COPPER SENSITIVITY OF PACIFIC HERRING, *CLUPEA HARENGUS PALLASI*, DURING ITS EARLY LIFE HISTORY

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ABSTRACT

Embryos and larvae of the Pacific herring, *Clupea harengus pallasi*, were exposed to copper, using a flow-through bioassay system. Herring embryos were exposed continuously from 12 h after fertilization until hatching, and larvae were exposed from the time of hatching until yolk sac absorption. Embryos were also exposed to 36-h duration pulses of copper in order to evaluate the sensitivy of different developmental stages of herring embryos to copper. Pulsed exposures started at 62, 98, or 136 h after fertilization. The following measurements were taken as indices of the toxic effects of copper: cumulative mortality, percent hatching, and larval length upon hatching.

The onset of mortality of herring embryos continuously exposed to copper began 90 h after fertilization, with deaths occuring over a short interval thereafter (response period). Significant embryo mortalities occurred at a copper concentration as low as 35 μg/l. Herring larvae continuously exposed to copper showed significant mortality at 300 μg/l copper, with no delay in the onset of mortality. Embryos exposed to 36-h pulses of copper during different developmental stages showed reduced sensitivity when exposed after the response period. Larvae that hatched from eggs exposed to a 36-h pulse of copper before the response period grew significantly less than those hatched from eggs exposed during later developmental stages.

Numerous studies have shown that many aquatic animals are adversely affected by increased levels of copper in water; most of the work on fishes has been restricted to freshwater species (Becker and Thatcher 1973; Brungs et al. 1976). Since 90% of the world's marine fish are taken from the continental shelf and nearshore upwelling areas (Waldichuk 1974), increases in copper pollution in coastal aquatic ecosystems are of particular concern.

The concentration of copper in unpolluted nearshore waters ranges from 0.3 to 3.8 μg/l (Chester and Stoner 1974). Increased concentrations of copper in coastal waters have resulted from the release of municipal waste waters (Mytelka et al. 1973; Mitchell and McDermott 1975) and of effluents from power plants (Hoss et al. 1975; Martin et al. 1977). In polluted waters, concentrations as high as 13,900 μg/l copper have been reported (Mitchell and McDermott 1975).

Examination of the toxic effects of copper on coastal marine fisheries is important for the establishment of water quality standards that will protect fishery resources of coastal zones. Eggs and larval stages of fish are reported to be the life history stages that are most sensitive to a variety of pollutants (Skidmore 1965; Pickering and Gast 1972; Struhsaker et al. 1974; Christensen 1975). The necessity of conducting toxicity tests during the most susceptible stage in the life history of an organism has been emphasized by Hynes (1970), and the sensitivity of vertebrate embryos to heavy metals has been suggested as a criterion for water quality by Birge and Just (1973).

While some work has been done to assess the toxicity of copper to the early life history stages of freshwater fishes (Mount 1968; Hazel and Meith 1970; McKim and Benoit 1971; O'Rear 1972; Gardner and La Roche 1973; Benoit 1975), little assessment has been made of toxic effects of copper on marine fishes. Such studies should be very important since mortalities that occur during the early life history stages of marine fish strongly influence the strength of a given year class of fish (May 1974; Bannister et al. 1974; Postuma and Zijlstra 1974; Cushing 1975; Vaughan and Saila 1976). Toxic effects that have an impact upon survival during early developmental stages would also act to reduce the strength of a given year class of fish. The embryos and larvae of the Pacific herring, *Clupea harengus pallasi*, represent a useful

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test organism for evaluating the toxic effect of copper upon the early life history stages of marine fish. The Pacific herring is a commercially important fish that spawns along both eastern and western Pacific coasts (Eldridge and Kail 1973; Hart 1973). Herring spawn great numbers of demersal, adhesive eggs on shallow intertidal substrates. The egg is relatively large, 1.3 to 1.6 mm in diameter, and is covered by a thick, three-layered, opaque chorion (Blaxter and Holliday 1963). Development of the embryo is comparatively slow, taking 7 to 9 days at 14°C (Alderdice and Velsen 1971). The tough chorion permits easy collection and handling, and the slow development of the embryo allows observation time not available in more rapidly developing species.

Three bioassays were conducted to evaluate the sensitivity of herring embryos and larvae to copper. The first two assays were designed to evaluate the sensitivity of embryos and larvae to continuous copper exposure, while the third examined the sensitivity of embryos to brief copper exposures. Since the form of copper to which the herring embryos and larvae were exposed may play a significant role in the toxic response (Pagenkopf et al. 1974), the partitioning of copper among the components of the water in the bioassay system was also determined.

MATERIALS AND METHODS

Collection and Handling of Test Organisms

Intertidal collections of Pacific herring eggs were made along the shore of Belvedere Island and the Tiburon Peninsula, San Francisco Bay, Calif. The eggs were collected directly into a 15-gal, insulated ice chest containing aerated seawater from the egg collection site and were transported to the laboratory within 2 h after collection. The water temperature at the collection site was 11.0°-11.5°C and upon arrival at the laboratory the temperature of the water increased to no more than 13.5°C. Only eggs deposited in single layers on *Fucus* sp., *Laminaria* sp., or *Gracilaria* sp. were chosen for testing. Before placing the eggs into exposure chambers, they were removed from the seaweed by bending the frond and then gently brushing them with a finger into a sorting dish containing seawater kept at 12°C. The eggs were examined with a microscope at 20×; only viable embryos at the same stage of development were chosen. No more than 51 embryos were placed in any exposure chamber. All transfers of embryos or larvae were carried out with a large-bore, polished glass pipette.

Embryos at two different stages of development were used for the tests. The age of the earlier stage embryos, collected 7 February 1975, was estimated to be 12 h after fertilization since they were undergoing epiboly (Ahlstrom's stage IV (Ahlstrom 1943)). These embryos were exposed to copper continuously, each of seven groups being exposed to a different copper concentration. The age of the later stage embryos, collected 26 February 1975, was estimated to be 48 to 50 h after fertilization (Ahlstrom's stage IX). These embryos were divided into four groups. Three groups were exposed for 36 h to the same copper concentration but during different developmental stages. The fourth group was maintained in flowing seawater from the time of collection to within 1 h after hatching, and then continuously exposed to different copper concentrations as larvae.

Bioassay System

The organisms were exposed to copper in 5-l clear plastic bowls (Figure 1). The exposure solution was introduced into each chamber by gravity flow from a mixing chamber into which seawater, at a rate of 11 ml/min, and copper chloride solution, pH 3, at a rate of 1 ml/h, were pumped continuously. Approximately 17 h was required for replacement of 90% of the water in the chamber. The height of water in the exposure chambers was maintained by a constant-level out-flow siphon. The diameter of the mouth of the out-flow siphon

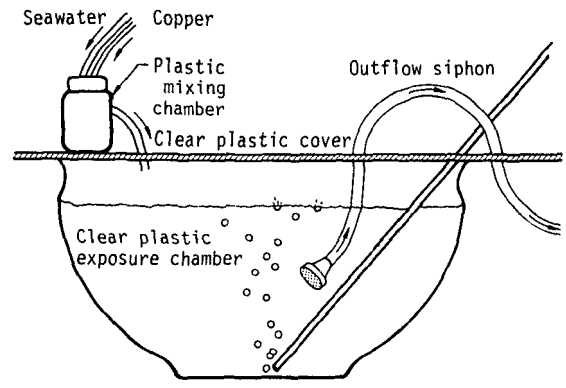

FIGURE 1.—Diagram of the exposure chamber and flow-through delivery system used to expose Pacific herring embryos and larvae to copper.

was greater than that of the tubing to reduce the flow velocity at the mouth of the siphon. The mouth of the siphon was covered with nylon netting (505-μm pore size) to prevent the loss of organisms from the chamber. A gentle stream of bubbles delivered to the bottom of the chamber provided aeration and mixing. All exposure chambers were immersed in a water bath whose temperature was monitored. Illumination was provided by the fluorescent lighting in the laboratory and followed the regular ambient photoperiod.

The exposure period, the nominal copper concentration, and the total number of embryos or larvae exposed during a typical experiment are given in Table 1. Each experiment was repeated at least once. All exposures were initiated by the addition of appropriate amounts of copper chloride to the chambers. The continuous exposure of the test organisms continued until all animals died or, in the case of embryos, until hatching occurred or, in the case of the larvae, until yolk sac absorption occurred. The pulsed exposures were terminated by transferring exposed embryos to an exposure chamber containing control seawater.

The following measurements were taken as indices of the toxic effect of copper: cumulative mortality with time, percent hatching, and larval length at hatching. The embryos or larvae were examined within the exposure chambers at each observation period with a 7× beam dissection microscope with a 21-cm depth of field. The criterion for embryo death was the lack of heart beat or body movement. Since the embryos were attached in clusters, dead embryos were not removed until the termination of a test. Larvae that hatched from pulse exposed embryos were collected, anesthesized with a 1% quinaldine solution, and preserved in 5% Formalin[2] in seawater. Measurements of the hatched larvae were made with an ocular micrometer and all obvious deformities noted. The criterion for larval death was a failure to respond to a gentle prod with a polished glass rod. Dead larvae were removed during each observation period and preserved in 5% Formalin in seawater.

Total copper concentrations were measured two

[2]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

TABLE 1.—Experimental conditions and median lethal times for bioassays determining the sensitivity of Pacific herring embryos and larvae to copper.

Experiment	Exposure period (h)	Nominal copper concentration (μg/l)	Number of organisms exposed	Mean total copper concentrations (μg/1 ± SD)	Number of water samples	Time to median lethal level (LT_{50} ± 95% confidence interval) (h)
Embryos, continuous exposure	180 (12 h after fertilization through hatching)	Control	150	4.3 ± 1.9	3	[1]
		25	48	27.9 ± 7.4	3	[1]
		35	49	38.1 ± 9.4	3	[2]144.9 ± 8.3
		45	50	44.1 ± 11.6	3	[2]134.4 ± 5.2
		55	49	51.2 ± 9.7	3	[2]134.8 ± 3.0
		100	53	127.9 ± 34.6	2	[2]115.4 ± 2.8
		200	49	235.5 ± 47.2	2	[2]98.7 ± 2.1
Larvae, continuous exposure	300 (Hatching through yolk sac absorption)	Control	100	2.5 ± 0.8	3	[1]
		300	49	274.0 ± 24.1	4	[1,3]
		600	49	572.1 ± 31.5	4	[1,2]
		1,400	50	1,349.0 ± 247.0	4	[2]41.7 ± 7.3
		2,000	51	1,969.0 ± 148.0	2	[2]23.8 ± 1.5
		2,500	49	2,425.4 ± 89.0	2	[2]20.9 ± 2.4
		3,500	51	3,430.5 ± 710.6	2	[4]15.6
						Time to median lethality following termination of pulse ± 95% confidence interval (h)
Embryos, pulsed exposures:						
Pulse I	36 (62 through 98 h after fertilization)	Control	49	3.0 ± 0.08	2	[1]
		100	44	93.8 ± 6.3	3	[2]38.4 ± 2.0
Pulse II	38 (98 through 136 h after fertilization)	Control	93	3.9 ± 1.6	3	[1]
		100	94	111.9 ± 13.7	4	[2]53.9 ± 6.1
Pulse III	36 (136 through 172 h after fertilization)	Control	46	6.1 ± 0.7	3	[1]
		100	48	101.6 ± 29.3	3	[1]

[1]50% mortality not achieved at this concentration.
[2]Slope significantly different from control slope ($P<0.01$) (Snedecor and Cochran 1967).
[3]Slope significantly different from control slope ($P<0.05$) (Snedecor and Cochran 1967).
[4]Determined according to the method of Litchfield and Wilcoxon (1949).

to four times during each bioassay to determine the actual concentrations to which the organisms were exposed. Water samples were collected in acid-washed polyethylene jars and acidified to pH 2 with concentrated HCl. Total copper was analysed by the APDC-DDDC-MIBK extraction method described by Kinrade and VanLoon (1974). The copper concentration in extracted MIBK solutions was determined with a model 303 Perkin Elmer atomic absorption spectrophotometer, using an HGA-2100 graphite furnace with a deuterium background corrector.

Since the chemistry of copper in seawater is complex, more than one form of copper may be present in the bioassay water. To examine the form of the copper in the bioassay system water, out-flow samples were collected from the bioassay system before organisms were introduced to determine the particulate-bound fractions (>0.45μm), ionic fraction (bound by Chelex-100 resin (Riley and Taylor 1968)), and complexed fraction (not bound by Chelex-100 resin). The analysis scheme is summarized in Figure 2. To monitor the partitioning of copper into each of these fractions, copper-64 was equilibrated with water samples after they were withdrawn from the bioassay system. The partitioning of stable copper in the seawater of the bioassay system was indicated by the percentage of the initial activity recovered in each of the described fractions.

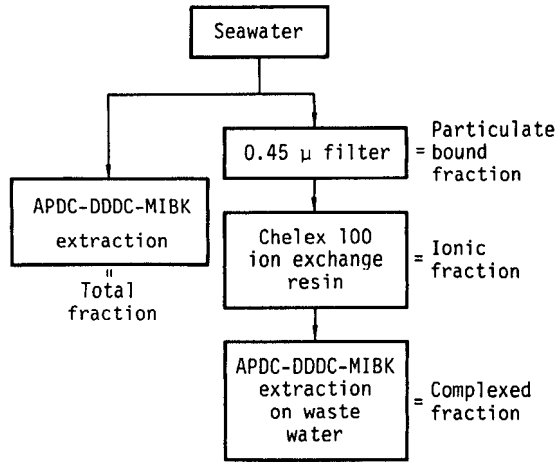

FIGURE 2.—Analysis scheme for the separation of copper fractions recovered from the bioassay system used to expose Pacific herring embryos and larvae to copper.

Statistical Analysis

The measures of toxicity determined in this study were the time to 50% mortality at each concentration of copper tested (median lethal time, LT_{50}) and the concentration of copper resulting in 50% mortality over a given time (median lethal concentration, LC_{50}). These toxicity measures were deterimed by performing a weighted linear regression analysis on the sets of cumulative mortality data using the logistic function. The straight line transform of the logistic function is: logit $P = \ln P/Q = \alpha + \beta x$, so that if logit P is plotted against x, the points will fall on a straight line with α as the intercept and β as the slope (Berkson 1953). In our calculations of LT_{50}, x represented the time from the onset of the reaction period in the case of continuous embryo exposures, from hatching in the case of continuous larval exposures, and from the termination of a given pulse in the case of pulsed embryo exposures. In our calculations of LC_{50}, x represented concentration, and our method followed that outlined by the American Public Health Association (1976) with logit analysis used in place of probit analysis.

A computer was used to calculate the LC_{50} and LT_{50} values, and for each fitted line the program determined: the LT_{50} or LC_{50}, the 95% confidence limits associated with the LT_{50} or LC_{50}, Pearson's rho (ρ), the slope (β) and the intercept (α), and the mean square error (EMS); no assumptions of homogeneity were made and the EMS was calculated in every case, rather than assuming an EMS of 1 for homogeneous data (Finney 1964).

In the case of embryos that were exposed continuously or exposed to pulses of copper, deaths prior to the delayed reaction period or the onset of the pulsed exposure, respectively, were not used in the data analysis. In no case were mortalities during these periods greater than 6%.

The relationship between time to 50% mortality during continuous exposure of both embryos and larvae and concentration was determined following the method outlined in the American Public Health Association (1976). The resulting toxicity curve was used to estimate the lethal threshold concentration (incipient LC_{50}) (Sprague 1969).

RESULTS

Physical Parameters of the Bioassay System

Mean copper concentrations measured during each test are reported in Table 1. The partitioning

of copper-64 among particulate-bound, ionic, and complexed fractions of copper recovered from the bioassay water indicates that the copper was primarily in the ionic form (Table 2). The mean pH of the water in exposure chambers in all tests was 8.08 (SD = ±0.024). The mean temperature for all tests was 13.3°C (SD = ±0.8°C).

TABLE 2.—Percentages of copper-64 in fractions of seawater recovered from the bioassay system used to expose Pacific herring embryos and larvae to copper.

Nominal copper concentration (μg/l)	Particulate bound	Ionic	Complexed	Total[1]
10	5.2	83.6	4.3	93.7
50	4.2	88.3	3.7	96.5
100	1.1	89.9	2.1	94.0
500	0.7	93.8	1.7	97.0
1,000	0.6	91.8	1.1	96.9
2,000	1.0	96.4	1.0	99.9

[1]Total includes copper-64 remaining in Chelex-100 resin after elution.

Continuous Exposures to Copper

Survival of embryos continuously exposed to copper was high at all concentrations of copper tested until 90 h of exposure, at which time dose-related mortalities occurred (Figure 3). The period during continuous exposure when embryo deaths begin is termed the reaction period. Mortalities of developing embryos at copper concentrations of 35 μg/l and higher were significantly different from controls ($P<0.01$). Virtually no hatching occurred at concentrations above 45μg/l copper. Developmental features observed in the control embryos during the onset of the reaction period included the appearance of eye pigmentation, the onset of coordinated body movements, and the initiation of heart beat. Embryos continuously exposed to copper concentrations of 100 μg/l copper and higher developed an opaque cast to the chorion, which was followed later by whitish discoloration of the body. Embryos continuously exposed to 200 μg/l copper developed an opaque change in the chorion at 60-72 h from fertilization, with body discoloration, spasmodic contractions, quiverings, and reduced fin fold development occurring at 84-96 h from fertilization.

Herring larvae continuously exposed to copper were many times less sensitive to copper than herring embryos. Larval mortalities differed significantly from controls at concentrations of 300 μg/l copper and higher ($P<0.05$) (Figure 4). Prior to death the larvae sank to the bottom of the exposure chamber and patches of whitish discoloration were observed over the bodies. Spasmodic quivering and whole body contractions were observed in larvae at concentrations of 1,400 μg/l copper and above.

The toxicity curves for continuously exposed herring embryos and larvae are shown in Figure 5. Median lethal times for each copper concentration tested and 95% confidence limits are detailed in Table 1.

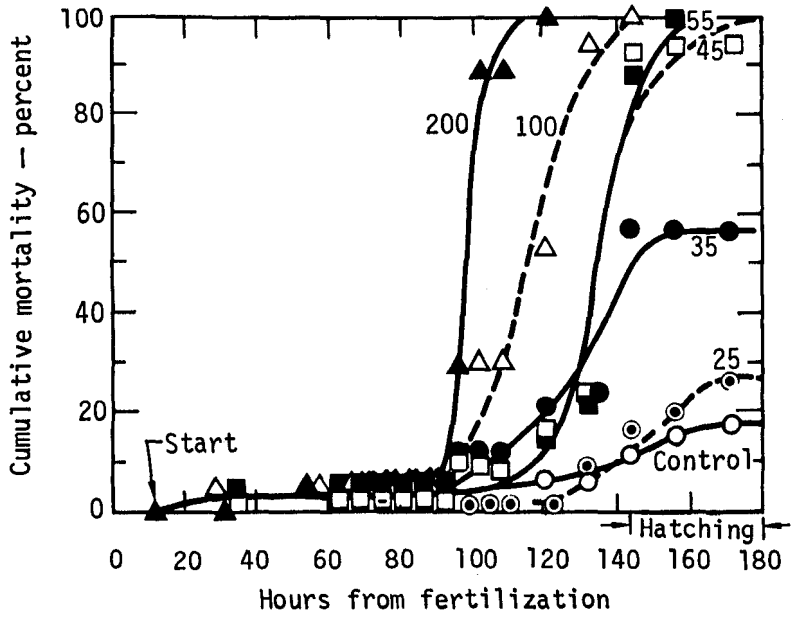

FIGURE 3.—Percent cumulative mortality of Pacific herring embryos continuously exposed to copper (micrograms per liter). Mortality curves shown are the fitted logit curves used to establish median lethal times.

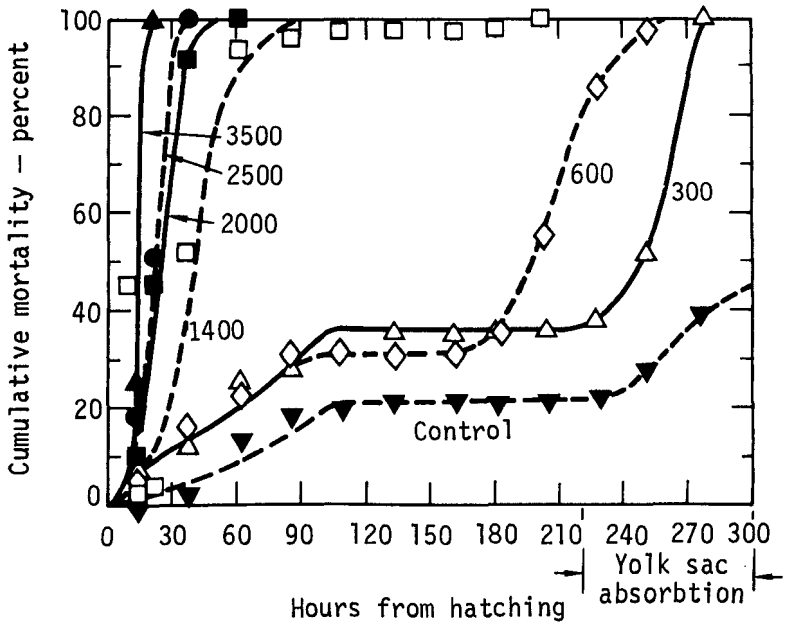

FIGURE 4.—Percent cumulative mortality of newly hatched Pacific herring larvae continuously exposed to copper (micrograms per liter). Mortality curves shown are the fitted logit curves used to establish median lethal times.

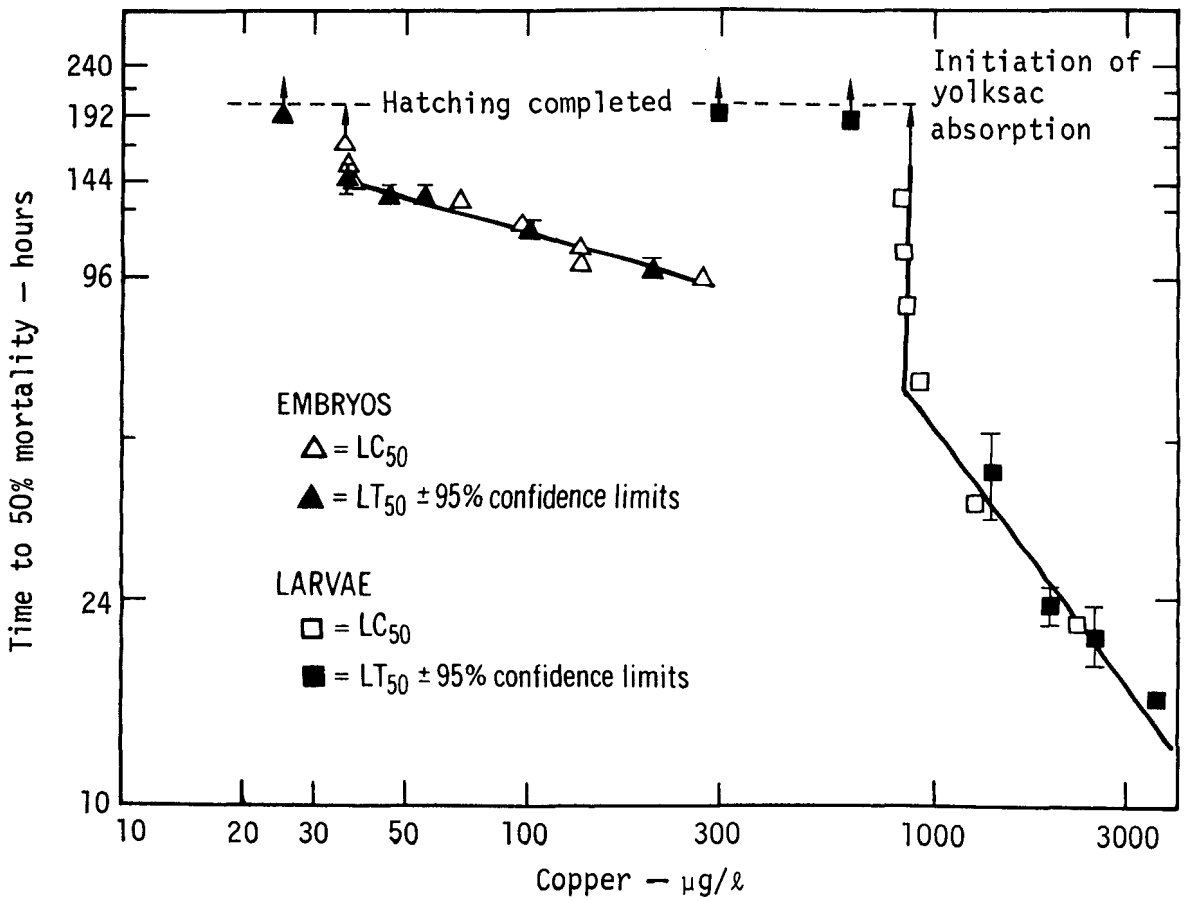

FIGURE 5.—Toxicity curves for Pacific herring embryos and larvae continuously exposed to copper.

The toxicity curve for herring embryos is presented for the purpose of discussion only, since the 90 h delay until the onset of mortality, regardless of concentration, biases the toxicity curve for comparison with other organisms without a reaction period. Sprague (1969) recommended that a concentration that killed 50% of the population during an exposure sufficiently long that acute lethal action has ceased (incipient LC_{50}) be used as the single most useful criterion for toxicity. The incipient LC_{50} is not influenced by the bias introduced by the reaction period. The estimated incipient lethal level for herring embryos was found to be 33 μg/l copper.

Only larval deaths earlier than 100 h after hatching were considered in the construction of the larval toxicity curve since larvae surviving beyond approximately 200 h after hatching have begun yolk sac absorption, and the apparently synergistic effects of copper stress and starvation can be observed in the larval time vs. percent mortality curves (Figure 4). The estimated incipient lethal level for herring larvae was found to be 900 μg/l copper.

Thirty-six Hour Pulsed Embryo Exposures

Pulses of copper exposure for 36 h showed that the sensitivity of herring embryos to copper changed as the embryos developed (Figure 6, Table 1). A 36-h pulse of 100 μg/l copper delivered during the reaction period (Pulse I) had the greatest effect upon hatching and the length of larvae at hatching (Table 3). A 36-h pulse of 100 μg/l copper delivered just before hatching (Pulse III) had a signficant effect on larval length at hatching, but the percentage of embryos hatching was actually greater than controls.

TABLE 3.—Percent Pacific herring embryos hatching and mean larval length at hatching for three groups of Pacific herring embryos exposed to 36-h pulses of 100 μg/l copper. Each group received a pulse at a different time during development.

Item	Mean larval length (mm ± SD)	Percent hatching
Controls	6.10 ± 0.47	92
Pulse 1	3.77 ± 0.2*	6
Pulse 2	4.23 ± 0.31*	47
Pulse 3	5.75 ± 0.62*	98

*Significantly different from controls ($P<0.01$) (Snedecor and Cochran 1967).

DISCUSSION

Several features of the toxic response of herring at various stages of their early life history are of interest. Previous tests examining the sensitivity of other fish embryos and larvae to copper have found that the larval stage is the more sensitive stage (Hazel and Meith 1970; McKim and Benoit 1971, O'Rear 1972; Gardner and La Roche 1973;

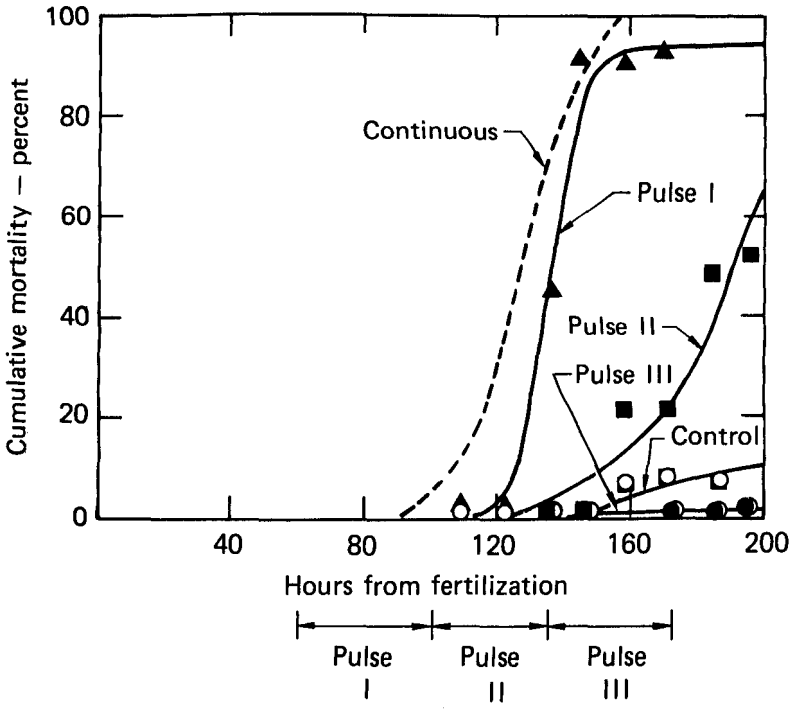

FIGURE 6.—Percent cumulative mortality of three groups of Pacific herring embryos exposed to 36-h pulses of 100 μg/l copper. Each group received a pulse at a different time during development. The cumulative mortality observed for Pacific herring embryos continuously exposed to 100 μg/l copper (See Figure 3) is shown for comparison.

Benoit 1975). Contrary to these findings, we found that embryos of the Pacific herring appear to be the stage that is more sensitive to copper. It should be noted that the fishes examined in previous studies spawn in fresh or brackish waters and cannot be considered true marine species as is the herring.

Another interesting feature of the toxic response of the herring embryos and larvae was that the behavior prior to death was similar to that of adult fish exposed to copper. Jerky, uncoordinated, and spontaneous movements were noted by Baker (1969) in the winter flounder, *Pseudopleuronectes americanus,* acutely exposed to 3,200 and 1,000 μg/l copper. Bluegill, *Lepomis macrochirus,* chronically exposed to 162 μg/l copper showed periodic involuntary spasms several weeks prior to death (Benoit 1975). The spasmodic contractions and quiverings noted in herring embryos and larvae prior to death might be of a similar nature. Baker noted that these symptoms are similar to those of Wilson's disease which also manifests spasmodic muscle contractions and quiverings in mammals. Wilson's disease is the result of an inborn error of metabolism that results in an excess of unbound copper in the blood stream (Adelstein and Vallee 1962). Goldfish, *Carassius auratus,* subjected to doses of 1,000 μg/l copper exhibit severe neurotoxic symptoms and accumulate copper in nervous tissues at levels similar to those seen in Wilson's disease (Vogel 1959).

Some of the toxic effects observed in herring embryos and larvae were similar to those reported for other heavy metals. Striped bass, *Morone saxatilis,* embryos exposed to copper or zinc (O'Rear 1972) and Baltic needlefish, *Belone belone,* exposed to cadmium (Dethlefsen et al. 1975) developed opaque discoloration of the chorion during exposure. In the present study, the chorion of the herring embryos became increasingly opaque as exposure to copper continued. Wedemyer (1968) found that in coho salmon, *Oncorhynchus kisutch,* 70% of the total zinc-65 uptake during exposure was firmly bound to the chorion, 26% was bound in the perivitelline space, and only 2% reached the yolk and 1% reached the embryo. Wedemyer (1968) also demonstrated that copper is bound by the salmon embryo's chorion. The opaque discoloration noted in herring embryos with continued exposure to copper may well be a reaction resulting from copper uptake by the chorion.

The observation of a reaction period during bioassays with herring embryos has been noted previously. A reaction period for herring embryos continuously exposed to cadmium (Rosenthal and Sperling 1974) and high temperatures and salinities (Alderdice and Velsen 1971) occurred at about the time of the onset of heart beat. The sensitivity of this developmental period in the herring was further borne out by our findings in which 36-h pulses of 100 μg/l copper during the reaction period caused higher mortalities than 36-h pulses during later developmental periods.

Pacific herring embryos may be vulnerable to toxic effects from effluents now being discharged into coastal environments. A survey of 108 municipal waste effluents on the Atlantic coast showed that 50% of the waste effluents contained >100 μg/l copper; some discharges were as high as 5,900 μg/1 copper (Mytelka et al. 1973). A survey of six municipal waste discharges along the southern California coast revealed concentrations ranging from 74 to 13,900 μg/l copper with an average annual mass emission rate of 532 t of copper during 1971-74 (Mitchell and McDermott 1975). While the amount of copper discharged in the ionic form was not reported, the potential for environmental exposure levels approaching the incipient LC_{50} of 33 μg/l copper found for herring embryos in the present study should be considered in establishing water pollution control standards.

Frequently authors conducting bioassays using copper or other heavy metals have not examined the chemical state of the metal in their bioassay system. Such characterizations are important since different chemical forms of metals may have different toxic effects (Lee 1973). The method outlined in this work for examining the particulate bound fraction, the ionic fraction, and the complexed fraction of metals in seawater provides a means of examining the important chemical forms of copper in aquatic bioassay systems. With the use of appropriate isotopes this method could easily be applied to other metals. In the case of the system used to expose Pacific herring embryos and larvae it appears that the ionic form of copper predominated. In freshwater the ionic form of copper seems to be the most toxic (Pagenkopf et al. 1974). This is probably also the case for Pacific herring embryos and larvae exposed to copper in seawater.

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