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An Evaluation of a Six Day Cyprinid Embryo-Larval Test for Estimating Maximum Allowable Toxicant Concentration of Pesticide Under Tropical Conditions

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Abstract

This study was designed to evaluate a six day embryo-larval test to arrive at a sensitive chronic effect threshold, (maximum allowable toxicant concentration or MATC) for pesticides. The embryo larval period of the Indian major carp *Labeo rohita* (Ham.) were exposed to sublethal levels of monocrotophos (an insecticide) during a 136 h exposure period. Among the end points observed, 'hatching' is found to be much less sensitive than 'survival.' Among the embryo life history phases exposed, the 'free embryonic' phase (22 to 96 h) is found to be as sensitive as the early apterolarval phase (96 to 136 h). A 96 h cyprinid embryo-free embryo toxicity test with "survival" as the end point is found to be a cost effective and rapid method for monitoring the chronic toxicity of pesticides to fishes under tropical conditions.

Introduction

The developing fish embryo and the larva are generally considered as the most sensitive stages in the life cycle of a teleost, being particularly sensitive to all kinds of low level environmental changes to which it might be exposed (von Westernhagen 1988). Reviews and studies on fish toxicology by McKim (1977, 1985); Macek and Sleight (1977); Woltering (1984); Kristensen (1994); Nagel (1994) and Meinelt and Staaks (1994) have shown that teleost early life-history stages are almost always the most sensitive to the impact of toxicants. McKim (1985) concluded that in 82% of the tests reported up to 1985, the lowest observable effect concentration (LOEC) for the early life history stages were identical to the LOEC values obtained for the complete life cycle studies of fish.

With regard to biological endpoints, Woltering (1984) in his review concluded that "survival" was as sensitive (or insignificantly less sensitive) as "growth." Kristensen (1994) concluded that in 75% of the reported experiments to date on early life history stages, 'survival' as a factor was within a factor of 3 of the lowest LOEC recorded for the different end-points studied.

Paucity of information based on evaluation of MATC (chronic effect threshold) for tropical species and toxicants initially necessitate relatively simple test techniques. Hence an attempt is made here to evaluate a cost effective short duration egg-embryo-larval test with 'hatching' and 'survival' as endpoints using *Labeo rohita* (Ham.Buch.) (*Cyprinidae*; *Cyprininae*) hatchery produced eggs and an organophosphate insecticide-monocrotophos, commonly used in the paddy fields of the tropics. *Labeo rohita* adults and juveniles have been found to be suitable test animals for toxicity monitoring (Ashraf et al. 1992, Nair and Sherief 1998).

Materials and Methods

Toxicant

The insecticide 'Nuvacron' is a water soluble organophosphate concentrate containing 360 g monocrotophos [0,0-dimethyl-0-(2-methyl-carbonyl-1-methyl vinyl) phosphate] as active ingredient in a kilogram of product (400 g·liter of product). It is a broad spectrum systemic and contact insecticide-cum-acaricide used against insect pests of paddy.

Test animals

The classification of the life history phases of rohu exposed to the toxicant are based on Balon (1975) as a consistant means of morphological identification of development levels.

EMBRYONIC PHASE OR INSIDE THE EGG EMBRYO (AGE 3 TO 22 H)

This phase encompasses the interval of intense organogenesis within the egg membrane and continues until hatching is completed.

ELEUTHEROEMBRYO OR FREE EMBRYO PHASE (AGE 22 TO 96 H)

This phase commences with hatching and lasts until all of the yolk is digested or absorbed and the fish begins to feed externally.

APTEROLARVAL PHASE OR PROTOPTERYGIAL LARVAL PHASE (AGE 96 TO 136 H)

This phase covers the interval between the transition to exogenous feeding and the commencement of differentiation of the embryonic median finfold (can be identified by the formation of the miniature dorsal fin).

The interval or age in hours was taken when the last of the test animals in the experiment has reached a particular threshold indicated. The fertilized live eggs were provided by the Fisheries College Carp Hatchery produced by standard induced breeding technique. The percentage fertilization for the particular spawning was around 75%. The fertilized eggs were collected with care and rinsed in dilution water to remove the debris and excrement if there is any. The exposure of eggs started approximately 3 to 4 h after fertilization at the end of the cleavage egg phase. The tests were terminated after 136 h at the end of the free swimming apterolarval phase. Control eggs were cultured simultaneously with the exposed eggs under identical conditions.

Eleven nominal concentrations (0.1, 0.2, 0.6, 1.2, 2.4, 3.0, 4.0, 6.0, 8.0, 10.0, and 15.0 $mg \cdot l^{-1}$) of monocrotophos were selected for the experiment. The 96 h LC 50 value for rohu juveniles is 46 $mg \cdot l^{-1}$ (Sulekha et al. 1999). One third of this value was taken as the maximum sublethal concentration (Konar 1969) while the lower concentrations were chosen according to Sprague (1973). The tests were conducted in triplicate in 4 l glass troughs, with 2 l dilution water and 50 eggs each (sample size 150 eggs in each test concentration and control). The troughs were partially covered to minimize evaporation The test concentrations were completely replenished every 12 h. The temperature, pH and DO levels were monitored before and after dosing with the toxicant to arrive at the desired range. The oxygen regime was 7.55 ± 0.3 $mg \cdot l^{-1}$ in the control replicates. The minimum dissolved oxygen level never went below 60% of the control value in any of the test concentrations. The pH ranged from 6.6 to 7.5 in the different treatments. Water temperature was maintained at 27 ± 1.5°C throughout the exposure period. Photoperiodicity was natural day-night cycle. The apterolarvae were fed on particulate egg yolk *ad libitum* twice a day. No incidence of any fungal infection was noted during the test period in any of the test concentrations. Embryonic and free embryonic phases of rohu appeared to show no adverse reactions to routine observations and disturbances due to replenishment of test solutions.

Hatching data was recorded at every one hour interval up to the time of complete hatching (11 to 22 h). Mortality data was monitored at 6 h intervals (22 to 136 h). The 50% hatching time (HT_{50}) for the eggs and the 50% mortality time (LT_{50}) for the different phases were calculated using linear regression analysis after probit transformation of mean cumulative hatching/mortality and the log 10 transformation of the test time based on the probit method (Finney 1971). Treatment means of the biological end points (percentage hatching and percentage survival) at the end of each phase were compared through one-way ANOVA following Snedecor and Cochran (1973) to arrive at the no-observable effect concentration (NOEC) and the least observable effect concentration (LOEC). The MATC is calculated based on these endpoints.

$$MATC = (NOEC \times LOEC)^{1/2.}$$

Results

The mean percentage hatching (cumulative) of the eggs of rohu in different concentrations of monocrotophos and control during the 11 to 22 h period are presented in figure 1. The 50% hatching time (HT_{50}) and the corresponding regression equations are given in table 1. The HT_{50} ranged from 12.93 h (1.2 $mg \cdot l^{-1}$) to 19.24 h (4 $mg \cdot l^{-1}$), the control eggs showing a value of 16.05 h.

The mean percentage mortality of the free embryo and apterolarval phases in the different test concentrations and control are presented in figure 2. The summarized results of the percentage hatching (at the end of 22 h), percentage survival of embryos (at the end of 94 h) and the percentage survival of

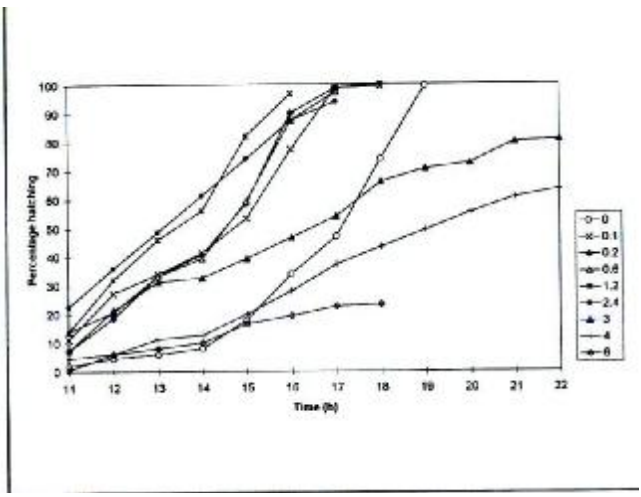

Fig. 1. The mean percentage hatching of the eggs of rohu exposed to different concentrations of monocrotophos (11 to 22 h).

Table 1. The incubation time or 50% hatching time (HT 50) of rohu eggs exposed to different monocrotophos treatments.

Nominal monocrotophos concentrations ($mg \cdot l^{-1}$)	HT_{50} (h)	r^2	Regression equation
0.0	16.05	0.820	Y = -14.236 + 15.971 log X
0.1	13.60	0.814	Y = -11.281 + 14.36 log X
0.2	13.67	0.890	Y = -15.385 + 17.947 log X
0.6	13.63	0.899	Y = -12.287 + 15.233 log X
1.2	13.00	0.942	Y = -13.486 + 16.593 log X
2.4	12.93	0.984	Y = -8.295 + 11.957 log X
3.0	16.19	0.974	Y = -3.6584 + 7.1587 log X
4.0	19.24	0.992	Y = -4.362 + 7.289 log X
6.0	only 23% of the eggs hatched (18h)		
8.0	None of the eggs hatched		
10.0	None of the eggs hatched		
15.0	None of the eggs hatched		

Y = % cumulative hatching (probit)
X = Hatching time (log_{10})

apterolarva (at the end of 136 h) in the different toxicant concentrations and control are presented in table 2. The MATC for the embryo phase based on 'hatching' is 2.68 $mg \cdot l^{-1}$, for the free embryo phase and apterolarval phase based on 'survival' is 0.346 $mg \cdot l^{-1.}$ Based on the 96 h LC_{50} value for rohu juveniles, the application factors are 0.06 and 0.008 respectively.

The mortality data of the embryos, free embryos and apterolarva were further analyzed to find out the lethal time at which 50% of the test animals died during the entire period of exposure. The LT_{50} occurred at 10.6 h in 6 $mg \cdot l^{-1}$ during the embryonic phase and at 71 h in 4 $mg \cdot l^{-1}$ during the free embryonic phase and at 118 h in 3 $mg \cdot l^{-1}$ during the apterolarval phase (Fig.3).

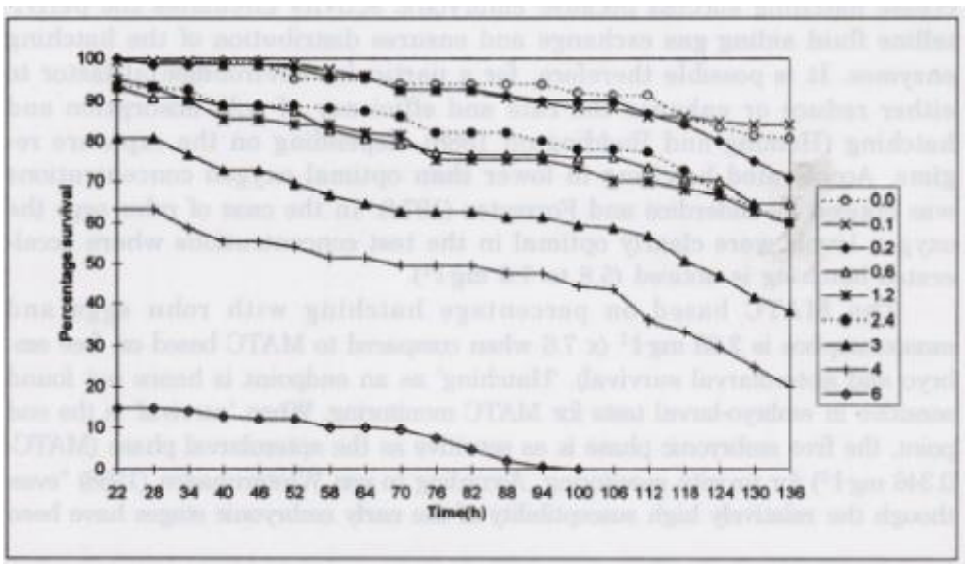

Fig. 2. The mean percentage survival of free embryo (22-94 h) and apterolarva (94-136 h) of rohu in the different test concentrations of monocrotophos.

Table 2. Hatching and mortality parameters (Mean ± S.D) for the rohu embryo, free embryo and apterolarva during 136 h exposure to various concentrations of monocrotophos ($mg \cdot l^{-1}$).

Parameters	Phase	Concentration $mg.l^{-1}$								
		0.0	0.1	0.2	0.6	1.2	2.4	3.0	4.0	6.0
Percentage hatching (at 22 h)	Embryo	99.33^{a} ± 0.94	99.33^{a} ± 0.94	100.0^{a}	93.33^{a} ± 4.12	95.33^{a} ± 2.49	93.33^{a*} ± 2.49	80.67^{b**} ± 7.36	63.33^{c} ± 14.07	14.67^{d} ± 5.25
Percentage survival (at 94 h)	Free embryo	94.0^{a} ± 3.26	89.33^{a} ± 1.88	90.0^{a*} ± 4.89	76.66^{b**} ± 7.36	75.33^{b} ± 5.73	80.0^{b} ± 5.88	61.33^{c} ± 6.59	47.33^{d} ± 9.84	0.66^{e} ± 0.94
Percentage survival (at 136 h)	Aptero larva	80.66^{a} ± 4.10	84.0^{a} ± 2.82	70.66^{a*} ± 6.59	64.66^{b**} ± 2.49	60.0^{b} ± 6.53	60.0^{b} ± 4.32	38.0^{c} ± 8.48	20.0^{c} ± 4.89	0.0

Values with different superscripts in the same row differ significantly ($P < 0.05$)
* No observable effect concentration (NOEC)
** Least observable effect concentration (LOEC)

Discussion

Hatching is not an instantaneous event but a process that occurs at various times in different individuals and is influenced by many epigenetic and environmental stimuli (Balon 1985). In the present study the lower concentrations (0.1, 0.2, 0.6, 1.2 and 2.4 $mg \cdot l^{-1}$ monocrotophos) show accelerated viable hatch (stimulated embryogenesis) while the higher concentrations (3, 4 and 6 $mg \cdot l^{-1}$ monocrotophos) show nonviable hatch (suppressed embryogenesis) when compared to control. The inside egg embryo showed increased activity in the lower concentrations and decreased activity in the higher concentrations when compared to embryo in the control. According to Rosenthal and Alderdice (1976) environmental factors that reduce embryonic activity might in some instances reduce yolk utilization and decrease hatching success because embryonic activity circulates the perivitelline fluid aiding gas exchange and ensures distribution of the hatching enzymes. It is possible therefore, for a particular environmental factor to either reduce or enhance the rate and efficiency of yolk absorption and hatching (Heming and Buddington 1988), depending on the exposure regime. Accelerated hatching in lower than optimal oxygen concentrations was noticed by Alderdice and Forrester (1974). In the case of rohu eggs the oxygen levels were clearly optimal in the test concentrations where accelerated hatching is noticed (5.8 to 7.8 $mg \cdot l^{-1}$).

The MATC based on percentage hatching with rohu eggs and monocrotophos is 2.68 $mg \cdot l^{-1}$ (x 7.6 when compared to MATC based on free embryo and apterolarval survival). 'Hatching' as an endpoint is hence not found sensitive in embryo-larval tests for MATC monitoring. When 'survival' is the end point, the free embryonic phase is as sensitive as the apterolarval phase (MATC-0.346 $mg \cdot l^{-1}$) for toxicity monitoring. According to von Westernhagen (1988) "even though the relatively high susceptibility of the early embryonic stages have been

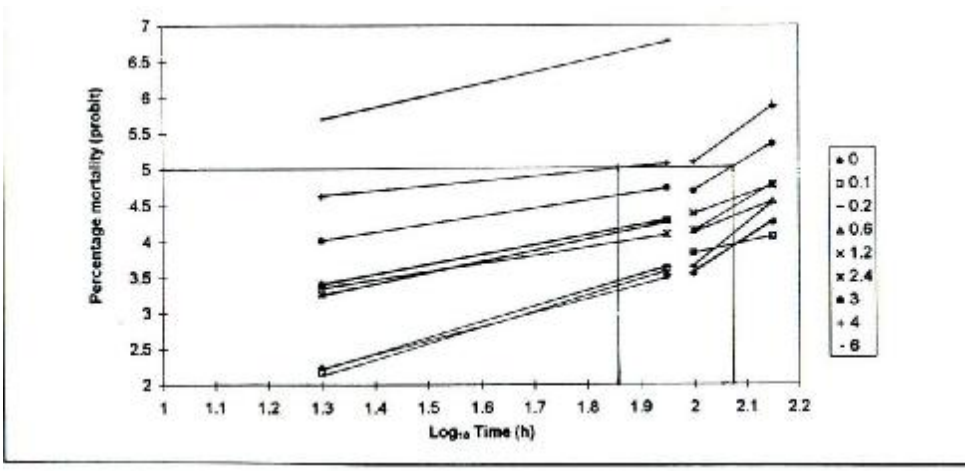

Fig. 3. The 50% mortality time (LT_{50}) for rohu embryo (22 to 94h) and apterolarva (100 to 136 h) exposed to different concentrations of monocrotophos.

well documented the yolk sac or alevin stage is known to be the most sensitive stage in the teleost life cycle." Kristensen (1994) in his review states that in 47% of the toxicity studies the inclusion of the juveniles (larva in the present context) did not produce a lower LOEC than that derived from FELS experiments terminated at the end of the sac-fry stage (free embryo in the present context). Van Leeuwan et al. (1985) as given in Whale et al. (1994) working with rainbow trout, thought that the increased sensitivity of fry stage (free embryo in the present context) was due to the accumulation of toxicants in the yolk sac which then becomes available to the fish when the yolk sac is utilized. Millet and Sims (1994) working with *Cyprinus carpio* and *Rutilus rutilus* early life history stages with ammonia as the toxicant observed that the more sensitive stage appeared to be the hatched larvae and early fry.

The authors consider it relevant to recommend 'survival' as a cost effective and sensitive endpoint and to avoid the critical free embryo-larval threshold of external feeding in the toxicity tests. The conclusion of Woltering (1984) that 'survival' as an endpoint was a cost effective replacement for 'growth' and Kristensen's (1994) observation that several authors have questioned the utility of using 'growth' as an endpoint at the juvenile (larval) stage, especially, as feeding is one of the complicating variables which might impair the reproducibility of the growth parameter, support our contention. The use of confusing nomenclature for early life history stages in fish toxicology studies by different authors greatly hampers further fruitful discussion.

The growing number of chemicals that need to be tested requires cheap and rapid test methods, coupled with a high quality and quantity of data, if possible (Meinelt and Staaks 1994), especially in the tropical developing countries of Asia. Here, a cost effective and rapid 96 h rohu embryo-free embryo subchronic toxicity test under tropical conditions is recommended with 'survival' as the endpoint. Some of the existing test procedures are 96 h DNA, RNA and protein test (Barron and Adelman 1984), a seven day fathead minnow larval test (Norberg and Mount 1985) and a 14 day zebra fish toxicity test (Dave 1986). All these test methods are developed with the philosophy of exposing only the most sensitive phases in the life cycle. Here the authors would like to reiterate Kristensen's (1994) observation that a relatively simple test technique will allow a number of laboratories other than fish specialists to generate data on chronic toxicity to fish, leading to a relatively high level of reproducibility and precision of the calculated toxicity. Other closely related carp species available in Asia could also be tested for their sensitivity and suitability to biocide toxicity monitoring.

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