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Combined Effects of Salinity, Temperature, and Copper on Embryos and Early Larvae of the American Oyster, *Crassostrea virginica*

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Abstract. The response of embryos and larvae of the American oyster, *Crassostrea virginica*, to copper at various salinity-temperature regimes was studied in the laboratory using response surface methods and factorial analysis. The experimental design was a 3 × 3 × 4 factorial experiment using temperatures of 20, 25, and 30°C, and salinities of 17.5, 22.5, and 27.5 ‰. It was carried out at copper concentrations of 0, 5, 10, and 20 ppb for the embryos and 0, 30, 60, and 90 ppb for the larvae. Statistical analysis indicated that salinity had the greatest effect on the embryos at 0, 5, and 10 ppb copper, but temperature had as great an effect as that of salinity at 20 ppb copper. The capacity of the embryos to adapt to the temperature-salinity changes was impaired when exposed to 20 ppb copper, as indicated by the shifting of the response center. Temperature had the greatest effect on the larvae when exposed to 30, 60, and 90 ppb copper. The interaction between temperature and salinity was significant only at the higher levels of copper. Low levels of copper may produce intolerable stress upon the recruitment of oyster embryos during periods of persistently low salinities and low or high temperatures.

In recent years, there have been numerous reports on the toxic effects of heavy metals on marine invertebrates, most of which describe the toxicity of a single metal pollutant to a single stage of a test organism's life history under optimal environmental conditions (Okubo and Okubo 1962; Wisely and Blick 1967, Connor 1972, Calabrese *et al.* 1973, 1977). In nature, however, marine organisms are subjected to fluctuating environmental conditions which are often not optimal and which affect the pollutant's toxicity; for example, temperature (oysters—MacInnes and Calabrese 1978) or salinity (polychaetes—Jones *et al.* 1976). Moreover, the toxicity of a pollutant to a test organism can also vary at different stages of its life history (echinoids—Heslinga 1976, lobsters—Thurberg pers. commun.[1]). To predict the level at which a marine organism is

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affected by a pollutant, therefore, one must test the organism at different life stages and under various environmental regimes.

Because a study of this nature had not been performed with the commercially important American oyster, *Crassostrea virginica*, we instituted work on heavy metals combined with natural variables. Both embryos and 24-hr larvae of the American oyster were exposed for 48 hr to copper as the chloride under various temperature-salinity regimes. Copper was chosen because previous investigations (Calabrese *et al.* 1973, 1977) have shown it to be fairly toxic to oyster embryos and larvae and because relatively high levels of copper may be found in waters which are nursery areas for oysters. Although several investigators have studied the influence of one environmental variable on copper toxicity to marine invertebrates (salinity—Olson and Harrel 1973, Jones 1975, Jones *et al.* 1976; temperature—MacInnes and Calabrese 1978), only a few papers have evaluated the effect of two or more variables, such as temperature and salinity, or of other metals on copper toxicity (marine protozoa—Gray 1974; lobsters—McLeese 1974). We report herein on the effects of these variables on copper toxicity in the oyster.

Materials and Methods

Adult oysters, obtained commercially from Long Island Sound in the vicinity of New Haven, Connecticut, were induced to spawn in the laboratory by thermal stimulation and the addition of sperm from one or more sacrificed males, as previously described by Loosanoff and Davis (1963). After the oysters began spawning, the eggs were fertilized and transferred to a 4-liter container where egg density was determined. To determine the effect of temperature, salinity, and copper on development of oyster embryos and their effect on 24-hr veliger larvae during a test period of 48 hr, approximately 20,000 fertilized eggs or 24-hr larvae were put into each of a series of 1-liter polypropylene beakers which contained filtered (1 μ) natural seawater prepared at the desired test conditions. The experimental design was a $3 \times 3 \times 4$ factorial experiment, using temperatures of: (a) 20, 25, and 30 ± 1°C; (b) salinities of 17.5, 22.5, and 27.5 ± 0.5 ‰; and (c) copper concentrations of 0, 5, 10, and 20 ppb for embryos, and 0, 30, 60, and 90 ppb for larvae. Concentrations reflect the addition of copper at the start of the experiment, not the salt ($CuCl_2 \cdot 2H_2O$), and do not include background levels in the seawater. These concentrations were chosen because of their known toxicities to oyster embryos and larvae (Calabrese *et al.* 1977, MacInnes and Calabrese 1978). Tests with embryos were initiated within two hr after the eggs were fertilized and terminated 48 hr later. Bioassay tests with larvae were also of 48-hr duration. Larvae for these tests were obtained by rearing fertilized eggs to the straight-hinge stage under normal conditions (26 ± 1 ‰ and 25 ± 1°C). They were then put into beakers for subsequent metal exposure. Embryos were unfed, but the larvae were fed a mixed algal diet of *Isochrysis galbana* and *Monochrysis lutheri*. Duplicate cultures were established in each test for each of the experimental conditions (72 beakers per test). Three tests were performed with embryos and two with 24-hr larvae.

The pH level in all test containers was maintained at 7.7 to 8.1, a range determined optimal for percent development of oyster embryos and larvae (Calabrese and Davis 1966). Whenever necessary, NaOH or HCl was added to the medium to bring the pH values within the desired range. Dissolved oxygen levels in the non-aerated beakers ranged from approximately 95% saturation at the beginning of each test to 90-95% saturation after 48 hr.

At the end of each 48-hr exposure test with either embryos or 24-hr larvae, the water in each beaker was mixed vigorously and 15-ml samples containing approximately 300 larvae were removed, preserved in 5% buffered formalin, and later examined under a compound microscope. To

determine the effect of copper on embryonic development of oysters at varying salinity and temperature regimes, the number of embryos that developed either normally or abnormally was counted. Similarly, in larval tests, those larvae that survived or died were counted. The results were expressed either as the percent abnormal development of embryos or the percent death of larvae in each of the test beakers. These values were not corrected for abnormal development or larval death in control cultures.

Factorial analysis was used to determine the significance of temperature and salinity at each of the three copper concentrations and controls in affecting the development of the oyster embryos and mortality of larvae. Response surface contours were calculated from a second order polynomial equation of the form:

$$Y = b_0 + b_1T + b_2S + b_3T^2 + b_4S^2 + b_5T \times S,$$

where $Y = \arcsin \sqrt{\%\text{ abnormal or dead}}$; b_0 = a constant; T and T^2 = linear and quadratic effects of temperature, respectively; S and S^2 = linear and quadratic effects of salinity, respectively; $T \times S$ = interaction between temperature and salinity. The b-values or regression coefficients were calculated using a stepwise regression computer program (BMD02R) (Dixon 1971). A computer program fitted the b-values to a full quadratic equation in temperature and salinity in order to print a contour diagram of the response surface, at 20% intervals.

Results

Tables 1 and 2 give the results of 48-hr exposures of oyster embryos and 24-hr larvae to various levels of copper at different temperature-salinity regimes. Figures 1a-d and 2a-b show response surfaces extrapolated from multiple regression equations (Table 3) of temperature-salinity effects on embryos and larvae at various copper concentrations. Without the addition of copper, embryonic development was markedly affected by low salinity at the lowest and highest temperatures tested, whereas survival of the 24-hr larvae was influenced neither by temperature nor salinity. Copper was most toxic to embryos at the lowest temperature and salinity tested, 20°C and 17.5 ‰, respectively, and was least toxic at the two higher salinities at 25°C. For larvae, the toxicity of copper at 60 and 90 ppb was greater at 25° and 30°C than at 20°C, at all salinities tested.

The optimal combination of salinity and temperature for normal development of oyster embryos, therefore, was 25°C and 26 ‰, as shown by the center of the response surface (Figure 1a). This environmental regime was also the point of greatest resistance to copper toxicity for embryos exposed to 5 and 10 ppb copper, as shown by the unchanged centers of response surface (Figures 1b, 1c). For those embryos exposed to 20 ppb copper, however, the center of the response surface (Figure 1d) lies beyond the experimental area and it would not be wise to estimate the temperature-salinity regime providing the maximal resistance to copper at concentrations of 20 ppb and above.

ANOVA results of temperature and salinity effects on oyster embryogenesis (Table 4) indicate that the linear effects of salinity were more pronounced than the quadratic effects at all copper concentrations tested, whereas the reverse was true for temperature. In other words, at all copper concentrations tested, abnormal development of embryos increased linearly as salinity decreased and curvilinearly as temperature either increased or decreased from the 25°C optimum. To a lesser extent, decreasing temperature had

Table 1. Mean percent abnormal development and standard deviation ( ) of embryos of the American oyster, *Crassostrea virginica*, exposed to copper for 48 hr at various temperature-salinity regimes. Each value represents the mean of three individual tests

	Copper concentration (ppb)											
	0			5			10			20		
Salinity ‰	17.5	22.5	27.5	17.5	22.5	27.5	17.5	22.5	27.5	17.5	22.5	27.5
Temperature °C												
20	31.8 (28.2)	2.1 (1.5)	3.1 (1.7)	63.9 (31.9)	7.4 (7.1)	4.9 (1.7)	99.3 (1.0)	40.0 (7.6)	11.7 (5.6)	99.6 (0.6)	96.9 (4.0)	94.8 (5.1)
25	7.0 (7.0)	1.6 (0.8)	1.4 (1.1)	11.0 (9.6)	1.5 (1.1)	2.6 (1.5)	49.7 (25.5)	4.5 (1.6)	4.4 (1.8)	92.7 (9.1)	60.2 (21.1)	30.8 (7.2)
30	25.2 (19.6)	7.1 (7.8)	6.0 (1.8)	40.0 (33.9)	11.7 (9.4)	9.4 (0.3)	62.2 (32.4)	23.1 (18.9)	15.6 (5.5)	92.9 (12.4)	69.5 (39.2)	73.9 (4.7)

Table 2. Mean percent mortality and standard deviation ( ) of 24-hr larvae of the American oyster, *Crassostrea virginica*, exposed to copper for 48 hr at various temperature-salinity regimes. Each value represents the mean of two individual tests

	Copper concentration (ppb)											
	0			30			60			90		
Salinity ‰	17.5	22.5	27.5	17.5	22.5	27.5	17.5	22.5	27.5	17.5	22.5	27.5
Temperature °C												
20	2.6 (2.0)	3.7 (1.7)	2.5 (1.8)	2.4 (1.1)	1.9 (1.0)	2.7 (0.8)	2.6 (1.4)	2.5 (2.0)	2.4 (0.6)	2.5 (0.9)	14.9 (10.0)	5.6 (4.6)
25	3.8 (4.7)	5.3 (3.3)	3.4 (3.6)	1.8 (0.5)	2.5 (1.0)	10.7 (9.9)	34.9 (20.7)	12.5 (3.0)	50.6 (43.5)	86.1 (11.8)	56.6 (23.0)	77.4 (32.4)
30	2.4 (1.1)	4.4 (5.0)	3.1 (4.2)	1.7 (0.5)	1.6 (1.5)	1.7 (0.9)	78.8 (23.2)	49.8 (16.1)	31.1 (8.1)	91.4 (10.2)	85.8 (11.7)	77.5 (16.0)

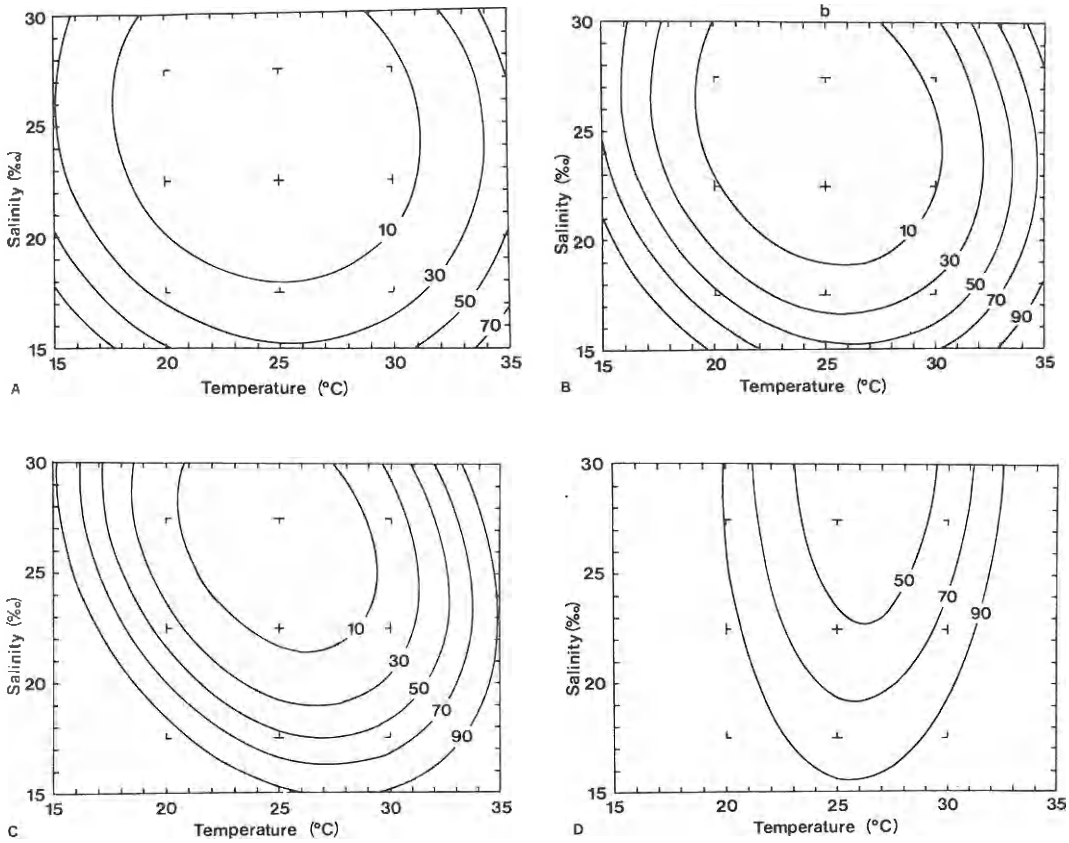

Fig. 1. *Crassostrea virginica*. Response surfaces for percent (10 to 90) abnormal development of oyster embryos exposed to different combinations of temperature and salinity: (a) without addition of Cu, (b) with addition of 5 ppb Cu, (c) 10 ppb Cu, (d) 20 ppb Cu. Inner tick marks represent experimental conditions used in this study

a linear effect on embryonic development at higher copper levels (10 and 20 ppb). Salinity had the greatest effect on control embryos and those exposed to the two lowest copper concentrations. The effect of temperature was as important as that of salinity at the highest level of copper tested. The interaction between temperature and salinity was not significant in the controls and 5 ppb copper-exposed embryos, but was significant at 10 ppb ($T_L \times S_L$) and at 20 ppb ($T_Q \times S_L$), as is evidenced by the skewed contours in Figures 1c and 1d.

In contrast to the embryos, larvae in controls and all copper-treated cultures were not significantly influenced by salinity (Table 5). The effect of temperature on larval survival was not significant in the controls, but became highly significant ($P < 0.01$) and was linear at 60 ppb and both linear and curvilinear at the 90 ppb copper-exposure level. This means that as temperature increased, larval mortality increased linearly or at a constant rate at 60 ppb copper, as indicated by the significance of T_L in Table 5, but the mortality

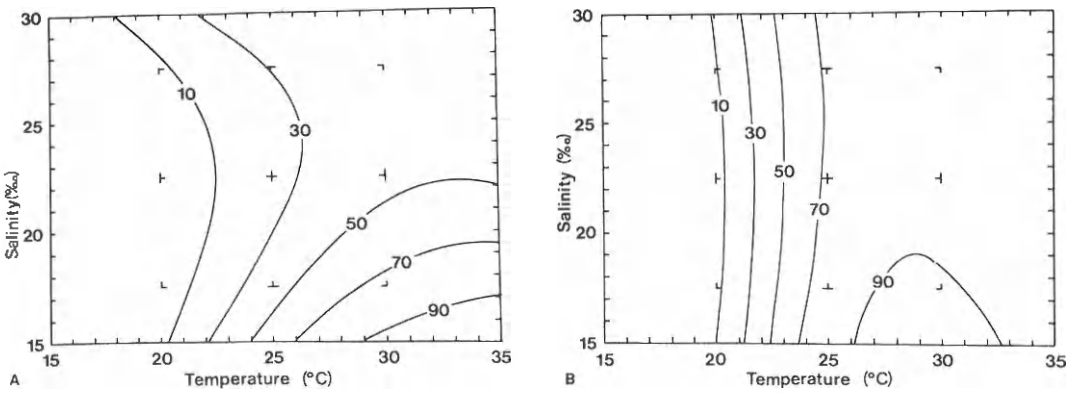

Fig. 2. *Crassostrea virginica*. Response surfaces for percent (10 to 90) mortality of oyster larvae exposed to different combinations of temperature and salinity: (a) with addition of 60 ppb Cu, and (b) 90 ppb Cu. Inner tick marks represent experimental conditions used in this study

Table 3. Equations of response-surface contours of combined effects of temperature and salinity on embryonic development and larval mortality of the American oyster, *Crassostrea virginica*, at various copper concentrations. Y = percent abnormality or mortality transformed to radians; T = temperature in °C; S = salinity in ‰; r = correlation coefficient

Oyster embryos	Oyster larvae
Controls	Controls
$Y = 8.20528 - 0.34103T - 0.31982S + 0.00625T^2 + 0.00568S^2 + 0.00144T \times S$	$Y = -1.17594 + 0.05202T + 0.06594S - 0.00113T^2 - 0.00160S^2 + 0.00025T \times S$
$r = 0.948$	$r = 0.983$
5 ppb Cu	30 ppb Cu
$Y = 13.17962 - 0.58517T - 0.46956S + 0.01017T^2 + 0.00767S^2 + 0.00330T \times S$	$Y = -0.92011 + 0.13298T - 0.5373S - 0.00271T^2 + 0.00135S^2$
$r = 0.948$	$r = 0.759$
10 ppb Cu	60 ppb Cu
$Y = 18.62057 - 0.80713T - 0.63604S + 0.01281T^2 + 0.00897S^2 + 0.00635T \times S$	$Y = -3.52420 + 0.38634T - 0.13335S - 0.00417T^2 + 0.00545S^2 - 0.00494T \times S$
$r = 0.990$	$r = 0.941$
20 ppb Cu	90 ppb Cu
$Y = 12.96981 - 0.78221T - 0.13260S + 0.01548T^2 + 0.00254S^2 - 0.00100T \times S$	$Y = -9.86400 + 0.78607T - 0 - 0.01269T^2 + 0.00131S^2 - 0.00268T \times S$
$r = 0.909$	$r = 0.975$

Table 4. Analysis of variance of temperature and salinity effects on development of embryos of the American oyster, *Crassostrea virginica*, at different concentrations using data in Table 1. Percent abnormality has been transformed to angular units; L = linear; Q = quadratic

Source of variation	F ratio			
	Copper concentration (ppb)			
	0	5	10	20
Salinity				
S_L	10.47[a]	17.07[a]	54.43[a]	18.65[a]
S_Q	4.12[b]	5.82[b]	7.21[a]	1.08
Temperature				
T_L	0.23	0.13	5.99[b]	7.63[a]
T_Q	4.57[b]	9.10[a]	13.58[a]	16.64[a]
Temperature × Salinity				
T_L S_L	0.42	2.42	6.95[a]	0.61
T_L S_Q	0.31	0.78	0.07	0.65
T_Q S_L	1.15	3.27	1.75	5.09[b]
T_Q S_Q	0.73	0.60	0.10	0.02
Mean square error	91.51	143.15	155.31	152.42

Statistical significance: [a]$P < 0.01$; [b]$P < 0.05$

Table 5. Analysis of variance of temperature and salinity effects on survival of larvae of the American oyster, *Crassostrea virginica*, at different copper concentrations using data in Table 2. Percent mortality has been transformed to angular units; L = linear; Q = quadratic

Source of variation	F ratio			
	Cu concentration (ppb)			
	0	30	60	90
Salinity				
S_L	<0.01	3.87	2.18	0.83
S_Q	1.55	1.69	3.48	0.26
Temperature				
T_L	0.01	0.80	56.68[a]	113.10[a]
T_Q	0.77	5.26[b]	1.26	17.65[a]
Temperature × Salinity				
T_L S_L	0.01	0.60	6.42[b]	2.08
T_L S_Q	0.01	6.51[b]	5.64[b]	0.02
T_Q S_L	0.06	0.01	0.20	0.85
T_Q S_Q	0.10	0.56	3.04	6.23[a]
Mean square error	25.61	16.97	167.48	166.67

Statistical significance: [a]$P < 0.01$; [b]$P < 0.05$

increased curvilinearly or at an increasing rate at 90 ppb, as shown by the significance of both T_L and T_Q. The interaction between temperature and salinity among the copper-exposed larvae is complex. Figures 2a and 2b, however, obviously show that there is an interaction between temperatures higher than 26°C and salinities lower than 20 ‰.

Table 6. Comparison by analysis of covariance of polynomial equations of effects of different copper concentrations on embryonic development and larval mortality of the American oyster, *Crassostrea virginica*. Hypothesis: No significant differences between control and treatment polynomials

Source of variation	F ratio	Significance
Oyster embryos		
Control vs 5 ppb Cu	1.00	N.S.
Control vs 10 ppb Cu	15.96	$P < 0.01$
Control vs 20 ppb Cu	35.68	$P < 0.01$
Oyster larvae		
Control vs 30 ppb Cu	1.82	N.S.
Control vs 60 ppb Cu	8.87	$P < 0.01$
Control vs 90 ppb Cu	46.67	$P < 0.01$

Discussion

Tolerance of oysters to various environmental factors was shown to differ greatly between the two early stages of oyster development used in this study. Veliger larvae were considerably more tolerant to temperature and salinity changes than were the developing embryos. Low salinity, coupled with low, as well as high temperatures, adversely affected the embryos, but did not influence larval survival within the conditions used in this study. When copper was added, embryos were significantly affected by a concentration as low as 10 ppb, but the 24-hr larvae were not significantly influenced by levels as high as 30 ppb, as indicated by a comparison of polynomial equations by analysis of covariance (Table 6). Increased tolerance of marine organisms with age to various environmental factors, as shown by this study, is well documented by others (Davis and Calabrese 1964, Vernberg and Vernberg 1972, Gray 1976, Heslinga 1976). This is not always the case; however, a study of the effect of copper on eggs and larvae of plaice and herring (Blaxter 1977) showed that older plaice larvae were more sensitive to copper than were the newly hatched larvae. Sublethal effects of copper on feeding in plaice were evident at about 1000 ppb in young larvae and as low as 90 ppb in older larvae.

In embryo tests, low salinity was shown to act synergistically with copper, increasing abnormality. Similar results have been reported by other investigators. Jones *et al.* (1976) found that synergistic stress caused by copper and low salinities affected survival of the polychaete worm, *Nereis diversicolor*. Thurberg *et al.* (1973) found that the greatest copper-induced disruption of osmoregulation in two estuarine crabs, *Carcinus maenas* and *Cancer irroratus*, occurred at lower salinities.

In response surface terms, capacity adaptation is measured by changes in the absolute magnitude of the minimal response of the response surface center (Alderdice 1972). When embryos were exposed to 0, 5, and 10 ppb copper (Figures 1a-c), the response surface center did not change very much (25°C and 26 ‰ salinity), but when the embryos were exposed to 20 ppb copper, the center was shifted to another area of the surface (Figure 1d). This finding

indicates that copper levels at 20 ppb or greater alter the salinity and temperature tolerance of the embryos.

In tests with larvae, low salinity acted synergistically with copper only at high temperature (30°C) (Figures 2a, 2b). Higher temperatures generally mean greater solubility of metal salts, as well as an increased water and solute movement across the cell membrane (Cairns *et al.* 1975); thus, there is the possibility of higher copper toxicity at higher temperatures. With embryos, however, higher copper toxicity occurred at 20°C rather than 30°C. The most likely reason for this seeming contradiction is that 20°C is closer to the temperature tolerance limit of the embryos than is 30°C, based on a more intensive temperature-salinity tolerance study of this species by Davis and Calabrese (1964).

Copper concentrations in near-shore waters of eastern Long Island Sound range from 0.6 to 8.7 ppb (Dehlinger *et al.* 1974), whereas copper levels are often much higher in heavily industrialized areas. A level of 65 ppb has been reported for the estuarine waters of Raritan Bay, New Jersey (Waldhauer *et al.* 1978). The salinity range for bottom waters of eastern Long Island Sound is between 22 and 30 ‰, with the lowest values occurring during late spring and early summer, the spawning season for oysters. The annual temperature range is between 1°C and 23°C (Dehlinger *et al.* 1974). Periods of persistently low salinities and low or high temperatures, both of which enhance copper's toxicity to oyster embryos, could impose intolerable stress upon this life stage when combined with high levels of copper, as in industrial estuaries. Larval survival would probably be less affected by similar levels unless water temperature is abnormally high.

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