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Effect of Intermittent Chlorination on Developing Zebrafish Embryos (*Brachydanio rerio*)

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Chlorine, a widely used disinfectant and anti-fouling agent, is recognized as a serious aquatic pollutant (TSAI 1968, ZILLICH 1972, BRUNGS 1973, MCKIM et al. 1973), and it is of special interest in the warm water effluents of electric generating plants that chlorinate to control fouling in their lines because these effluents may provide protected areas for controlled fish culture, particularly in temperate areas. Several investigations have shown that chlorine is not equally toxic to all portions of the fish life cycle (MCKIM et al. 1973, BRUNGS 1973), and in particular the embryos and young larvae of marine and estuarine fish may be extremely sensitive to low levels of chlorine (ALDERSON 1974, MIDDAUGH et al. 1977). But many oviparous fish now used extensively in fish culture such as salmon, trout, goldfish, and catfish, are freshwater species, and little is known about the effect of chlorine on the embryos of freshwater fish. Both in the environment and in culture, the early life stages of a fish population ultimately determine productivity, and therefore, it is necessary to know more about the potentially detrimental effects of pollutants on these stages.

In adult and larval fish, chlorine damages the gills (VALENZUELA 1976, MIDDAUGH et al. 1977) and it is thought to cause death either by suffocation due to excessive mucus secretion (BASS et al. 1975) or by impairment of normal iono- and/or osmoregulatory functions (COHEN 1977, COHEN and VALENZUELA 1977, VALENZUELA 1976). The fact that mortalities can be altered by the ionic composition of the water supports the latter contention (COHEN 1977, VALENZUELA 1976), and may well be a factor in determining the survival of embryonic and larval fish exposed to chlorine. The primary objective of this study is to determine whether chlorine adversely affects the hatchability of freshwater fish embryos and to what extent, if any, chlorine toxicity is modified by the ionic composition of the water.

MATERIALS AND METHODS

Chemical Methods: Large quantities of tapwater used in the holding and breeding tanks were dechlorinated by adding sodium thiosulfate (STANDARD METHODS 1971) since this method dechlorinates large quantities of water quickly. However, the tapwater used in

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rinsing eggs, or in experimental solutions, was dechlorinated by charcoal filtration. Natural seawater (33°/oo) obtained from the Atlantic ocean, was filtered through Whatman No. 1 filter paper and stored in glass bottles. Artificial seawater solutions were made isotonic to 35°/oo in distilled deionized water (MARINE BIOLOGICAL LABORATORY 1956), and final dilutions were made with organic-free commercial water to produce 1% or 20% seawater equivalent solutions (SWE).

Commercial bleach (5.25% NaOCl) served as the source of chlorine for the experiments. A stock solution (100 mg/L) was prepared and quantitated immediately before use of iodometric acid titration (STANDARD METHODS 1971). Each experimental solution was dosed with chlorine (TRC), and the subsequent dissipation rates determined by the orthotolidine-arsenite method (STANDARD METHODS 1971), using a Bausch & Lomb Spectronic 70 spectrophotometer. All glassware used in the experiments was rinsed thoroughly in distilled deionized water immediately prior to use, following overnight soaking in a chlorine solution (10 mg/L) to eliminate chlorine demand. It was also found that this treatment effectively retarded the growth of bacteria and fungus in the cultures.

Biological Methods: Male and female zebrafish, maintained separately in dechlorinated tapwater (pH 7.5-8.3) in aerated gallon jars, were fed twice daily with frozen brine shrimp. Temperatures in the holding tanks ranged from 20-29°C over the period of study, fluctuating not more than 4°/day. The breeding chamber consisted of a plastic box (6.5 x 30 x 8 cm) filled with dechlorinated tapwater and containing a net fitted with a screened aluminum bottom. Two males and one female were placed inside the net. At each spawning, 600-1200 clear, demersal eggs dropped through the screen, thereby preventing cannibalsim by adult fish, and could be easily siphoned from the chamber.

The eggs were transferred to a round culture dish, rinsed, and then dipped in a 0.05% formalin solution for 10 min, which decontaminated the chorion (RUGH 1962) and greatly improved hatchability. To insure a more homogeneous population, the eggs from only one female were used for each experiment, and all eggs were stages microscopically. Fifty stage I embryos (Table 1) were randomly selected and placed into each separate culture dish. Each subsequent day, all eggs were transferred to new solutions and any dead eggs (determined by chorionic opacity) were counted and removed prior to chlorination. Newly hatched larvae were counted and removed within 2 to 8 h after hatching, and preserved in 5% buffered neutral formalin for subsequent length determinations. Incubation temperatures were maintained at 26 ± 1°C. Since oxygen tension did not fall below 8 mg/L, no aeration was provided.

TABLE 1: Normal Developmental Stages of Zebrafish as 26±°C*

Stage	Description	Approximate time after fertilization (hours)
I	flat bastula to optic cup formation and muscular movements	3 - 24
II	optic cup formation to body pigmentation over head and yolk (circulation also evident)	24 - 48
III	body pigmentation to xanthophore development (diffuse yellow coloration)	48 - hatching
H	hatching	66 - 70
L	free swimming larva	post hatching

*adapted from HISAOKA and BATTLE (1958).

RESULTS

The TRC was measured daily at 0, 1, 3, and 6 h after chlorination, and the average chlorine dissipation rate determined (Table 2). Approximately half of the chlorine had dissipated within the first two hours, and less than 15% remained at the end of 6 hours. The dissipation rate was slightly greater in more concentrated solutions.

TABLE 2: Dissipation Rate of Total Residual Chlorine (mg/L)

A.	Hrs	Control	1% SW	1% SWE			
				$CaCl_2$	$MgCl_2$	KCl	NaCl
	0	.86	.90	.91	.89	.88	.90
	1	.40	.61	.58	.63	.54	.64
	3	.14	.30	.31	.32	.25	.36
	6	.06	.12	.09	.12	.09	.13

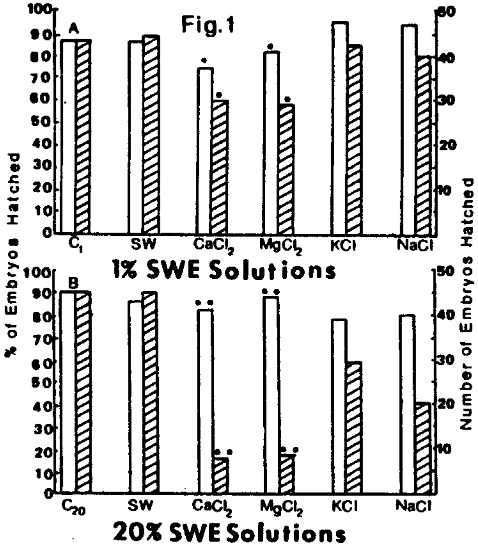
B.	Hrs	Control	20% SW	20% SWE			
				$CaCl_2$	$MgCl_2$	KCl	NaCl
	0	.85	.88	.84	.87	.81	.82
	1	.33	.36	.33	.49	.47	.41
	3	.10	.13	.11	.17	.11	.13
	6	.06	.05	.06	.07	.05	.06

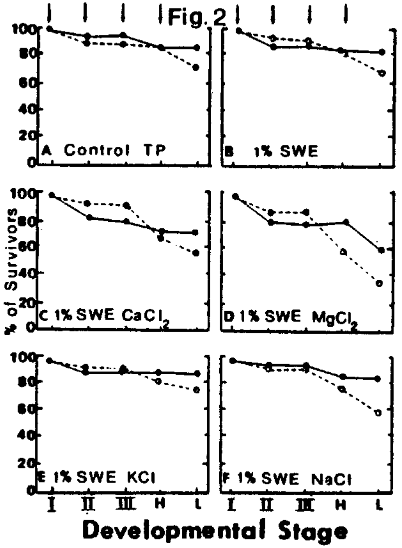
In order to determine the effect of intermittent chlorination, hatchability was divided into three components: 1) hatching success; 2) rate of development (h_{50}), or time needed for 50% of the embryos to hatch; and 3) standard length of hatched larvae. The latter two measurements were included to provide an indication of sub-lethal effects of chlorine. The results of these experiments indicated that under normal circumstances (tapwater), intermittent chlorination does not influence hatching success of freshwater zebrafish embryos (Fig. 1). However, chlorine in the presence of a mild external ionic stress (1% SWE solutions of $CaCl_2$ and $MgCl_2$) significantly reduced the number of embryos that hatched (Fig. 1A), based on the binomial test comparing each non-chlorinated solution with its respective chlorinated solution. Mortalities due to chlorine increased when external stress increased (Fig. 1B), and again $CaCl_2$ and $MgCl_2$ caused the most mortalities. Furthermore, it was apparent that ionic stress and not simply osmotic stress caused the mortalities, since nearly osmotically equivalent solutions of seawater did not reduce hatching success (Fig. 1).

Of the larvae that hatched, no developmental abnormalities were detected, although no histological studies were conducted to assess internal damage. The developmental rate (h_{50}) in the two controls ranged between 67 and 72 h, and no significant differences were detected between chlorinated and non-chlorinated solutions in a two-way analysis of variance, even though development appeared to be slower in the 20% SWE solution of $CaCl_2$ and $MgCl_2$. The standard lengths at hatching for the two control populations (n=38, n=40) were mean standard lengths of 2.88± .19 mm and 2.90±.10 mm, and again, no differences were detected between chlorinated and non-chlorinated populations in a two-way analysis of variance. There was a greater standard deviation in populations from 20% SWE $CaCl_2$ and $MgCl_2$, but this could be attributed to smaller population size (n=21 and n=28 respectively).

Based on these results, it became apparent that the freshwater zebrafish embryo was resistant to chlorine throughout the developmental period, but that an external ionic stress could potentiate chlorine toxicity. On the premise that chlorine may not be equally toxic to each developmental stage, the developmental sequence was divided into five stages (Table 1), with each stage corresponding to the time of chlorination, and the cumulative survival was plotted (Figs. 2-3). It was found that 82 to 96% of the embryos reached stage II, with the greatest survival at this stage randomly distributed between chlorinated and non-chlorinated solutions. However, beginning at stage III and continuing to hatching, chlorine elevated the number of mortalities. For example, a 34% and 30% reduction in survival occurred in the 1% SWE $CaCl_2$ and $MgCl_2$ solutions, respectively, compared to a maximum 8% reduction in other solutions (Fig. 2). As expected, the increased concentration of external ions increased the number of mortalities only between stage III and hatching (Fig. 3). Therefore, stage III was judged to be the critical developmental stage.

Chlorine was toxic to newly hatched larvae in all solutions tested, whereas no mortalities of the newly hatched larvae occurred in any of the solutions in the absence of chlorine. Based on the previous results, it was expected that chlorine would be more toxic in each of the 20% solutions, with $CaCl_2$ and $MgCl_2$ causing the most mortalities. To confirm this, the number of hatched larvae that died immediately after hatching was converted to per cent mortality of hatched embryos, so that any deaths occurring prior to hatching would not enter into the comparison. A larva was considered hatched it it had broken through the chorion whether it had completely freed itself or not; if no heartbeat were detected, it was considered dead. The





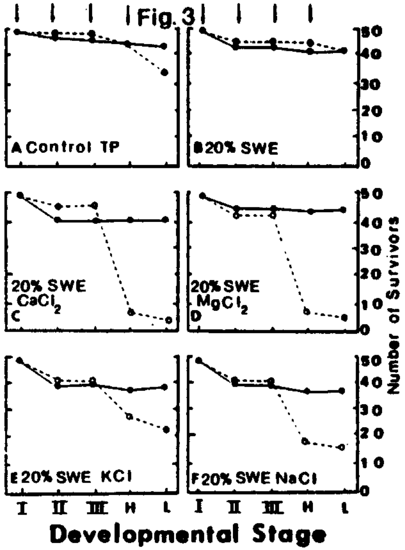


Fig. 1. Hatching success of zebrafish embryos in 1% and 20% SWE (seawater equivalent) solutions. A. Hatching success is significantly reduced only in $MgCl_2$ (* = $p < .05$). B . Hatching success is significantly reduced not only in $CaCl_2$ and $MgCl_2$ but also in KCl and NaCl (** = $p < .01$). C_1 and C_{20} = tapwater controls. Non-chlorinated ☐; chlorinated ▨.

Fig. 2. Cumulative survival of zebrafish embryos at developmental stages I-L when incubated in control and 1% SWE solutions A-F (TP = tapwater). Decreased survival in presence of chlorine occurs only after stage III. Points of chlorination ⟶ ; non-chlorinated ●—●; chlorinated ○- - -○.

Fig. 3. Cumulative survival of zebrafish embryos at developmental stages I-L when incubated in control and 20% SWE solutions A-F. Decreased survival in presence of chlorine occurs only after stage III. Points of chlorination ⟶ ; non-chlorinated ●—●; chlorinated ○- - -○.

expected results were not entirely achieved (Table 3). Mortalities increased in chlorinated 20% SWE $MgCl_2$ and $CaCl_2$ as expected, but surprisingly fewer deaths occurred in chlorinated 20% seawater and 20% SWE NaCl than in chlorinated tapwater. Clearly, external ions alter chlorine toxicities differently in larvae from prehatched embryos.

TABLE 3: % Mortality of Hatched Larvae

Medium	# Hatched Larvae	# Dead Larvae	% Mortality	Medium	# Hatched Larvae	# Dead Larvae	% Mortality
Control	43	6	14	Control	45	8	18
1% SW	44	9	20	20% SW	45	3	7
1% SWE				20% SWE			
$CaCl_2$	30	6	20	$CaCl_2$	7	3	43
$MgCl_2$	29	10	35	$MgCl_2$	8	3	38
KCl	42	3	7	KCl	29	5	17
NaCl	40	7	18	NaCl	20	1	5

DISCUSSION

The objectives of this study were to determine the effect of intermittent chlorination on zebrafish eggs, and to what extent, if any, the toxicity is modified by ionic and osmotic stresses. Intermittent chlorination, with an initial concentration of 1 mg/ml TRC (TABLE 2), was chosen because this level is high enough to be toxic to most fish (BRUNGS 1973), yet also represents a closer approximation to the changing levels of chlorine likely to be encountered in the environment (BRUNGS 1973, DICKSON et al. 1974) than does continuous chlorination.

In adult fish, chlorine disrupts gill integrity by cleaving intercellular junctions (COHEN and VALENZUELA 1977, MIDDAUGH et al. 1977) and thereby impairs iono- and/or osmoregulation (COHEN 1977). In fact, when mortally intoxicated mosquitofish (*Gambusia affinis*) were placed in waters approximately equivalent to physiological saline, they recovered at least temporarily and subsequent mortalities were markedly lower (COHEN 1977). In the present experiments, the newly hatched zebrafish larvae, which have already begun to osmoregulate, were the most sensitive developmental stage (TABLE 2) and exhibited the same pattern of susceptibility to chlorine intoxication as adult fish.

Contrary to initial expectations, chlorine, even at this relatively high concentration (1 mg/mL TRC), is not toxic to developing zebrafish embryos prior to hatching (Figs. 2-3), in contrast to the embryos of plaice (ALDERSON 1974), a marine teleost, and striped bass (MIDDAUGH et al. 1977), an estuarine species, both of which succumed to chlorine levels much lower than 1 mg/L during continuous exposure. A possible reason is that chlorine chemistry differs in saline water (JOHNSON 1977). Within seconds after mixing with seawater, chlorinated compounds are converted into bromoform analogs which may be the actual toxic agents. However, because of the instability, they are not present in great amounts. On the other hand, free chlorine and chloramines form in both seawater and freshwater and are toxic to freshwater fish populations (BRUNGS 1973, MCKIM et al. 1973). Therefore, it is more likely that species differences rather than water chemistry account for insensitivity of zebrafish eggs to chlorine. In turn, the basis of this resistance may be attributable to the characteristics of the two physical barriers that surround zebrafish from at least stage I to hatching: the chorion and the blastodermal layer. The chorion is a non-living proteinaceous membrane surrounding the egg that protects it from bacterial and fungal infection (RUGH 1962) and may also be reacting with at least some of the chlorine present in solution (YOSHA 1976). However, because the chorion is known to be porous (SHANKLIN 1959, POTTS and RUDY 1969), it offers negligible protection against the diffusion of a small molecule such as chlorine. Furthermore, dechorionated zebrafish eggs are not harmed by intermittent chlorination in tapwater for up to three days (YOSHA UNPUBLISHED DATA). On the other hand, the blastodermal layer, which forms between 2 and 10 h after fertilization (HISAOKA AND BATTLE 1958) and corresponds to stage I embryos (TABLE 1), consists of flattened cells that completely surround the embryo and yolk. Although this layer is disrupted shortly before hatching (SHANKLIN 1959) when the mouth and gills begin to function, the blastodermal layer normally maintains a very low permeability to water and salt while it is intact (PRESCOTT and ZEUTHEN 1953). This layer's low permeability has been confirmed for several other species, including trout (PRESCOTT and ZEUTHEN 1953, ZOTIN 1965), Fundulus (SHANKLIN 1959), and salmon (HAYES and ARMSTRONG 1942, RUDY and POTTS 1969). It is not known whether the permeability characteristics are attributable to the plasma membrane, cell coat, or surface charges, but as a living cellular membrane, the blastodermal layer must retain its integrity in order to fulfill its protective function. Accordingly, its damage by chlorine and/or ionic stresses could subject the embryo to serious physiological insults. Since the blastodermal layer normally begins to disrupt and allow ion exchange the time of hatching (HAYES 1946), it is not surprising that initial sensitivity of hatching larvae to ionic and toxic stresses coincides with commencement of ionoregulation. However, not all blastoderms are impermeable. The pike embryo, for example, is freely and continuously permeable to water (LOEFFLER and LØVTRUP 1969). It is therefore postulated that the zebrafish embryo is relatively resistant to chlorine because it performs a minimal amount of iono- and osmoregulation, if at all, and those fish embryos with more permeable blastodermal membranes will be more susceptible to chlorine intoxication. By the same reasoning, it is expected that continuous exposure to chlorine would be more harmful than intermittent exposure due to continued osmotic stress placed on the embryos.

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