-4). The embryos took two different forms at mortality. The first form (Type I) was observed predominantly during the initial 8-10 h of exposure and accounted for varying proportions of the total mortality, depending on the copper exposure concentration (Table 3). These embryos appeared to have had epiboly disrupted; the yolk was naked and a deformed opaque mass of protoplasm was found at the animal pole. The second form (Type II) appeared similar to normally developing embryos (the embryo encircling the yolk sac), except for an opacity of the embryo. In em-

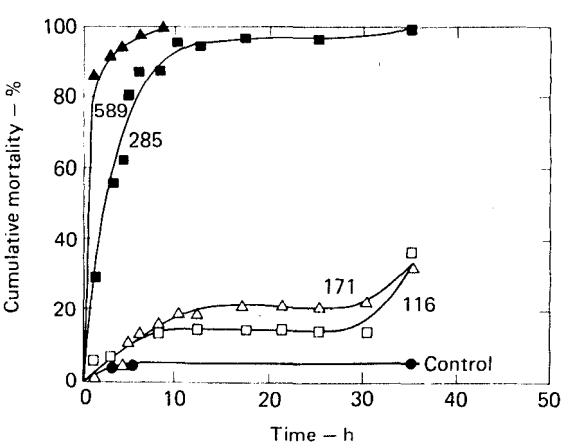

FIGURE 2.—Percentage cumulative mortality of northern anchovy embryos continuously exposed to copper during test I: numbers next to curves are the exposure concentrations in μg Cu/l.

[10]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA, or the University of California.

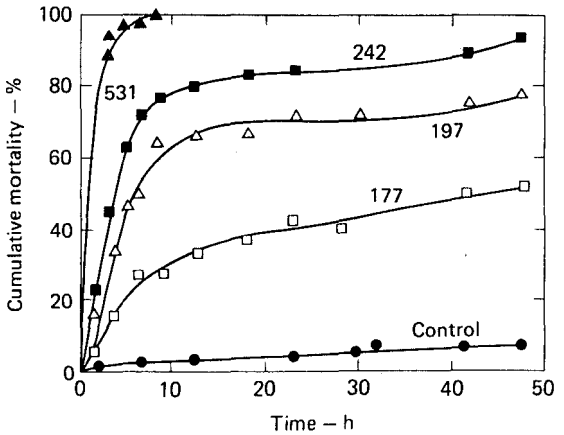

FIGURE 3.—Percentage cumulative mortality of northern anchovy embryos continuously exposed to copper during test II: numbers next to curves are the exposure concentrations in μg Cu/l.

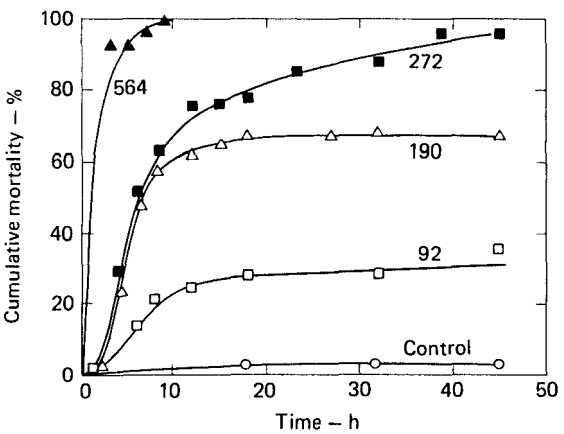

FIGURE 4.—Percentage cumulative mortality of northern anchovy embryos continuously exposed to copper during test III: numbers next to curves are the exposure concentrations in μg Cu/l.

TABLE 3.—Types of mortality and the percentage hatching of northern anchovy embryos exposed to different concentrations of copper.

Mean Cu concentration (μg/l)	Tests pooled	Embryos showing type of mortality (%)		Hatching (%)
		Type I[1]	Type II[2]	
Control	I, III	3	4	93
92	III	4	32	64
177	II	34	20	46
194	II, III	63	17	20
257	II, III	60	35	5
548	II, III	96	2	2

[1]Epiboly disrupted, the yolk naked and a deformed opaque mass of protoplasm at the animal pole.
[2]Dead after epiboly, embryo appears normally developed.

bryos with either type of mortality the chorion was clear at the time the embryos were removed from the exposure chambers. However, during preliminary testing, we noted that the chorion became opaque when dead embryos were allowed to remain in copper concentrations as low as 100 μg Cu/l for a period of time. Embryo mortalities of both types were found at the bottom of the exposure chambers whereas normal embryos were found at or near the surface of the water, except just before hatching when they tended to sink. The estimated mean hatching time from the start of copper exposure was 32, 33, and 37 h for embryo tests I, II, and III, indicating that in each test the embryos were exposed during similar developmental periods. Hatching success was high for controls and decreased with increases in copper exposure concentration.

Larval control mortalities were high, but followed the general pattern for larvae not fed during yolk-sac absorption (O'Connell and Raymond 1970; Lasker et al. 1970). Northern anchovy larvae continuously exposed to concentrations <200 μg Cu/l consistently showed better survival than did the controls (Table 3) (Figures 5, 6). Though he offered no explanation, Benoit (1975) found bluegill, *Lepomis macrochirus*, larval survival greater at 12 μg Cu/l than in the controls. It is possible that low levels of copper exposure increased survival of both the northern anchovy and bluegill larvae by inhibiting harmful microbial populations. The period of yolk absorption was estimated to be between 24 and 30 h from the start

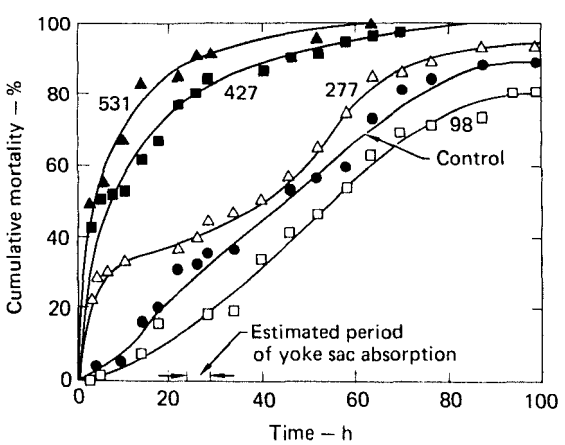

FIGURE 5.—Percentage cumulative mortality of northern anchovy larvae continuously exposed to copper during test I: numbers next to curves are the exposure concentrations in μg Cu/l.

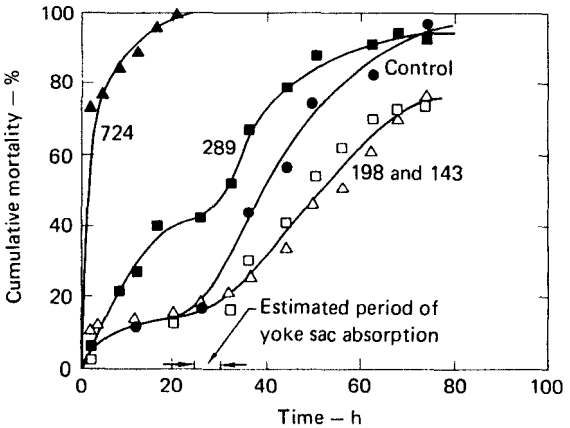

FIGURE 6.—Percentage cumulative mortality of northern anchovy larvae continuously exposed to copper during test II: numbers next to curves are the exposure concentrations in μg Cu/l.

of larval exposure. During exposures >200 μg Cu/l, synergism between copper toxicity and starvation may have played a role in the mortality and the shape of the 277 and 289 μg Cu/l mortality curves (Figures 5, 6) may show this effect.

No obvious abnormalities were noted in the dead larvae. Before death, larvae tended to sink to the bottom of the exposure chambers and often exhibited head shaking movements and whip movements in which head and tail met.

Examples of the cumulative mortality data used to calculate LC_{50} values and to generate the toxicity curves (Figure 7) are given in Tables 1 and 3. Chi-square values for embryo cumulative mortality curves at every observation time were significant. This variation from the logit model may possibly be due to changes in copper sensitivity as the embryos developed. Chi-square values for larval cumulative mortality curves at different observation times indicate a better fit to the logit model.

The embryonic and larval toxicity curves reflect several developmental changes in sensitivity (Figure 7). A slight increase in copper sensitivity can be seen in the embryos during hatching. When we estimated the embryo ILC_{50}, we considered only mortalities before hatching. For embryos, the estimated ILC_{50} was found to be 190 μg Cu/l, and was reached approximately 24 h after the start of copper exposure. The sudden increase in mortality of the larvae at about 40 h probably was the result of starvation. Only mortalities before this time were considered in the larval estimated ILC_{50}. The ILC_{50} for the northern anchovy larvae was found to be higher than for embryos: 370 μg Cu/l copper, and was reached about 32 h after the start of copper exposure. The estimated 24-h LC_{50} was 398 μg Cu/l.

DISCUSSION

We found the embryonic stage of the northern anchovy to be more sensitive to copper than the larval stage. This is in keeping with the majority

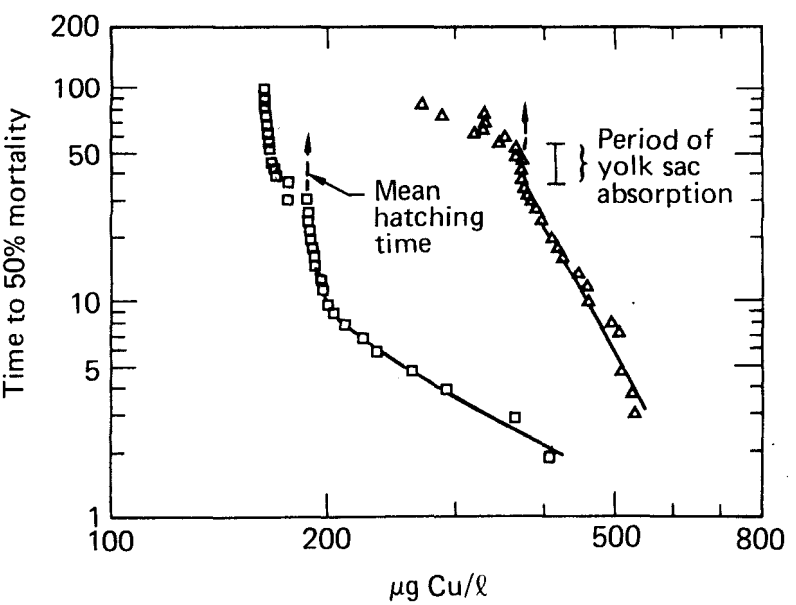

FIGURE 7.—Toxicity curves for nothern anchovy embryos and larvae continuously exposed to copper.

of previous studies examining the sensitivity to copper of marine fishes' early life history stages (Engle et al. 1976; Blaxter 1977; Rice and Harrison 1978). In contrast, studies examining the copper sensitivity of various life stages of freshwater fishes revealed that the larval stages are the most sensitive to copper (Hazel and Meith 1970; McKim and Benoit 1971; Gardner and LaRoche 1973; Benoit 1975; McKim et al. 1978; O'Rear[11]). This difference in comparative sensitivity between embryos and larvae of freshwater and marine fishes suggests that caution should be exercised in applying the extensive results of toxicity tests on freshwater fishes to marine fishes.

The adult northern anchovy and Pacific herring are similar in form and in behavior, but their reproductive strategies are quite different. The northern anchovy spawns pelagic eggs into offshore waters; the 1.0 × 0.5 mm diameter egg is covered by an elliptical, transparent chorion. Northern anchovy eggs hatch in about 48 h at 17° or 18° C into fragile, unpigmented larvae, 2.5-3.0 mm long (Ahlstrom 1956). The Pacific herring spawns demersal, adhesive eggs on shallow intertidal substrates; the 1.3-1.6 mm diameter egg is covered by a thick, three-layered, chorion (Blaxter and Holliday 1963). Herring eggs hatch in 7-9 d at 14° C into pigmented larvae 5.0-6.0 mm long (Alderdice and Velsen 1971). Comparisons of the sensitivities of the early life stages of these two fish may prove useful for predicting the impact of copper on broad groups of fishes. For comparisons between the copper sensitivity of northern anchovy and Pacific herring embryos and larvae, the data on herring sensitivity are taken from our earlier study (Rice and Harrison 1978).

It might be expected that the fragile northern anchovy embryo would be more sensitive to copper than the larger, tougher Pacific herring embryo; in fact, however, the opposite appears to be the case. The ILC_{50} for northern anchovy embryos was approximately six times higher than that for Pacific herring embryos. The results of Engel and Sunda (1979) showed a similar pattern; relatively tough benthic spawned eggs of the silverside, *Menidia menidia*, were found to be more sensitive to copper than the more fragile pelagic eggs of the spot.

The differences in sensitivity seen in the two embryos may be the result of differences in the chorionic structure and the developmental period during copper exposure. The chorion of Atlantic herring, *C. h. harengus* (Rosenthal and Sperling 1974), and another demersal adhesive egg, the Baltic garpike, *Belone belone* (Dethlefsen et al. 1975), have been shown to concentrate cadmium. The chorion of the Pacific herring may be the site of mechanisms to accumulate metals, mechanisms that may be reduced or lacking in the northern anchovy. Changes in sensitivity during development were seen in both the northern anchovy and Pacific herring embryos. The high percentage of northern anchovy mortalities during epiboly indicates that this period of development might be more sensitive to copper than the later developmental periods. Increased copper sensitivity during this period also was found for winter flounder, *Pseudopleuronectes americanus*, (Cardin[12]). The sensitive period for the Pacific herring embryo appeared to be about 96 h after fertilization, well beyond epiboly.

Differences in sensitivity were also seen between the two larvae. The fragile northern anchovy larvae were about three times more sensitive to copper than the Pacific herring larvae.

Both northern anchovy and Pacific herring larvae displayed spasms before death at the higher copper concentrations to which they were exposed. Such spasms during copper poisoning have been suggested to be similar to those seen in Wilson's Disease (Baker 1969).

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