

Life Cycle Stages and Length of Zebrafish (*Danio rerio*) Exposed to DDT

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Long-term toxicity growth tests were conducted with DDT using zebrafish (*Danio rerio*). The fish were exposed to concentrations of 0.05 µg/l, 0.5 µg/l, 5 µg/l and 50 µg/l DDT under flow-through conditions. The effect of the compound on the ecologically important parameters hatching rate, duration of the developmental stages and growth were the focus of interest. When exposed to 50 µg/l DDT, the hatching rate was 2 to 3 times lower than in embryos exposed to 0.05 µg/l DDT. This study demonstrates that the rearing zebrafish in the pesticide causes a reduction in body length and the durations of the life cycle stages (LCS) of the offspring are extended. These effects are increased when the fish are exposed to higher DDT concentrations.

Key words — *Danio rerio*, early life stage, life cycle stages, growth, F₁-generation, DDT

INTRODUCTION

DDT persists for a long time in the environment. It can harm beneficial neutral species (non target) and hence significantly disturbs the ecosystem. Due to its persistence, the ecological damage of this chemical is still imminent in many parts of the world. It is nearly 40 years after the first evidence was presented that organochlorines accumulate via the food chain and at different trophic levels in the clear lake California which has been treated several times with DDT.^{1–7)}

Fish represent important organisms for the evaluation of toxicity in the hydrosphere. One of the most important effects of the xenobiotics on fish population is the impairment of reproduction. Ecotoxicants can have an impact on reproduction at many levels, including effects on clutch size and egg quality, development of the embryos and larvae, the rate of hatching and development to sexual maturity. Tests limited to early life stages have been shown to be inadequate to investigate these parameters comprehensively.⁸⁾ A full life cycle assessment has to be carried out instead.

Although level two of the German chemical act requires a long term test that has to include reproduction,⁹⁾ no satisfactory test guidelines are avail-

able up to now. The zebrafish [*Danio rerio* (*D. rerio*)] is a suitable model for full life cycle assessments because zebrafish takes only 3–4 months to reach the sexual maturity. Moreover, they can be maintained in a laboratory environment at low cost and remain sexually active throughout the year.

The test employed in this study involved exposure of zebrafish to the toxin from the fertilized egg up to the mature animal. The effect on reproduction was assessed by examination of mortality, hatching rate, body length and development of the offspring. This study encompassed the examination of two generations of fish over a period of approximately 8 months. This was regarded necessary to obtain sufficient information on the duration of each life stage and the deleterious effect of the chemical on the subsequent generation. The parameters were also investigated in the offspring of exposed parents that were subsequently reared in a toxin-free environment. The second generation was assessed up to six weeks and the difference with offspring which remained exposed to DDT was determined.

MATERIALS AND METHODS

Test Species — In Europe zebrafish (*D. rerio*) is increasingly used in toxicological studies. The fish well known to breeders measures 3–5 cm in the adult state. It thrives both in soft and hard waters, grows quickly at temperatures around 26°C and reaches

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the sexual maturity within 3 months. Zebrafish produces gametes throughout the year, and easily reared under laboratory conditions.^{10,11)}

Egg Collection — The fish of the F₀-generation were obtained from a zoological store in Saarbrücken, Federal Republic of Germany (FRG). They were 4–5 months old at the time of arrival. Mature females were mated with males in glass aquaria. Eggs were collected in glass dishes covered with stainless steel wire mesh. The dishes were placed at the bottom of the glass aquaria. Green glass trees served as spawning substrate. Immediately after switching on the light in the morning, mating began and two hours later, glass dishes containing eggs were removed from the aquaria.

Egg Exposure — Fertilized eggs were exposed under flow-through conditions to the different concentrations of DDT ranging from 0.05 µg/l to 50 µg/l and separated by a factor of 10. An untreated group was kept under the same conditions as control. This stage was considered to be the early life stage (ELS) of the F₁-generation of test organisms. The flow-through system (Fig. 1) consisted of a water tank in which charcoal filtered tap water was aerated. The temperature was kept constant at 26°C (± 1°C), the pH value 7.9 ± 0.2 and O₂ content 5.9 (± 0.3) mg/l. Dilution water was pumped into the glass vessels. The toxicant was added to the test solution using a micropipette and a photoperiod of 12 hr was maintained.

Start of the Life Cycle — The life cycle experiment was started with 90 fertilized eggs per glass petridish (8 cm height, 15 cm in diameter, 500 ml test solution). Eggs were obtained according to the method of Nagel.¹²⁾ From day 6 young larvae were fed twice a day with AZ25 (Tetrawerke, Melle, FRG). From day 8 newly hatched brine shrimp (*Artemia* species) and pulverized dry food (Tetramin) were added. Two weeks later after the evaluation of the rate of hatching, the larvae were separated into 2 groups. The first group (Group I) was transferred into glass vessels (20 × 15 × 30 cm, H × w × L, 8l test solution) filled with uncontaminated solution and the second group (Group II) of larvae remained exposed to DDT during their whole life time. The experiment was conducted subsequently for both cases.

Feeding with Tetramin AZ25 was gradually reduced. Excrement and surplus food were removed daily. Hatching rates, morphological abnormalities and mortality during the test were recorded.

Post-Larval Stages — After the embryonic stage, body length and survival rate were determined dur-

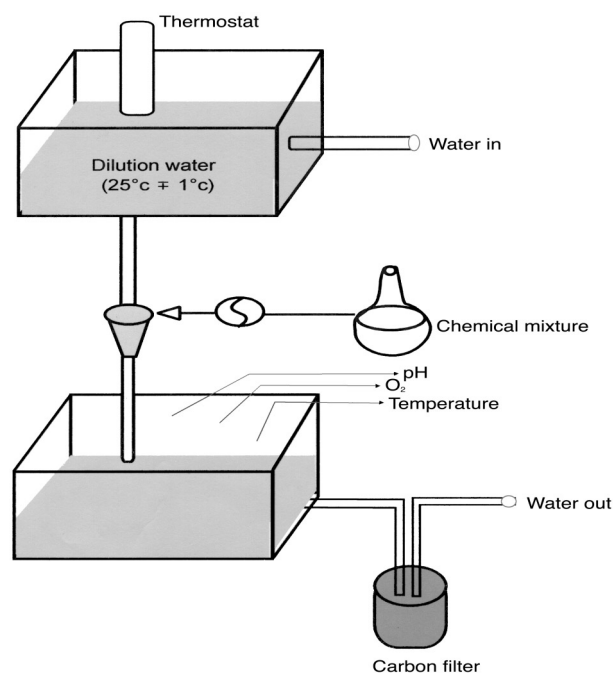


Fig. 1. Flow Through Aquarium

ing 60 days. Surviving fish were kept in larger glass aquaria (30 × 40 × 25 cm, H × W × L, 28-l test solution) until sexual maturity was reached. Water exchange was once a day.^{13,14)} Depending on the level of DDT in the aquaria, sexual maturity was reached after 12–17 weeks. From day 125, 10 male and 5 female fish of each test chamber were mated. The eggs laid were used for the investigation of the ELS of the F₂-generation in the same way to that of the F₁-generation. To assess the quality of the offspring whose parents were long term exposed to the pesticide, an evaluation of the body length was carried out during the ELS of the F₂-generation.

Chemical and Chemical Analysis — DDT (CAS no. 50–29–3), CA 1,1,1-trichloro-2,2-bis (*p*-chlorophenyl) ethane (mixed isomers)- was obtained from the company Prochem GmbH in Wesel, FRG.

DDT was extracted with ethyl acetate from water between day 65 and 125 during the washing phase of Group I fish. The samples were first filtered and the extraction was performed according to a column type CHROMABOND® C18ec / 6 ml / 500 mg. The extracts were analyzed by the Standard Operating Procedure (SOP) for analysis of organochlorine pesticide residues in biological matrices according to the US-EPA method 608.¹⁵⁾ The extraction procedure was the same for each liter of all the water samples. All equipment was disinfected before use.

The mean duration of the stages was determined

and length of fish was evaluated on fixed specimens each in triplicates. All equipment was disinfected. Comparison of body length and duration of the LCS between the tested concentrations and control was performed using the statistical *t*-test and analysis of variance (ANOVA).

RESULTS

Rate of Hatching, Development to Sexual Maturity and Survival Rates

Hatching did not happen, when fertilized eggs were initially exposed to 500 $\mu\text{g/l}$ DDT and the rate of hatching decreased with increasing concentrations (5 $\mu\text{g/l}$ and 50 $\mu\text{g/l}$). The dose-dependant reduction of the hatching rate varied from 4 to 58% between the lowest and the highest concentration. Premature hatching occurred at 50 $\mu\text{g/l}$ and the larvae died. At none of the tested concentrations was a 100% hatching rate recorded and it was reduced already to less than 30% at 5 $\mu\text{g/l}$.

In Group I, from day 44 to 120, the development was inhibited according to the concentration of DDT. However, 0.05 $\mu\text{g/l}$ causes already a 50–55% effect. Therefore 0.05 $\mu\text{g/l}$ could be regarded as Low Observed Effect Concentration (LOEC). The rate of hatching of Group II fish in the F_2 -generation where all the fish remained exposed to DDT was lower than that were lower than that in the F_1 -generation (Fig. 2). In Group II, a survival rate of more than 50% was recorded only at the concentration 0.05 $\mu\text{g/l}$ after 6 weeks of treatment. All the fish exposed to the highest tested concentration (50 $\mu\text{g/l}$) succumbed before day 44. Most of the fish died within the second week. In Group I, where the fish were transferred to DDT-free tanks, more than 50% of fish survived although the survival rate was inversely correlated with the DDT concentrations (Fig. 3). Moreover, the rate of mortality decreased slightly, but remained higher in treated tanks than in controls.

Length of Fish Emerged and Duration of the Life Cycle Stages

A dose-dependant reduction of the body was recorded in Group I and Group II fish. The body length of fish was reduced by 5–25% and 1–21% in Group II and Group I, respectively (Fig. 4). A complete life cycle test starting with fertilized eggs lasted 136 days. After treatment, the life cycle experiment

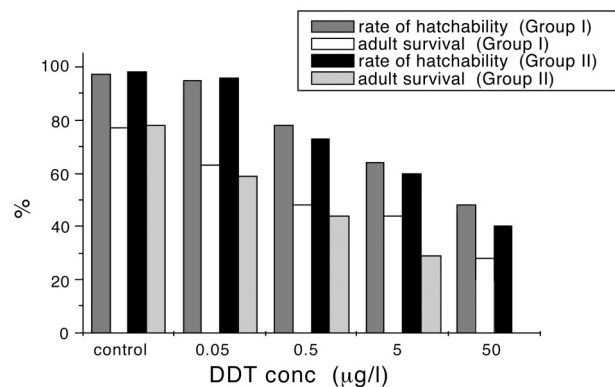


Fig. 2. Rates of Hatchability and Adult Survival of Groups I and II Fish in the F_1 -Generation

The absence of the adult survival at 50 $\mu\text{g/l}$ in Group II fish is due to the death of all juvenile fish within a short time after hatching.

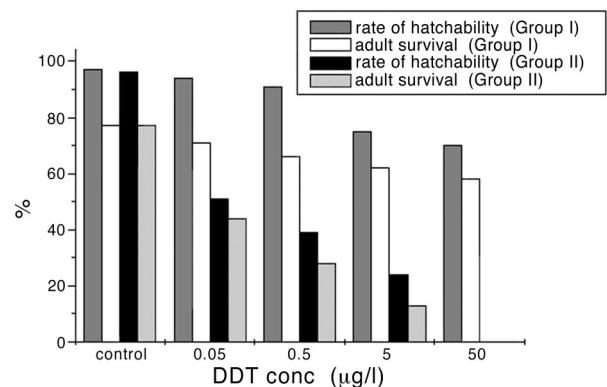


Fig. 3. Rates of Hatchability and Adult Survival of Groups I and II Fish, 6 weeks after Hatching in the F_1 -Generation

At 50 $\mu\text{g/l}$ no hatching occurred in Group II fish.

starting with fertilized eggs showed a dose-dependant increase of the duration of the life stages. However, a 6–33 days and a 10–38 days delay of development up to the next generation was noted in Group II and Group I, respectively (Table 1, Table 2). The delay in growth in each life stage was found to be dependant on the dose of the pesticide.

In Group I, a few days delay was also recorded during the hatching stage of eggs produced in the F_1 -generation and for the F_2 -generation. It is clear from these data that the effects of DDT were widespread up to the ELS of the F_2 -generation. This result was emphasized by the evaluation of body length in the F_2 -generation of Group I, six weeks after hatching which also demonstrated a dose-dependant reduction (Fig. 5).

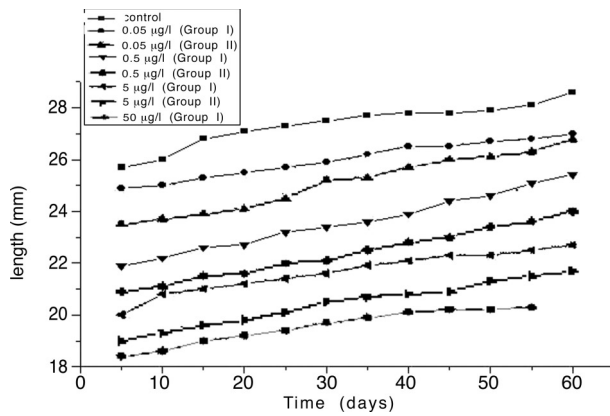


Fig. 4. Length of Groups I and II Fish Emerged within 60 days after the Larval Stage at all the DDT Tested Concentrations

For Group I: N = 3; F-ratio = 0.13 at degree of freedom (4.40); $p < 0.05$. For Group II: N = 3; F-ratio = 0.09 at degree of freedom (3.33); $p < 0.05$. At 50 µg/l all the fish succumbed.

Table 1. Mean Duration in Days (\pm Standard Error) of the Life Cycle Stages up to 6 weeks in the F₂-Generation of Group I Fish

	Control	0.05 µg/l DDT	0.5 µg/l DDT	5 µg/l DDT	50 µg/l DDT
A	0	0	0	0	0
B	44 ± 1	45 ± 1	48 ± 2	52 ± 2	55 ± 1
C	87 ± 2	90 ± 2	94 ± 3	101 ± 3	106 ± 4 ^{a)}
D	108 ± 3	114 ± 4	121 ± 3	131 ± 5	134 ± 2
E	175 ± 4	182 ± 3	191 ± 5	206 ± 7	212 ± 5

A: Start with 90 fertilized eggs. B: Development of embryos and larvae. C: Sexual maturity. D: Egg production. E: Larval stage of the F₂-generation. ^{a)} indicates more than 10% mortality between the larval development and sexual maturity.

Quantification of DDT in Test Chambers

The analysis of water samples in test vessels of Group I fish on day 125 demonstrated the presence of DDT residues. The higher the initial dosed concentration of the pesticide, the higher was the level of residues detected (Table 3).

DISCUSSION

All the tested concentrations of DDT influenced the rate of hatching, growth and duration of the LCS and therefore no No Observed Effect Concentration (NOEC) could be established.

In Group II fish, contrary to the F₁-generation, F₂-eggs were directly spawned into DDT treated water. That influenced the rate of hatching, which

Table 2. Duration in Days (\pm Standard Error) of the Life Cycle Stages at the DDT Tested Concentrations up to 6 weeks in the F₂-Generation of Group II Fish

	control	0.05 µg/l DDT	0.5 µg/l DDT	5 µg/l DDT
A	0	0	0	0
B	44 ± 1	47 ± 1 ^{a)}	60 ± 3 ^{a)}	68 ± 3 ^{a)}
C	87 ± 2	97 ± 2 ^{a)}	111 ± 4 ^{a)}	128 ± 5 ^{a)}
D	108 ± 3	115 ± 4	137 ± 5	163 ± 4
E	175 ± 4	190 ± 5	220 ± 7	243 ± 8

A: Start with 90 fertilized eggs. B: Development of embryos and larvae. C: Sexual maturity. D: Egg production. E: F₂-generation. ^{a)} indicates more than 10 % mortality between larval stage and sexual maturity.

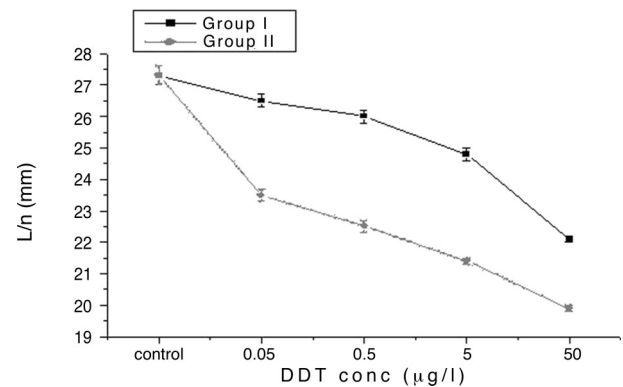


Fig. 5. Mean Length (L/n \pm S.E.) of Juvenile Fish Taken Randomly in the ELS of the F₁-Generation

Where n = 6 is the number of fish measured at each tested concentration. S.E.: standard error.

was reduced to 10–65%. Relative to controls, the reduction of the rate of hatching recorded during this study agreed with the report of Woltering¹⁶⁾ in 1984 in which he reviewed a total of 173 tests including exposure to metals, pesticides and xenobiotics and found that the hatching rate was reduced to 19%.

But in all the cases, an increase in developmental abnormalities was not observed. In all tested concentrations and controls, all rates were less than 3%. Eight larvae exposed to 50 µg/l DDT in Group II showed edema and died before day 14. It could be assumed that the observed deformation rate is natural agreeing with data from Bresh *et al.*¹⁴⁾ who found less than 5% deformations during an experimental time over two fish generations.

On the other hand, the growth of juvenile fish was greatly reduced at 0.5 µg/l and 5 µg/l for Group II. These reductions of the growth were not negligible. The effect of DDT on growth was evident at the higher concentrations where the fish density was

Table 3. Evaluation of DDT Residues (ng/l) in Test Chambers of Group I Fish from Day 65 and 125 post Hatching

Time (days)	0.05 $\mu\text{g/l}$	0.5 $\mu\text{g/l}$	SE	5 $\mu\text{g/l}$	SE	50 $\mu\text{g/l}$	SE
2	—	5.36	2.13	23.81	9.48	131.55	52.37
5	—	5.07	2.01	24.25	9.65	134.61	53.57
10	—	4.15	1.65	25.64	10.2	136.2	54.21
15	—	3.2	1.27	25.76	10.25	137.21	54.61
20	—	2.59	1.03	25.54	10.16	133.85	53.27
25	—	2.53	1.01	23.02	9.16	125.53	49.96
30	—	2.51	1	22.57	8.98	123.35	49.09
35	—	2.09	0.83	22.34	8.89	120.51	47.96
40	—	—	—	22.11	8.8	119.26	47.46
45	—	—	—	21.81	8.68	119.01	47.36
50	—	—	—	21.76	8.66	118.93	47.33
55	—	—	—	21.48	8.55	118.89	47.31
60	—	—	—	21.25	8.46	118.17	47.07

— : indicates where residues were not detectable. S.E.: Standard Error.

lower due to the high rate of mortality. Our study suggests that the effect of DDT on the growth of zebrafish is independent from their density, which does not agree with the report of Ensenbach and Nagel.¹⁷⁾ However, the body length of fish was reduced by 1–24% in Group I. This reduction was higher at 50 $\mu\text{g/l}$ suggesting a dose dependant reduction of the body length.

Although in some reports, for example Nagel¹⁾ reported no effect on growth of zebrafish exposed to Lindane and Dichlormethane up to 150 $\mu\text{g/l}$. We observed in our study that DDT alone at 5 $\mu\text{g/l}$ already alters the growth of zebrafish. Moreover, the body length of fish was also reduced in Group I fish. The reduction was also dependant on the initial level of the tested chemical and was therefore higher at the highest tested concentration and up to ELS of the F_2 -generation. This could be explained by the detection of DDT residues in test chambers filled with untreated water in which the larvae initially exposed to DDT were transferred two weeks after hatching. In general, it could be proposed firstly that the ELS of zebrafish is very sensitive to DDT and secondly that the effects of DDT could be widespread to the offspring of the next generation. Comparing the effects of DDT on the early life stage tests of F_1 -generation and that of F_2 -generation, it was demonstrated that raising of the parent fish in DDT influenced the quality of their offspring.

The developmental stages of the fish lasted longer in Group II animals, which remained exposed to DDT during their whole life time. The time taken was found dependant to the initial level of the pesticide. It was also the case for Group I fish although

the difference in time was not that significant as in Group II. It was concluded that the effect of DDT is irreversible and the occurrence of this effect is accelerated in case of whole life time exposure which can lead to the death of the fish. In such a situation, when the fish survived, the complete life cycle lasted longer than expected. For example in Group II, at 0.5 $\mu\text{g/l}$ the production of eggs started on day 145 whereas in the Group of untreated fish it started on day 108.

The reduced number of eggs reported during this experiment demonstrates that the reproduction and the rate of fertility of fish were disturbed by DDT.

Conduction of a life cycle test with zebrafish could be a way to get toxicological data of parameters relevant for the population. Fish were exposed for at least seven months to DDT and most of the developmental stages were recordable. In comparison to experiments with adult fish, we hope much more that toxicological data can be collected, which leads to a better assessment of the toxic potential of the chemicals.

Exposure time and DDT level were two factors which influenced the development of zebrafish. It can be concluded that timing, duration and amount of exposure are each important determinants of the outcome. There are windows of vulnerability during larval development in which exposures to DDT may have profound effects also observed in adults. Another question remains how to proceed if a test performed for the chemicals Act does not result in a NOEC value as it is the case in the present study. If this value is absolutely needed, it may be advisable to test another concentration 10-fold under the low-

est amount applied in the former test.

A test over two generations using a suitable species, certainly is the most precise method at this time to estimate the hazard potential of a substance on representative species. Any laboratory tests, of course, yield only estimates of the effect of a substance in nature. A strong mechanistic case can be made to explain how exposure of the ELS in the F₁-generation could disturb the LCS, the population size, the growth, reproduction and life span of zebrafish up to the ELS of the F₂-generation. The suggested mechanism whereby exposure to DDT could induce the quality and quantity of offsprings produced can be offered as a hypothesis on which to focus discussion and research.

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The experiments involved in the present work comply with the current laws of the Federal Republic of Germany (FRG).

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