*Dar*T: The Embryo Test with the Zebrafish *Danio rerio* – a General Model in Ecotoxicology and Toxicology

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Summary
The acute fish test is an animal test whose ecotoxicological relevance is worthy of discussion. The primary aim of protection in ecotoxicology is the population and not the individual. Furthermore the concentration of pollutants in the environment is normally not in the lethal range. Therefore the acute fish test covers solely the situation after chemical spills. Nevertheless, acute fish toxicity data still belong to the base set used for the assessment of chemicals. The embryo test with the zebrafish Danio rerio (DarT) is recommended as a substitute for the acute fish test. For validation an international laboratry comparison test was carried out. A summary of the results is presented in this paper. Based on the promising results of testing chemicals and waste water the test design was validated by the DIN-working group "7.6 Fischei-Test". A normed test guideline for testing waste water with fish is available. The test duration is short (48 h) and within the test different toxicological endpoints can be examined. Endpoints from the embryo test are suitable for QSAR-studies. Besides the use in ecotoxicology the introduction as a toxicological model was investigated. Disturbance of pigmentation and effects on the frequency of heart-beat were examined. A further important application is testing of teratogenic chemicals. Based on the results DarT could be a screening test within preclinical studies.

Zusammenfassung: *Dar*T: Der Embryotest mit dem Zebrabärbling *Danio rerio* – ein Modell zur Abschätzung ökotoxikologischer und toxikologischer Wirkungen
Bei dem akuten Fischtest handelt es sich um einen Tierversuch, dessen ökotoxikologische Relevanz diskussionswürdig ist. Das primäre Schutzziel in der Ökotoxikologie ist die Population und nicht das Individuum. Außerdem liegen die Konzentrationen von Schadstoffen in der Umwelt normalerweise nicht im letalen Bereich. Folglich bildet der akute Fischtest nur die Störfallsituation ab. Trotzdem gehört das Ergebnis dieses Tests nach wie vor zum Grunddatensatz in der Chemikalienbewertung. Der Embryotest mit dem Zebrabärbling Danio rerio (DarT) wird als Ersatzmethode für den akuten Fischtest vorgeschlagen. Zur Validierung wurde erfolgreich ein internationaler Laborvergleichsversuch durchgeführt, dessen Ergebnisse vorgestellt werden. Ausgehend von den positiven Ergebnissen zur Chemikalienprüfung und zur Abwasserprüfung, hat der DIN-Arbeitskreis „7.6 Fischei-Test" den Test validiert und eine Normvorschrift für die Abwasserprüfung vorgelegt. Die Testdauer ist kurz (48 h) und es können verschiedene toxikologische Endpunkte innerhalb des Tests erfaßt werden. Endpunkte des Embryotestes sind auch eine gute Basis für die Betrachtung von quantitativen-Struktur-Wirkungs-Beziehungen (QSAR). Neben diesen Einsatzmöglichkeiten in der Ökotoxikologie wurde auch der Einsatz als ein Modell in der Toxikologie geprüft. Störungen in der Pigmentierung oder die Veränderung der Herzschlagfrequenz wurden untersucht. Eine weitere wichtige Einsatzmöglichkeit ist die Prüfung von teratogenen Substanzen. Basierend auf den Ergebnissen könnte DarT ein Screening-Test im Rahmen der präklinischen Studien sein.

Keywords: zebrafish, embryo test, replacement, acute fish test, QSAR, teratogenic, heart-beat, pigmentation

1 Introduction

The acute fish test is the initial step in testing chemicals with fish according different laws, for example the German chemicals act or the plant protection act. Usually, acute toxicity of chemicals is determined as a LC_{50} (96 h) value (e.g., according to OECD guideline 203), i.e., as the concentration of the test substance resulting in 50% mortality of the experimental fish over a period of 96 hours. There is an extensive data base for acute toxicity. Different fish species may vary by orders of magnitude with respect to their sensitivity in acute tests to environmental contaminants. Generally, salmonid fish are more susceptible than cypriniformes or cyprinodontiformes.

Death as an endpoint in toxicological research represents an unambiguous parameter for the individual. However, the environmental significance of death of individuals after short-term exposure to high concentrations, except in the case of accidental spills (e.g. as observed in the river Rhine in November 1986), is questionable. More frequently, chronic effects resulting from long-term exposure to low concentrations are relevant and should be investigated.

Moreover, the ecotoxicological relevance of death is limited by the fact that,

at least in my opinion, extrapolation of chronic effects from acute toxicity data is, by principle, impossible (Nagel and Isberner, 1998). However, it is chronic toxicity which represents the ultimately important endpoint in ecotoxicology.

In recent years acute toxicity tests with fish have also aroused considerable ethical concern. Since acute toxicity to fish is determined in tests with juvenile or adult animals, intact fish are subjected to considerable pain and suffering, which is clearly in conflict with current Animal Rights Welfare legislation. Since results of LC_{50} tests are only of minor ecotoxicological significance, acute toxicity tests with fish should be replaced in terms of the 3R. Possible alternatives to the acute fish test might be the embryo test with zebrafish *Danio rerio* (*Dar*T) and cytotoxicity tests with fish cells.

Lange and colleagues (1995) related results of the zebrafish embryo test to those of cytotoxicity tests with the permanent cell line RTG-2 derived from rainbow trout (*Oncorhynchus mykiss*) for 10 selected compounds with different modes of action. In most cases the zebrafish embryo was more sensitive than adult zebrafish and the RTG-2 cells.

In another study for 17 compounds, results of the embryo and the RTG-2 cytotoxicity tests were compared to LC_{50} data from tests with golden ide (*Leuciscus idus melanotus*) (data evaluated by F. Moldenhauer and H. Spielmann, personal communication). For either alternative test system, linear regression analysis documented satisfactory correlation. The correlation coefficient for the comparison of zebrafish embryo test and juvenile golden ide fish test proved to be somewhat better (0.991) than the correlation between RTG-2 cytotoxicity and juvenile golden ide toxicity (0.796). A comparison of y-axis intercepts revealed that the zebrafish embryo test (y = -0.13) is more sensitive and reflected the acute toxicity more accurately than the RTG-2 cytotoxicity test (-0.89). These data indicate that the embryo test (*Dar*T) is a particularly suitable candidate to replace the acute fish test at the base level for testing chemicals with fish.

Furthermore the embryo test has the potential to be a substitute of fish test in routine waste water control and it could be also a model for testing chemicals in toxicology. Therefore the method will be described and an overview of the state of the art of the zebrafish embryo test is given.

Tab. 1: Stages of embryonic development of the zebrafish *Danio rerio* at 26±1°C

Time (h)	Stage	**Characterisation** (after Kimmel et al., 1995)
0	Fertilisation	zygote
0	Zygote period	cytoplasm accumulates at the animal pole, one-cell stage
0.75	Cleavage period	discoidal partial cleavage
		1. median vertical division: two-cell-stage
1		2. vertical division: four-cell-stage
1.25		3. vertical and parallel to the plane of the first: eight-cell-stage
1.5		4. vertical and parallel to the second plane of division: 16-cell-stage
2	Blastula period	start of blastula stage
3		late cleavage; blastodisc contains approximately 256 blastomers
4		flat interface between blastoderm and yolk
5.25	Gastrula period	50 % of epibolic movements; blastoderms thins and interface between peribalst and blastoderm become curved
8		75 % of epibolic movement
10		epibolic movement ends, blastopore is nearly closed
10.5	Segmentation period	first somite furrow
12		somites are developed, undifferentiated mesodermal component of the early trunk, tail segment or metamere
20		muscular twitches; sacculus; tail well extended
22		site to side flexures; otoliths
24	Pharyngula period	phylotypic stage, spontaneous movement, tail is detached from the yolk; early pigmentation
30		reduced spontaneous movement; retina pigmented, cellular degeneration of the tail end; circulation in the aortic arch 1
36		tail pigmentation; strong circulation; single aortic arch pair, early motility; heart beating starts
72-96	Hatching period	heart-beat regularly; yolk extension beginning to taper; dorsal and ventral stripes meets at tail; segmental blood vessels: thickened sacculus with two chambers; foregut developmental; neuromasts

2 Animals, materials and methods

2.1 The zebrafish *Danio rerio*

The zebrafish *Danio rerio* (Hamilton-Buchanan, 1922; formerly *Brachydanio rerio*) is a small cyprinid found in tributaries and branches of the Ganges River in South-East Asia (Eaton and Farley, 1974). This species measures 3-5 cm as an adult and thrives in both soft and hard waters. At 26°C the zebrafish grows quickly and reaches maturity within three months. This species is easily obtainable, inexpensive, readily maintainable and, under appropriate conditions, will provide a large number of non-adherent and transparent eggs (Laale, 1977). One female lays approximately 50-200 eggs per day. The zebrafish is a r-strategist (Nagel, 1993).

The embryonic development was described in numerous studies (Roosen-Runge, 1938; Hisaoka and Battle, 1958; Laale, 1977; Thomas and Waterman, 1978; Kimmel et al., 1988; Kimmel et al. 1995) and is the basis for the interpretation of effects caused by environmental pollutants.

The *Danio rerio* egg is telolecithal, and cleavage is meroblastic and discoidal. Shortly after fertilisation, cytoplasm of the teleost egg accumulates at the animal pole where it surrounds the nucleus of the zygote. Only this portion of egg cytoplasm, the so called blastodisc undergoes cleavage, whereas the yolk rich zone is excluded from cleavages. In Table 1 the stages of embryonic development of zebrafish embryos are summarised and will give an impression of the complexity of the early development.

Selected stages of the embryonic development of the zebrafish are shown in Figure 1–4.

The zebrafish has been used as a model in numerous studies in the fields of molecular genetics, vertebrate biology as well as in developmental, neurobiology and transgenic research (Roosen-Runge, 1938; Hisaoka and Battle, 1958; Laale, et al., 1977; Sander and Baumann, 1983; Nagel, 1988; Kimmel et al., 1995; Westerfield, 1995; Lele and Krone, 1996; Goolish et al., 1999; Wixon, 2000). Furthermore, zebrafish has undoubtely become the most important model in developmental biology of vertebrates (Ekker and Akimenko, 1991; Nüsslein-Volhard, 1994; Westerfield, 1995).

2.1.1 Culture, egg production and differentiation

A breeding stock of non-treated, mature zebrafish is used for egg production. Females and males are kept at a ratio of 1:2 in a glass aquarium filled with charcoal filtered tap water with an oxygen saturation of more than 80%. The culture conditions are 26 ± 1°C at a 12 hour day/night light regime. Optimal filtering rates should be adjusted using a filter system. The fish are fed with dry flakes twice per day, and *ad libitum* with nauplia larvae of Artemia spec. once a day. To ensure optimal water quality remaining food should be removed daily.

To prevent the eggs from cannibalism by the adult zebrafish the spawn traps are covered with a stainless steel mesh. Plant imitations made of green glass are used as spawning substrate. The spawning and fertilisation take place within 30 minutes after light is turned on in the morning. 30–60 minutes after spawning the egg

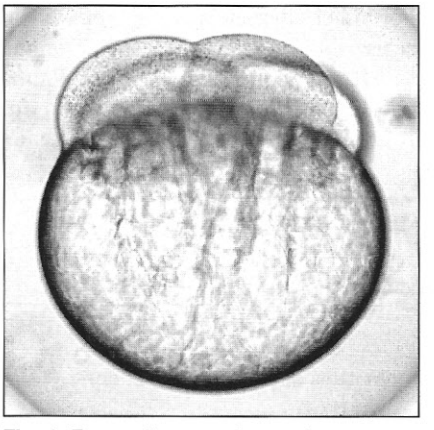


Fig. 1: Four-cell-stage of an embryo of *Danio rerio* approximately 1 h after fertilisation (from Zeller, 1995).

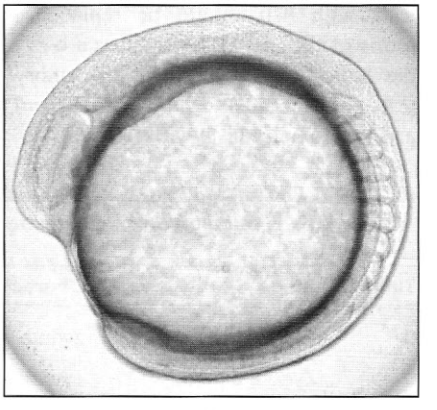


Fig. 2: Segmentation phase of an embryo of *Danio rerio* approximately 12 h after fertilisation (from Zeller, 1995). The head and tail region as well as the somites are visible.

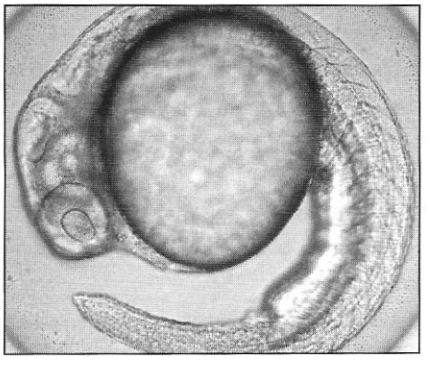


Fig. 3: Normally developed embryo of *Danio rerio* after 24 h (from Zeller, 1995). The tail is detached from the yolk and spontaneous movement starts at this time.

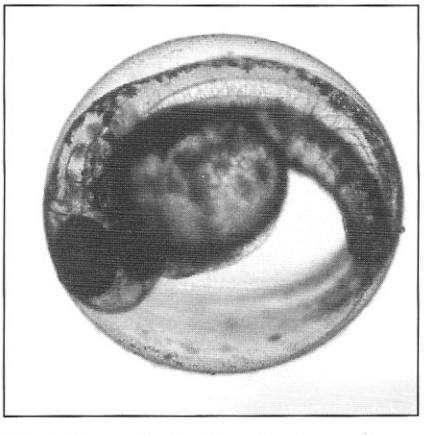


Fig. 4: Normally developed embryo of *Danio rerio* after 48 h (from Zeller, 1995). Eye and skin pigmentation due to melanophores, the sacculus containing two otoliths as well as the completely developed and well structured spine are visible. At this stage blood circulation and regular heart beats can be observed.

traps can be removed and the eggs are collected in a plastic mesh sieve. A single mature female lays 50-200 eggs per day. At the culture conditions described above, fertilised eggs undergo the first cleavage after approximately 15 min and consecutive synchronous cleavages form 4, 8, 16, and 32 cell blastomeres. At this stages (4-32 cells) eggs can be identified clearly as fertilised and only these should be used for the experiments.

2.2 *Dar*T – The *Danio rerio* toxicity assay/ *Danio rerio* teratogenicity assay

The embryo test procedure is described in Schulte and Nagel (1994) and Nagel (1998) in detail. Following initial range-finding experiments, the toxicity of a chemical substance can be determined by using 24-well multiplates. After preparing a stock solution of the test substance, typically five concentrations are tested.

Information about the use of solvents can be found in Maiwald (1997) and in Nagel (1998).

40 eggs are transferred to the test solutions about 60 minutes after light has been turned on to initiate spawning. Fertilised eggs are separated from the non-fertilised and placed in the multiplate wells with a pipette using a stereo microscope. 20 fertilised eggs are placed individually in 2 mL of the respective test solutions to exclude mutual influences. The remaining four wells of each plate are used as internal control filled with dilution water amounting to a total of 20 controls per test. The dilution water corresponded to the reconstituted water according to ISO–standard 7346/3, which is diluted 1:5 using deionised water. After this procedure the multiplates are covered with a self-adhesive foil and incubated at 26°C ± 1°C. Lethal, sublethal and teratogenic endpoints are recorded using a dissecting microscope within 48h. A test is classified as valid, if 90% of the embryos in the control treatments showed neither sublethal nor lethal effects.

In Table 2 lethal and sublethal endpoints for both the *Dar*T toxicity assay and the teratogenicity assay are summarised.

3 The embryo test, a model in ecotoxicology

3.1 *Dar*T, *Danio rerio* toxicity test, a substitute of the acute fish test in chemical testing

The method and preliminary results for 6 chemicals were published by Schulte and Nagel (1994).

In three laboratories (University Mainz, FHG-IUCT Schmallenberg, IGB Berlin), 21 reference chemicals were investigated. Consistency of the results was confirmed by the relatively low coefficients of variation between the laboratories (highest coefficient of variation: 36%). The average value from the inter-laboratory study and LC_{50} data for 16 additional chemicals were compared to LC_{50} values resulting from fish acute toxicity tests with zebrafish (*Danio rerio*) or, if not available, with golden ide (*Leuciscus idus melanotus*). Regression analysis with data for these 37 chemicals revealed a good correlation with a slope of 0.81 and a regression coefficient of 0.87 (Schulte et al., 1996).

Based on these results an international inter-laboratory study with 12 laboratories, funded by the German Environmen-

Tab. 2: Lethal and sublethal endpoints for evaluating the toxicity and teratogenicity of chemicals on the embryo of *Danio rerio* (according to Schulte and Nagel, 1994; Nagel, 1998; Bachmann, 2002).

Toxicological endpoints	**Exposure time (h)**			
Lethal*	**8**	**24**	**48**	**120**
Coagulation	●	●	●	
Tail not detached		●	●	
No somites		●	●	
No heart-beat			●	
Sublethal/Development				
Completion of gastrula	●			
Formation of somites		●		
Development of eyes		●	●	
Spontaneous movement		●	●	
Heart beat/blood circulation			●	
Pigmentation				
Oedema			●	
Teratogenic				
Malformation of head		●	●	
sacculi/otoliths		●	●	
tail		●	●	
heart		●	●	
modified structure of the corda		●	●	
scoliosis		●	●	
rachischisis		●	●	
deformity of yolk		●	●	
growth-retardation		●	●	
Length of tail**				●

* After 48 h the four endpoints were assessed to be lethal. Within the teratogenicity test for a better comparison with mammalian data only the endpoint "coagulated" is used as a lethal effect.

** There is the option to measure the length of tail after 120 h. In this case the eggs are transfered into water without the test compound after 48 h. After natural hatching the larvae is straightened and the length can be determined.

tal Protection Agency (UBA), was initiated (Fußmann and Nagel, 1997). Three test substances (EDTA-Na_2·$2H_2O$, benzoic acid, 2,4-dinitrophenol) with different toxic potential were repeatedly blind-tested in twelve laboratories. Lethal and sublethal effects were surveyed within the first 48 hours of embryonic development. The twelve laboratories, which participated in this comparative study, performed 61 valid tests for the three test substances altogether.

LC_{50} values for each valid test were calculated using identical software (data documentation and probit analysis in MS-Excel ©Fußmann). Each laboratory performed at least one valid test for each test substance. One laboratory delivered three replicate test results and five laboratories two results for EDTA. Ten and nine laboratories delivered two replicate test results for benzoic acid and 2,4-dinitrophenol, respectively. The toxicity of the three test substances, as revealed by the LC_{50}, varied over three orders of magnitude. Mean LC_{50} (of the average value for each laboratory) were 727 mgL^{-1}, 36.6 mgL^{-1}, and 1.13 mgL^{-1} for EDTA-Na_2·$2H_2O$, benzoic acid, and 2,4-dinitrophenol, respectively. All laboratories found EDTA-Na_2·$2H_2O$ to be the least toxic substance followed by the other two substances. Variance among replicate tests performed by a single laboratory was always small compared to differences between substances. Laboratories 11 and 12, which used different strains of *Danio rerio*, consistently found higher LC_{50}-values than the other ten laboratories for benzoic acid and 2,4-dinitrophenol (lab 11) or for all three test substances (lab 12).

The factor "laboratory" had a significant effect on toxicity assessment (LC_{50}) in this inter-laboratory study, when all twelve participating laboratories were considered (Friedman's χ^2=22.2, $\chi^2_{crit,\alpha=0.05}$=19.7, p=0.02). Specifically the differences of LC_{50}-values between laboratory 1 and 12 (and only between these two) were statistically significant (Wilcoxon-Wilcox sum of ranks difference = 29, critical difference$_{\alpha=0.05}$ = 28.9). The LC_{50}-values for the remaining ten laboratories are no longer significantly different at the 5%-level, if the two laboratories were omitted from the analysis, which used different fish strains (for lab 1 through lab 10: Friedman's χ^2=16.2, $\chi^2_{crit,\alpha=0.05}$=16.9, p=0.06). The differences between the LC_{50} values for the three compounds were about a factor of 10 between the two laboratories.

A fairly homogenous body of test results could be received upon reducing the data set to the results of the eight intermediate laboratories (for lab 3 through lab 10: Friedman's χ^2=7.1, $\chi^2_{crit,\alpha=0.05}$=14.1, p=0.42).

Toxicity estimates derived from the embryo test are in good accordance with the acute toxicity test with fish. Together with results of the previous study (Schulte et al., 1996) this study may be considered as an important contribution to the validation of the embryo test. Furthermore a draft testguideline was fromulated based on the results and experiences of this international inter-laboratory study (Nagel, 1998).

By now the database has been extended to 58 compounds. The correlation is given in figure 5. The data corroborate the fact that toxicity estimates derived from the embryo test are in good accordance with the acute toxicity test with fish. Consequently, from our point of view and in correspondence with the animal protection law, the acute fish test has to have substituted by the zebrafish embryo test (*Dar*T). Although we have no information about the ability to feel pain and passion of the early developmental stages, we are convinced this will be lower as for juvenile or adult fish.

Therefore, and based on a careful assessment of the data from the various tests the following test concept with fish is suggested (for details see Nagel and Isberner, 1998):

Base level. For ethical reasons, the conventional acute toxicty test with juvenile or adult fish should be replaced by the embryo test with zebrafish (*Dar*T).

Level 1. At level 1 the early life-stage test (OECD guideline 210) deserves priority over the prolonged fish test according to OECD guideline 204.

Level 2. Only a complete life-cycle test fulfills the requirements of a chronic toxicity test. It cannot be substituted by an early-life-stage test and extrapolation

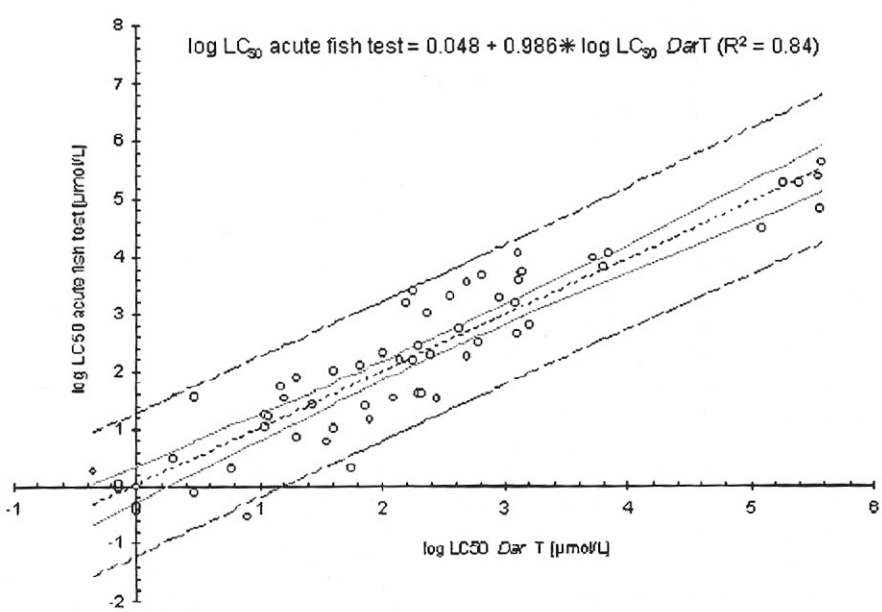


Fig. 5: Correlation of LC_{50} acute fish toxicity and LC_{50} embryo toxicity (DarT). 58 data points are from Schulte et al. (1996), Bachmann (1996), Maiwald (1997) and Brust (2001).

from ACR data (**A**cute **C**hronic **R**atio; see also Nagel and Isberner, 1998) is not possible. Therefore, only a complete life-cycle-test can be recommended.

Using zebrafish exclusively is advantageous, because in a tiered approach the simpler tests can serve as reliable range finders for the next study, thus saving animals and costs.

3.2 The embryo test, a model in routine waste water control

The zebrafish embryo test could also be an alternative to fish acute toxicity tests in routine waste water control. Friccius and coworkers (1995) tested 29 samples of industrial effluents from 11 different sewage plants. The embryo toxicitiy test was as or more sensitive than the fish test according German DIN-Norm 38 412, L 31.

Based on these results and influenced by the perception that the cytotoxiciy test with permanent fish cells was no longer a candidate to substitute the fish acute toxicity test in waste water control the "DIN AK 7.6 Fischei-Test" was established.

27 institutions and 17 laboratories participated in this working group. First the available draft guideline from the embryo test for testing chemical (Nagel, 1998) was adapted to the particular conditions testing waste water in different dilutions. The same lethal endpoints as in the embryo test (*Dar*T) were defined as lethal effects (48 h): coagulated, no somite, tail not detached, no heart beat.

Subsequently comparison tests with reference chemicals and ringtests with waste water of different toxicities were carried out. All these studies were successful and therefore a guideline was formulated which is now available as the national normed guideline DIN 38415-T6 "Bestimmung der nicht akut giftigen Wirkung von Abwasser auf die Entwicklung von Fischeiern über Verdünnungsstufen".

In Germany this test is called the "fish egg test". The use of this name is not unproblematic. Eggs are exposed but the embryo will be effected and therefore from a biological point of view this test is also an embryo test.

3.3 *Dar*T, a model in QSAR

The relationship between the biological activity of molecules (e.g. 96-h LC_{50}) to their chemical structures and corresponding chemical and physicochemical properties can be described by mathematical models in quantitative structure-activity relationships (QSARs) (Lipnick, 1995). The ultimate rationale is the establishment of causal relationships between features of the chemical structures and the observed effects or activities (Nendza, 1998). The effects of toxicants on biota depend on their hydrophobic, polar and electrostatic character and can be determined by reactive transient interactions, hydrogen bonding, covalent binding and /or steric fit to the interaction site (Nendza and Russom, 1991).

These relationships can be used to predict the behaviour, accumulation potentials, and toxicity of chemicals so far untested, and further to rationalise future experiments (Könemann, 1981; Saarikoski and Viluksela, 1982; Nendza, 1991).

For two groups of aliphatic amines, the primary and secondary amines, a clear coherency between lipophilicity and toxicity can be seen (Brust, 2001). The toxicity of aliphatic amines increases with increasing lipophilicity (Fig. 6) and can be described using a simple linear regression model:

$\log(1/LC_{50}) = 0.467 \cdot \log K_{ow} - 3.429$
($n = 13$; $R^2 = 0.91$)

Also for the secondary amines a linear regression model could be found:

$\log(1/LC_{50}) = 0.278 \cdot \log K_{ow} - 3.370$
($n = 13$; $R^2 = 0.85$).

Both regressions showed that the relationship between toxicity and lipophilicity of the primary and secondary aliphatic amines is significant. However, the slope of the regression model for the secondary amines differs from that of the model for the primary amines by approximately a factor of two.

In the case of tertiary amines the toxicity increased with increasing lipophilicity up to a log K_{ow} of approximately 2 and then the toxicity of higher lipophilic tertiary amines decreased. (A more detailed discussion, also on polar and non polar narcotics, can be found in Brust, 2001).

The possibiltiy to predict the toxicity of chemicals from homologous series based on their properties – or in general QSAR–studies-should play a much greater role in terms of the 3R. Also in "The European Commission White Paper on the Strategy for a Future Chemicals Policy" the reduction of tests with animals and in this context the use of QSAR is an important aspect.

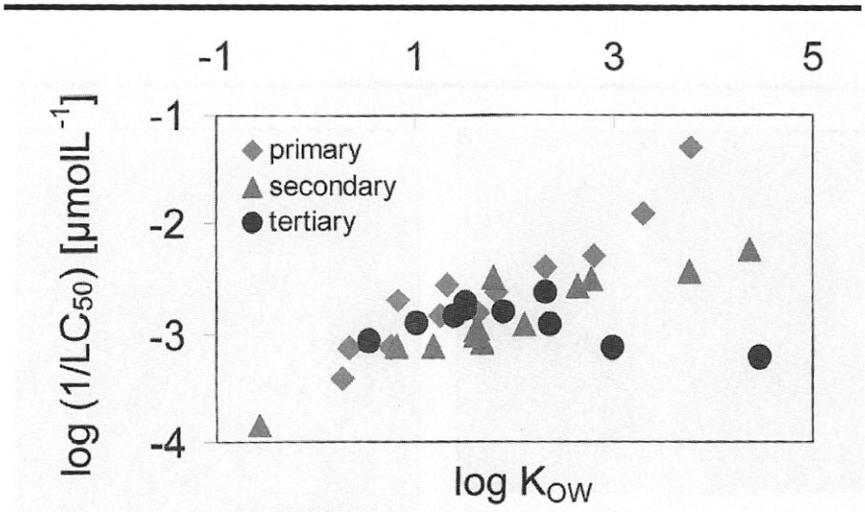


Fig. 6: Relationship between toxicity (LC_{50}) and lipophilicity (log Kow) of primary, secondary and tertiary aliphatic amines. (from Brust, 2001).

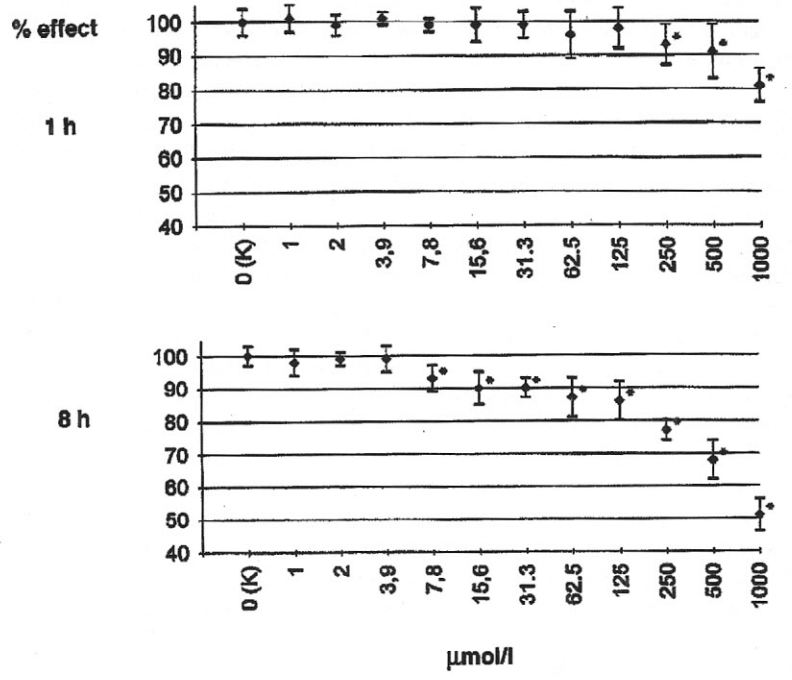


Fig. 7: Influence of propanolol on the frequence of heart-beat of the zebrafish Embryo. Exposure time 1 and 8h after 48 h of development. (* significantly reduced; from Zeller, 1995).

4 The embryo test, a model in toxicology

Based on the manifold observations within the embryo test we proved the applications of *Dar*T in toxicology.

4.1 Effect of drugs on heart-beat

Heart-beat is an important sublethal endpoint which is routinously measured within *Dar*T. Therefore ist was obvious to test the effects of drugs which are used to influcene the heart-beat therapeutically. We selected four drugs: Verapamil is a blocker of the calcium channel which reduces the frequency of heart-beat and the power of contraction. Propanolol is an antagonist of adrenaline/noradrenaline, at the β-adrenergen receptors and reduces also the frequency of heart-beat and the power of contraction. Theophyllin, an inhibitor of the phosphodiesterase, and antagonist at receptors of adenosin, increases the heart-beat and the power of contraction. This applies also to isoprenalin an agonist of adrenalin/noradrenalin at the β-adrenergen receptors.

*Dar*T showed the results which we expected according to the different modes of action (Zeller, 1995). Figure 7 shows the reduction of heart-beat during exposure to propanolol exemplary. The reduction depends on exposure time and concentration of test substance. Based on these results we think, *Dar*T could be a model to detect compounds which influence the cardiac functions of vertebrates.

4.2 Effects of chemicals on pigmentation

Schulte (1997) found a reduced pigmentation of zebrafish embryos for several anilines and phenols. Therefore we looked for compounds which are known to influence the pigmentation in humans. One candidate was p-tert-butylphenol. Maiwald (1997) observed a concentration-dependent hypopigmentation of all pigment cells of the fish embryo (Fig. 8a,b).

Hypopigmentation was also observed in tests with primary, secondary and tertiary amines, except for cyclohexylamine and dimethylbutylamine (Brust, 2001).

As far as we know there is no toxicological model to detect effects on pigmentation, and therefore *Dar*T could be a tool.

4.3 *Dar*T, *Danio rerio* Teratogenicity Assay

"*Spina bifida*" was described by Sander (1983). He tested ethanol and colcemide and proposed that this phenomenon can be caused by teratogenic substances within the most sensitive developmental phase of ontogenesis. The first sign of aberrations can be seen in a slower epibolic movement and later in a dumb-bell shaped yolk.

Zeller (1995) observed this phenomenon in embryos exposed to high

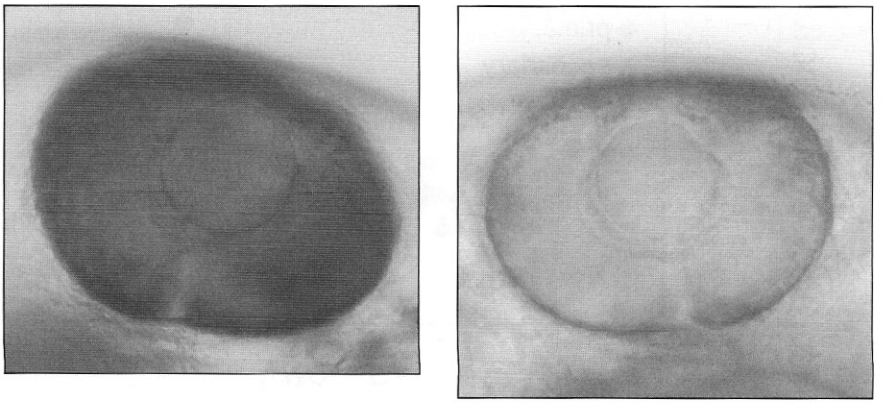
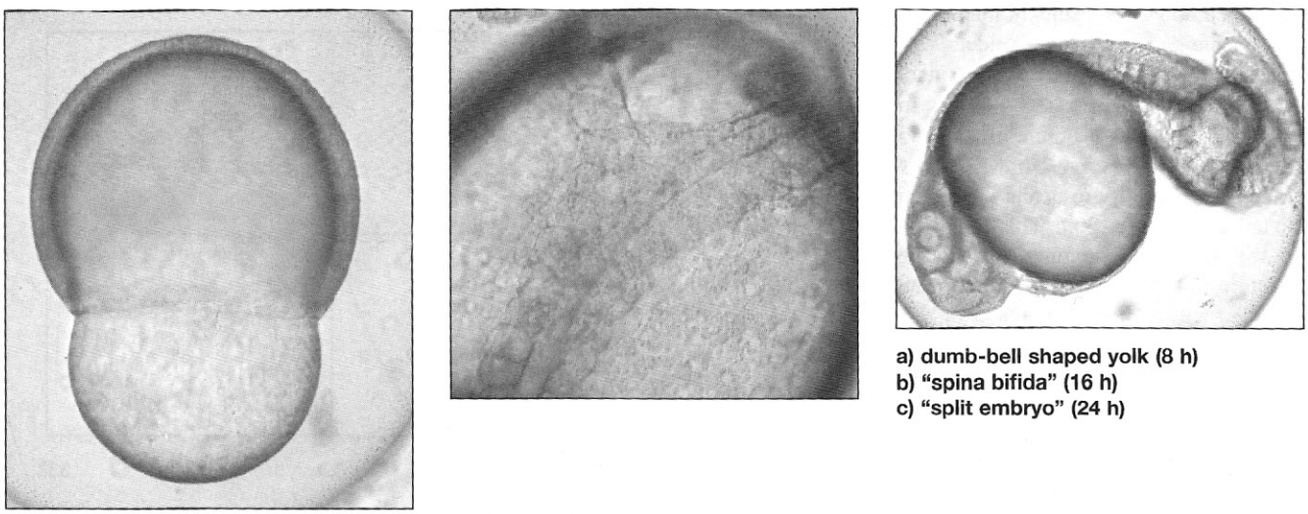


Fig. 8a, b: Influence of p-tert-butylphenol on the pigmentation of the eyes of the embryos of zebrafish. (a: control, b: 1.2 mg/L; from Maiwald, 1997).



a) dumb-bell shaped yolk (8 h)
b) "spina bifida" (16 h)
c) "split embryo" (24 h)

Fig. 9a, b, c: "Spina bifida" one example of malformations which can be seen within *Dar*T; during a exposure to a high concentration of propanolol (1,000 mmol/L; from Zeller, 1995).

concentrations of propanolol, a β-receptor blocking drug. Maiwald (1997) found this effect in embryos which were exposed to acetone and Schulte (1997) observed "*Spina bifida*" after exposure to malathion. In very few cases, this phenomenon was also observed in the toxicity tests performed with hexylamine, diisobutylamine, dibutylamine and dimethylbuytlamine (Brust, 2001). The phenomenon is shown in Figure 9.

The observation of this malformation and many other teratogenic effects within the many tests carried out in my working group led to a research project funded by BASF AG. The goal was to develop a screening assay to predict the teratogenic potential of chemicals in mammals (*Dar*T). The tested teratogenic toxicological endpoints are included in table 2.

41 chemicals were selected based on following criteria: Mammalian teratogens, with or without metabolic activation. For example diethylene glycol dimethyl ether, N,N-dimethylfromamide and all-trans retinoic acid, Methylmercury chloride and valproic acid, respectively. Mammalian non-teratogens

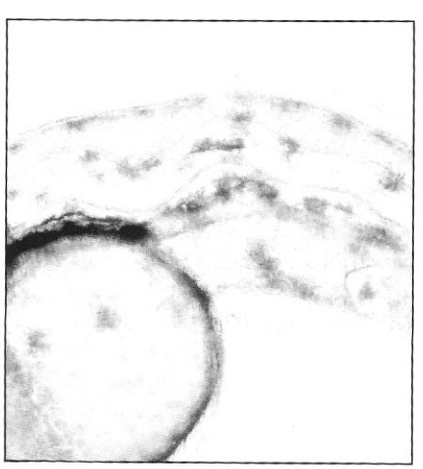


Fig. 10: Scoliosis after exposure to methylmercury chloride (0.24 mmol/L, 48 h, from Bachmann, 2002).

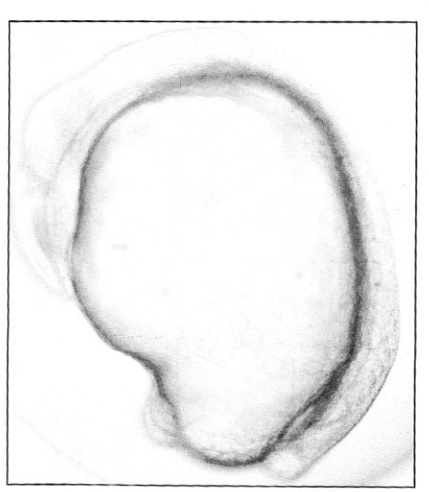


Fig. 11: Deformations of tail and head (Rachischisis) after exposure to valproic acid (0.12 mmol/L, 24 h, from Bachmann, 2002).

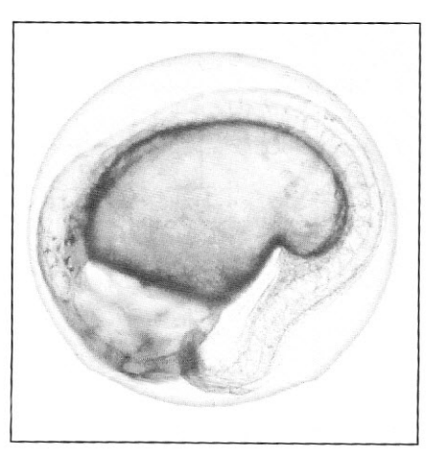


Fig. 12: Growth-retardation in larvae after exposure to diethylene glycol dimethyl ether (62.2 mmol/L, 120 h, * significantally reduced ((p = 0.05)), from Bachmann, 2002).

as diethyl glycol and glucose. Substances showing ambiguous results in mammals, e.g. colcemide and cycloheximide.

All the data and a detailed assessment of the test will be found in Bachmann (2002). In Figure 10, 11 and 12 selected effects are shown. *Dar*T offers the possibility to detect effects on the tail length after 120 h. This extension of the test is recommended if the structure of the chorda seems to be modified within 48 h. One result of this procedure is given in Figure 13 for diethylene glycol dimethyl ether exemplary.

All the effects observed within *Dar*T, lethal and malformations, are evaluated by probit analysis. An example is given in Figure 14. The quotient between LC_{50} and $EC_{50\ malformation}$ is an indicator of the teratogenic potential of the compound and can be calculated as Teratogenic Index (TI-Value). In the case of all-trans retionic acid the LC_{50} is 3.6 µmol/l and the $EC_{50\ malformation}$ 0.011 µmol/l and the TI-Value consequently 327.3. For this compound the teratogenic potential, expressed by the TI-Value, is very clear. But for other compounds the significance of this index is worthy of discussion (for details see Bachmann, 2002).

For the 41 substances tested and evaluated up to now the results of 88% of chemicals are in agreement with findings in mammals. In 10% (4 compounds) a false positive result was found. For salicyclic acid the result was false negative in comparison with the effects in mice and rats. For thalidomide the result was false negative if compared to the effects in humans. But the result compared with the results of other toxicological models, for example with rats, is not false negative but in accordance.

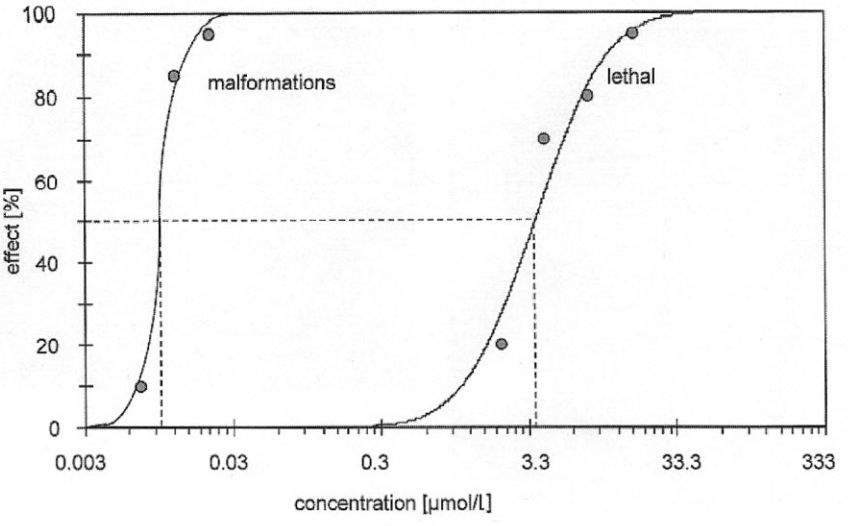


Fig. 14: Concentration-effect-relationships of lethality and malformations for all-trans retinoic acid (48 h) (from Bachmann, 2002).

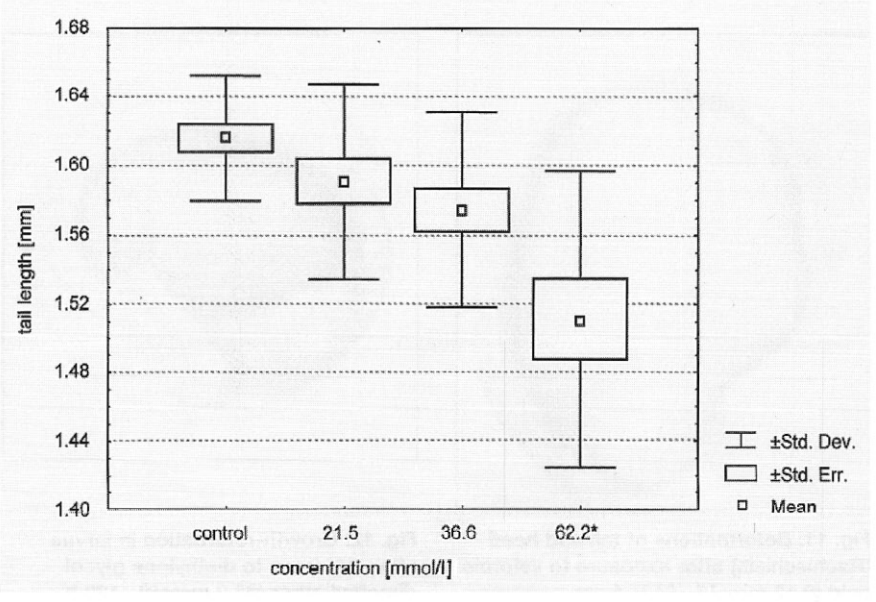


Fig. 13: Growth-retardation in larvae after exposure to diethylene glycol dimethyl ether (62.2 mmol/L, 120 h, * significantally reduced ((p = 0.05)), from Bachmann, 2002).

Even for teratogens with metabolic activation within *Dar*T malformations could be detected. But the effects were not as strong as assumed. For example for diethyleneglycol dimethyl ether (diglyme) the $LOEC_{length\ of\ tail}$ was 62.2 mmol/l and for metoxyacetic acid the $LOEC_{length\ of\ tail}$ was 0.11 mmol/l. Therefore it seems to be meaningful to investigate the metabolic potential of the zebrafish embryo and if necessary to optimise the test for example by the use of S 9 mammalian liver homogenate.

In summary, the *Danio rerio* Teratogenicity Assay can be a screening test to investigate drugs and other chemicals in an early inventory phase. In terms of the 3R *Dar*T is an alternative method to reduce the number of common laboratory tests and replace screening assays with laboratory animals (Bachmann, 2002).

5 Perspectives

*Dar*T is a rapid, simple and cost-effective test. The embryos are available permanently and timely. Only small amounts of test substance are required.

Furthermore *Dar*T is an alternative test, because within the test period the embryos will not hatch. According to the German Animal Welfare Act, studies performed on embryos are classified as non-animal-test. The study is primarily limited to the first 48 hours of development and therefore, in our opinion, can be justified ethically. Furthermore from a biological point of view we think that the use of less far developed stages of animals is an important issue in terms of 3R.

The number of advantages will enormously increase in the near future. The zebrafish is the "*Drosophila*" of vertebrates. The genome is or is mostly known. There are thousands of mutants available and companies are founded to use this potential commercially. The existing phenotypes can be compared which chemotypes and the phenomenon can be assessed from a genetic, molecular and developmental point of view.

This development makes great progress and could not be foreseen when we started ten years ago to substitute the acute fish test by the embryo test with zebrafish.

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