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Early Life-Stage Test in Zebrafish versus a Growth Test in Rainbow Trout to Evaluate Toxic Effects

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Different test procedures to evaluate the hazard potential of anthropogenic xenobiotics on fish have been proposed and are applied in practice. In the simplest tests acute toxicity is determined, and mostly concentrations leading to lethality within a short time only are of interest. However, from the ecotoxicological point of view, lethality is of minor significance as most xenobiotics usually occur in subacute concentrations in the environment. To reveal actions of low substance concentrations, finer test procedures have to be applied; effects on ecologically important factors such as growth and reproduction are of interest. Long-term tests, i.e. life-cycle tests extending over one or two generations, are indicated to scrutinize a substance thoroughly. Such tests have been conducted occasionally. As internationally recognized guidelines for such tests are not available, everyone performing a long-term test uses a method of his own. One of the most serious problems in planning such a test is choosing the adequate concentration range. Within this range concentrations should be graduated in such a way that under the highest concentration distinct effects are observed, while the lowest does not cause any effects. As no general relation exists between the LC50 and threshold concentrations obtained from long-term studies, it is not possible to deduce the concentration range for a long-term test from acute toxicity values alone. More information for better range finding is obtained from subchronic tests. Guidelines for subchronic test procedures were published, e.g. for early life-stage tests, (US-EPA 1982; ASTM 1988) and for a test regarding prolonged toxicity studies in adult fish (OECD 1983).

A test especially following growth of fish for a certain period and worked out for rainbow trout was proposed by Crossland (1985). In this test trout fingerlings are incubated for a period up to 4 wk. In the case of

rainbow trout and other salmonids, fish of a practicable size unfortunately are not available throughout the year. Tests using these sensitively reacting fish therefore are limited to certain periods. An alternative is the early life-stage test conducted with a species developing and growing rapidly and available in all life stages throughout the year. The zebrafish (Brachydanio rerio), for instance, meets these requirements. It grows rapidly, reaches sexual maturity within 3 mon and thereafter delivers gametes of excellent quality throughout the year. The test hence may be conducted whenever needed.

The aim of the work presented in this paper was to compare toxic threshold concentrations of three substances obtained from growth tests in rainbow trout (Salmo gairdneri) with data from early life-stages in zebrafish. The growth test was conducted over a period of 7 wk in case of 4-chloroaniline and 4 wk in case of 3,4-dichloroaniline and diazinon. The data from the experiment in zebrafish originate from life-cycle studies; here, only the results obtained within the first 6 wk of development after fertilization are considered. These time limits have been set, as in the FRG a growth test in rainbow trout extending over 4 wk and an early life-stage test in zebrafish extending over 6 wk are being discussed for the Chemical Act.

MATERIALS AND METHODS

Fish were kept and reared, and the tests were conducted in tap water, total hardness 360 mg/L (as $CaCO_3$), ions (mg/L): Ca 117, Mg 13, Na 10, K 2, Cl 19, SO_4 70, NO_3 5.5, PO_4 0.01; pH 7.4 under flow-through conditions. Oxygen levels were never below 60% of saturation in the case of zebrafish and 70% in case of rainbow trout. For zebrafish temperature was kept at 26 ± 1°C and for rainbow trout at 15 - 17°C. Adult zebrafish were fed three times per d with dry food (TetraMin) corresponding to 3% of average body weight and once per d with Artemia salina nauplii (brineshrimp nauplii) in such quantities that nauplii were still present 1 hr after feeding time. Larvae were fed once a d with egg yolk in the tests with 4-chloroaniline, as was described (Bresch et al. 1990) and in the tests with 3,4-dichloroaniline and diazinon three times per d with an aqueous suspension of a new breeding food (Tetra Werke, Melle, FRG) which is commercially not yet available. Brineshrimp nauplii were offered once a d in addition. When the fish had reached an age of 4 wk, the breeding food was replaced by TetraMin. Further details on conducting the test in zebrafish have already been described (Bresch et al. 1990).

Rainbow trout weighing 1-3 g were obtained from commercial breeders. Twenty animals were kept in 35-L all-glass aquaria under a flow rate of 600 mL/min. The animals were fed commercial trout food pellets at a total quantity of 3% of mean body weight twice a d. Before the tests trouts were allowed to acclimatize for at least 2 wk. Before the weight of fingerlings was determined the fish were dried quickly between several sheets of Kleenex paper.

To prepare the test waters, stock solutions and tap water were mixed and transferred to the aquaria as described earlier (Bresch et al. 1990). The fish were grown as described in the same publication. Stock solutions were prepared by dissolving of the compound in hot water under stirring and quick cooling to room temperature when the substance had dissolved. Chemicals used were of analytical grade. Test concentrations were verified once a week by HPLC: 4-chloroaniline was analysed in a system as described earlier (Bresch et al. 1990); whereas 3,4-dichloroaniline was directly determined by injection of a 20 µl water sample, diazinon was extracted with dichloromethane first in order to get rid of interferring substances; the hydrophobic phase was dried, evaporated and the residue dissolved in acetonitrile-water was injected. Recovery rates of diazinon were between 84 and 87%. Both substances were chromatographed on a Nucleosil C_{18}-column (Macherey-Nagel, Düren, FRG)in an acetonitrile-water mixture and detected at 245 nm. Triplicate determinations of one sample differed by less than 5%. The analytical determinations have shown that real concentrations in the test vessels were kept within a limit of ± 20% of theoretical values.

For statistics the data were evaluated by analysis of variance and by Scheffé's test (1953). Rejection level was $P > 0.05$.

RESULTS AND DISCUSSION

The effect of the substances 4-chloroaniline, 3,4-dichloroaniline and diazinon on early development of zebrafish and growth of the rainbow trout was investigated. The compounds had been chosen for different reasons: 4-chloroaniline is one of the test compounds the Federal Environmental Agency of the FRG has proposed for comparative studies according to Level 1 of the German Chemicals Act (Rudolph and Boje 1987); 3,4-dichloroaniline was investigated in a collaborative study in which early development and growth of zebrafish were studied over 6 wk (Nagel et al. 1990); diazinon, a phosphorester insecticide, had already been tested in long-term experiments in several fish species years ago

by several laboratories (Allison 1977; Allison and Hermanutz 1977; Jarvinen and Tanner 1982); it seemed interesting to learn about the response of zebrafish.

The action of 4-chloroaniline on zebrafish was investigated in a long-term experiment. Concentrations of 1.0, 0.2 and 0.04 mg/L were tested. Under the influence of the highest concentration, development was abnormal; deformations, specially of the backbone, became clearly visible when the fish were 5 wk old. Under the lower concentrations no effects on early development and growth were observed (Bresch et al. 1990).

Growth of trout under the influence of 4-chloroaniline was followed for a period of nearly 2 mon. The same concentrations were applied as in the zebrafish test. Growth was measured by weight. In Figure 1 growth of the trouts is shown. First weight measurements already, 14 d after the beginning of incubation in chloroaniline, have shown that growth was inhibited under the highest concentration. After 4 wk, the average increase in weight of the animals kept under 1 mg/L was 50% lower than the weight of fish of other groups, and the fish were deformed in addition. Obviously the influence of 4-chloroaniline is the same in both species of fish. As in zebrafish, the lower concentrations did not influence growth of the trout. As both test methods delivered the same numerical toxic threshold concentrations - LOEC 1 mg/L, NOEC 0.2 mg/L - both test procedures are equivalent in case of 4-chloroaniline.

To investigate the action of 3,4-dichloroaniline on zebrafish, concentrations of 0.2, 0.02 and 0.002 mg/L were tested. Under 0.2 mg/L development after hatching was abnormal and death rate was high. Whereas in the control group and under the lower concentrations more than 85% of fish survived the first 6 wk, only 62% survived under the high concentration of dichloroaniline. Nearly all fish kept under this concentration showed anomalies, especially in the head region whereas no anomalies were observed in fish grown under the lower concentrations. Further details are described elsewhere (Nagel et al. 1990).

The concentrations of 0.2, 0.02 and 0.002 mg/L 3,4-dichloroaniline did not influence growth of trout: the mean weights of fish of the different groups did not differ and increase in weight over 30 d was not influenced under the applied concentrations. Two values demonstrate this result. At the end of the experiment the average weight of control fish was 6.5 g versus 6.6 g of fish kept under the highest concentration. Anomalies were not observed in any group of fish; there

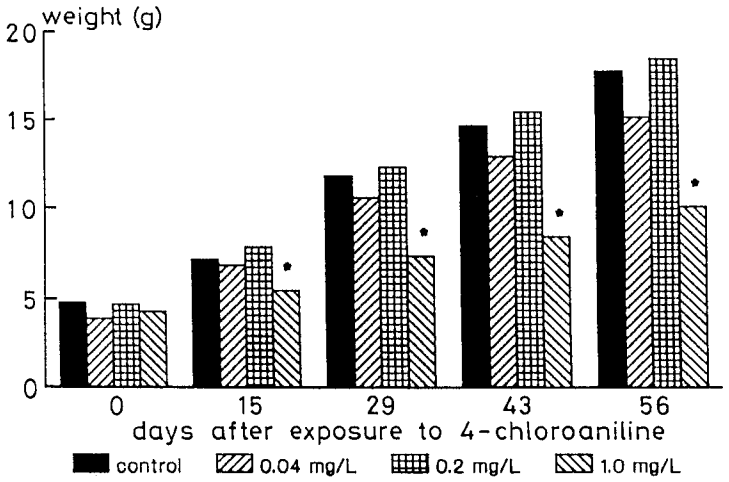

Figure 1. Growth of rainbow trouts kept in 4-chloroaniline for 56 days. One bar represents the mean weight of 20 animals. *Significantly different from other groups.

were no differences in lengths either. In case of 3,4-dichloroaniline the early life-stage test in zebrafish was more sensitive than the growth test in trout.

Zebrafish kept under diazinon concentrations of 0.2, 0.04 and 0.008 mg/L obviously grew alike as no different effects among the groups were observed. Survival rates of animals of different groups did not differ either, as demonstrated in Table 1.

In the experiments with diazinon in trout, fish of all groups kept under the same concentrations and the control group grew alike: differences among mean weights could not be verified statistically; so, at the end of the experiment, after 28 d, mean weight of control fish was 9.5 g, mean weight of fish kept under 0.2 mg diazinon was 9.1 g. In all groups no abnormal fish could be seen. Since the concentrations applied did not lead to any effect in either test, one cannot conclude which method is the more sensitive. However, the fact that the weights of 8 wk old zebrafish grown under the highest diazinon concentration were significantly lower than the weights of fish of the other groups may suggest that the early life-stage test in zebrafish is not less sensitive than the growth test in trout. The differences in weight probably existed already when the animals had reached the age of 6 wk. No weight determination was made at that time because zebrafish of this age are very sensitive to any manipulation and it was intended to continue the test beyond that stage.

Table 1. Early development of zebrafish grown in water (control) and under diazinon.

	Diazinon (mg/L)							
	0		0.008		0.04		0.2	
Group	a	b	a	b	a	b	a	b
Survival (d 1) %	75	73	70	73	71	67	75	70
Hatching (d 8) %	95	99	97	100	99	98	98	99
Survival (d 20)%	88	92	93	97	96	90	89	85
Survival (d 42)%	84	89	91	97	93	89	87	85

All eggs originated from one spawn. Per concentration, two groups consisting of 150 eggs each were analysed. Incubation in diazinon was started 2-3 hr after spawning. After 1 d 100 embryos per group were randomly taken and their development was further studied.

Dichloroaniline and diazinon were evaluated in early life-stage tests by other laboratories and in other fish species. Does the zebrafish react as sensitive in the presence of these compounds? A dichloroaniline concentration of 0.02 mg/L influenced growth and survival of the fathead minnow (Pimephales promelas) within the first 28 d after fertilization, 0.005 mg/L were harmless (Call et al. 1987). Different results were reported from tests with diazinon. Whereas growth and survival of fathead minnows were not influenced at concentrations below 0.2 mg/L in a test described by Allison and Hermanutz (1977), the minnows responded more sensitively in a similar test by another laboratory: effects were observed below 0.08 mg/L (Jarvinen and Tanner 1982). Allison (1977) found reduced larval growth of the flagfish (Jordanella floridae) if reared under 0.014 mg/L. According to these results zebrafish seem to respond less sensitively to the presence of these compounds than fathead minnows and flagfish. However, this statement may be provisional as all tests were performed under different water conditions, and the diazinon samples tested might have contained different quantities of a highly toxic contaminant, as discussed by Jarvinen and Tanner (1977). To compare the sensitivity of species accurately, the fish should be examined by one and the same laboratory under the same conditions.

The results presented here support the assumption that an early life-stage test in zebrafish or another rapidly growing species, e.g. fathead minnow or flagfish,

delivers the same toxic threshold concentrations or is even more sensitive than the growth test in rainbow trout. The zebrafish is preferred here, because keeping and breeding are easier. Even if rainbow trout may be a more sensitively responding species than zebrafish in a long-term test, it is not surprising that an early life-stage test in zebrafish conducted over 6 wk is the more sensitive procedure as it includes early development and growth as well. The zebrafish test is more time-consuming than the proposed test in trout. However, this disadvantage is compensated by the fact that the test can be performed whenever needed. In addition it is easier to keep zebrafish in good health in the laboratory than rainbow trout. These fish are often contaminated by pathogenic microorganisms upon delivery by the breeder. An infection might become manifest during the test. In contrast to trout, zebrafish are easily grown in the laboratory free of pathogenic microorganisms and the background of the animals is known. Whereas it is not an easy task to perform a growth test in trouts over a period considerably longer than 4 wk without application of antimicrobial substances, a test in zebrafish may be extended to a life-cycle test if the procedure is continued for another 6 wk. In rainbow trouts, such a test would take two years at least.

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