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Oral examination:.....

Refinement of the fish embryo toxicity test (FET) with zebrafish (*Danio rerio*): Is it a real replacement of the acute fish toxicity test?

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Hiermit erkläre ich, dass ich die vorliegende Dissertation selbst verfasst und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe. Des Weiteren erkläre ich, dass ich an keiner Stelle ein Prüfungsverfahren beantragt oder die Dissertation in dieser oder einer anderen Form bereits anderweitig als Prüfungsarbeit verwendet oder einer anderen Fakultät als Dissertation vorgelegt habe.

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Eva Lammer

Dedicated to my parents.

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Contents

Summary

Alternative methods in ecotoxicology play an increasingly important role in chemical and risk assessment. Already in 1986, the Directive 86/609/EEC stated that an alternative method, once it is "practicably and reasonably available", should replace the corresponding animal method. In 2007, REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) came into force to systematically evaluate the risk to human health and the environment of approx. 30,000 chemical substances produced, used or imported in quantities of 1 - 100 t/a. Within the framework of REACH, animal testing should be reduced or even replaced by alternative methods.

The 96 h acute fish test, however, is still a mandatory component in the base set of data required for chemical risk assessment. With mortality as the exclusive endpoint, it is not compatible with most current animal welfare legislations in Europe. Furthermore, there is increasing concern that some form of pain perception, similar to what is present in mammals, may be present in bony fish, and adult fish exposed to acute toxic concentrations of chemicals may at least be suspected to suffer severe distress and pain.

A promising candidate to replace the acute fish test in chemical assessment is the fish embryo toxicity (FET) test. In Germany, the FET has already replaced the acute fish test in whole effluent testing since 2005. A major advantage of the FET concerning animal welfare is the fact that embryos may have not yet developed the same complex mental processes and may have not pain perception identical to adult fish.

Since the correlation between the alternative and the test to be replaced is one of the most important facts to accept a test as alternative, a detailed literature research and a thorough re-evaluation of FET and acute fish test data has been conducted. The analyses resulted in an overall R^2 of 0.90 for the comparison of FET data and acute fish test data. Restricting the data to zebrafish (*Danio rerio*) FET data and all fish data, the correlation resulted in an R^2 of 0.87. In general, all correlations with different restrictions of the complete dataset (e.g. only zebrafish FET and zebrafish acute fish test data, only 48 h FET and 96 h fish toxicity data, etc.) showed no R^2 lower than 0.81. For comparison between different biotests, these are almost perfect correlations, and compared to correlations of 96 h acute fish data among different fish species, they are in the same range or even better.

However, outliers are inevitable. In order to investigate if the static procedure of the FET may be the reason for outliers, a flow-through protocol was developed. The applicability of the flow-through system in chemical assessment was checked by testing four different "outlier chemicals" (hydroquinone, 4-chlorophenol, pyraclostrobin and α-endosulfan), which were selected according to increasing log P_{ow}. Independent of chemical nature and lipophilicity, the flow-through and the static FET gave almost identical results for all substances. Thus, at least for these chemicals, the differences between the results from the FET and the acute fish toxicity test could not be traced back to reduced bioavailability due to adsorption to the polystyrene plates. Further investigations are necessary to identify reasons for the poor correlation between FET and acute fish test results for specific chemicals. However, the static protocol seems to be a sufficient testing procedure and should definitely be used in further chemical assessment.

Zusammenfassung

In der Chemikalien- und Risikobewertung spielen Alternativmethoden eine zunehmend wichtige Rolle. Bereits 1986 forderte die Direktive 86/609/EEC, dass praktikable und vernünftige Alternativmethoden den entsprechenden Tierversuch ersetzen sollten. Im Januar 2007 trat REACH (Registrierung, Evaluierung, Autorisation und Restriktion von Chemikalien) mit dem Ziel in Kraft, das Risiko für Mensch und Umwelt durch ca. 30.000 Chemikalien, die in Mengen von 1 - 100 t/a produziert oder importiert werden, abzuschätzen. Im Rahmen von REACH sollen Tierversuche soweit wie möglich durch Alternativmethoden ersetzt werden.

Der akute Fischtest als grundlegender Test in der Risikobewertung ist jedoch immer noch gesetzlich vorgeschrieben. Mit dem exklusiven Endpunkt Mortalität ist der Test mit den meisten Tierschutzgesetzen in Europa nicht mehr zu vereinbaren. Ebenso gehen viele Wissenschaftler davon aus, dass Knochenfische ein ähnliches Schmerzempfinden besitzen wie Säugetiere, und dass adulte Fische, die mit akut toxischen Konzentrationen belastet werden, leiden und mit großer Wahrscheinlichkeit Schmerz empfinden.

Der Fischembryotest (FET) ist eine Alternative, um den akuten Fischtest in der Chemikalienbewertung zu ersetzen. Bereits seit Januar 2005 ist der FET in der Abwasseruntersuchung in Deutschland als Ersatz für den akuten Fischtest gesetzlich vorgeschrieben. Unter Tierschutzaspekten hat der FET u.a. den Vorteil, dass man davon ausgehen kann, dass Embryonen im Vergleich zu adulten Tieren noch nicht die gleichen komplexen mentalen Prozesse und dementsprechend noch nicht das gleiche Schmerzempfinden entwickelt haben.

Einer der wichtigsten Faktoren für die Akzeptanz von Alternativmethoden ist deren Korrelation zu dem zu ersetzenden Tierversuch. Daher wurden in einer detaillierten Analyse Daten aus FET und Fischtest verglichen; die Gesamt-Korrelation ergab ein R^2 von 0,90. Eine Reduktion auf den Zebrabärbling (*Danio rerio*) ergab ein R^2 von 0,87. Unabhängig von den unterschiedlichen Reduktionen der Datensätze konnte kein R^2 kleiner als 0,81 berechnet werden. Die Korrelationen führten zu exzellenten Ergebnissen und verglichen mit Korrelationen zwischen Daten des akuten Fischtests verschiedener Fischarten ist die Korrelation mit den FET-Daten mindestens genauso gut, wenn nicht sogar besser. Daher kann der FET bedenkenlos als Ersatzmethode für den akuten Fischtest in der Risikobewertung empfohlen werden.

Die Korrelationsanalyse identifizierte allerdings auch einige Chemikalien als Ausreißer. Um zu prüfen, ob das statische Prinzip des Testsystems für lipophile Ausreißer verantwortlich sein könnte, wurde ein Durchflusssystem für den FET entwickelt. 4 nach steigendem log P_{ow} ausgewählte Chemikalien (Hydrochinon, 4-Chlorphenol, Pyraclostrobin und α-Endosulfan) wurden im Durchfluss-FET getestet, um die Anwendbarkeit des Durchflusstestsystems für die Chemikalienbewertung zu prüfen. Unabhängig von Wirkungsweise und Lipophilität der Chemikalien konnten keine signifikanten Unterschiede zum statischen FET nachgewiesen werden. Unterschiede in den Ergebnissen von FET und akutem Fischtest können daher nicht auf Absorption an das Polystyrol zurückgeführt werden. Weitere Tests sind notwendig, um das unerwartete Verhalten der Embryonen gegenüber manchen Chemikalien zu erklären. Es konnte gezeigt werden, dass der statische FET mit voriger Absättigung der Testplatten ein adäquater Test für die Prüfung von Chemikalien ist und damit den akuten Fischtest ersetzen kann.

Chapter 1

Introduction

1.1 Pollution of aquatic ecosystems and chemical hazard and risk assessment

The global production of chemicals has increased from 1 million tons in 1930 to 400 million tons today, and about 100,000 different substances have been registered in the EU market, of which 30,000 to 70,000 are in daily use (EC 2001; Schwarzenbach et al. 2006). In particular, most of the chemicals enter the aquatic environment *via* wastewater from industrial, agricultural and domestic sources, including municipal sewage treatment plants (Ohe et al. 2004). Industries and municipalities use about 10 % of the globally accessible runoff and generate a stream of wastewater, which flows or seeps into rivers, lakes, groundwater or the coastal seas. These wastewaters contain numerous chemical compounds in varying concentrations. Thus, about 300 million tons of synthetic compounds annually used in industrial and consumer products partially find their way into natural waters. Additional pollution arises from diffuse sources from agriculture, where 140 million tons of fertilizers and several millions of tons of pesticides are applied each year. Other notable sources of contamination are the intrusion of salty water into groundwater due to over-exploitation of aquifers; the human-driven mobilization of naturally occurring geogenic toxic chemicals, including heavy metals and metalloids; and the biological production of toxins and malodorous compounds (Schwarzenbach et al. 2006).

However, little is known about the toxic potential or the environmental fate of the vast majority of these chemicals. This can be explained by the fact that prior to REACH (Registration, Evaluation, Authorization and Restriction of Chemicals; EC Directive 1907/2006; EC 2007c) all chemicals on the market in the EU before 1981 were “grandfathered”: They could be used further without any (additionally) testing according to the regulations by national legislations to prove if they were safe for humans and the environment (Brown 2003; EC 2001; Rogers 2003). These so-called “existing substances” amount to more than 99 % of the total volume of all substances on the market (EC 2001), and since earlier testing regimes were less rigorous in their testing requirements, there is concern that a substantial number of the existing chemicals, which are currently commercialized, may have been inadequately tested and could therefore be harmful due to so-called data gaps in the information relating to their hazard potentials (Combes et al. 2003). Chemicals produced at high volumes (above 1,000 tons per year) had been examined more in depth (EC 2001). Even so, there are no data for about 21 % of these high production volume chemicals, and another 65 % come with insufficient data (Fig. 1.1).

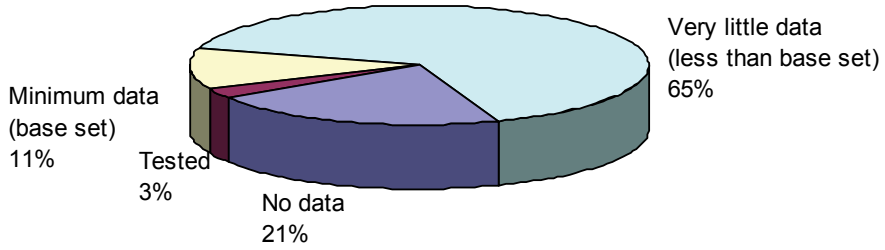

Fig. 1.1: Data available for high production volume chemicals; cf. EC (2001).

Only for approx. 2700 so-called “new substances”, i.e. substances commercialized after publication of national regulations for chemicals at volumes > 10 kg, the risk to human health and environment had to be tested and assessed; for higher volumes, more in-depth testing with a focus on long-term and chronic effects was required. Thus, for “new substances” sufficient data about the toxic potential and environmental risk are available (EC 2001; Rogers 2003).

REACH eliminates the distinction between existing and new substances and replaces over 40 different EU Directives and Regulations (Brown 2003; Petry et al. 2006). The major aim of REACH is to systematically evaluate the risk to human health and the environment of the approximately 30,000 chemical substances produced, used or imported in quantities of 1 - 100 tons per year (Combes et al. 2003; Lilienblum et al. 2008). Themed with “no data, no market”, only substances with an adequate database depending on their production volumes will be given authorization for commercialization. REACH is a “volume-triggered notification system” approach and it is based on the traditional assumption that the higher the level of production of a substance, the greater is the potential level of exposure to humans and the environment (Combes et al. 2003).

Prior to REACH, governments had been responsible for the determination of chemical toxicity, mutagenicity, potential for endocrine disruption, carcinogenicity, persistence and bioaccumulation, as well as the degree and likelihood of exposure to humans and the environment. Under REACH, the burden of proof of a chemical's safety is transferred primarily to manufacturers. If a chemical is used in a way unanticipated by the manufacturer, downstream users will have to show proof of safety (Brown 2003).

For all substances that are produced or imported in volumes of 1 ton or above per year, manufacturers or importers have to provide a registration dossier to the European Chemical Agency (EChA) at Helsinki. Failure to register means that the substance does not (no longer) have permission to be manufactured or imported. The technical dossier should contain fundamental information on the chemical’s (thermo)physical, (thermo)chemical and toxicological properties and uses (Lewis et al. 2007).

For substances in quantities of 10 tons or more, a chemical safety report has to be prepared. It documents the hazards and classification of a substance and the assessment if the substance is PBT (persistent, bioaccumulative and toxic) or vPvB (very persistent, very bioaccumulative). Moreover, exposure scenarios for risk assessment for specific uses of substances that are classified as dangerous or are PBT or vPvB substances should be described (EC 2007b).

In general, environmental chemical risk/safety assessment usually proceeds in the following sequence (Fig. 1.2): hazard assessment, exposure assessment and risk characterization.

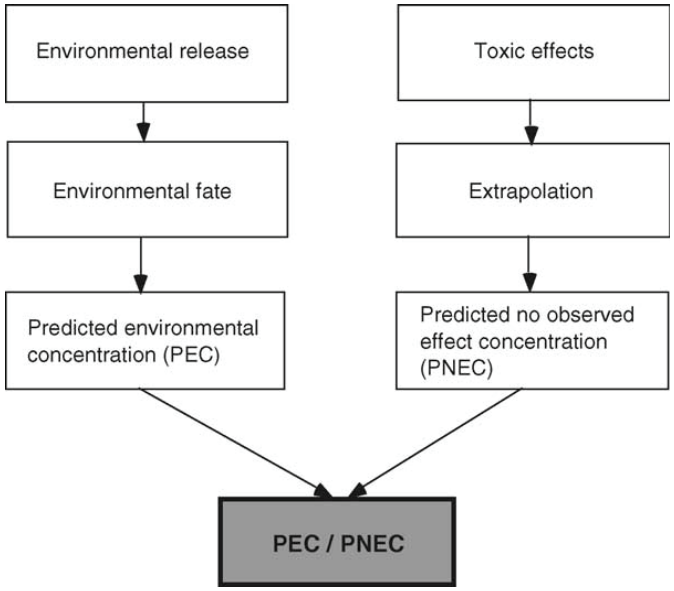

Fig. 1.2: Environmental risk assessment (Fent 2004).

In hazard assessment, reliable and relevant acute and long-term (chronic) ecotoxicity data for organisms belonging to different trophic levels are gathered to calculate the predicted no effect concentration (PNEC). The PNEC is a concentration below which unacceptable effects on organisms up to the ecosystem level most likely will not occur (Ahlers et al. 2006). It is derived by dividing the single lowest EC_{50}/LC_{50} or NOEC value obtained from single-species tests by an appropriate "assessment factor'' (Girling et al. 2000). Assessment factors should ensure that the results of mono-species laboratory tests will also protect the multi-species ecosystem (Ahlers et al. 2006; Girling et al. 2000). In establishing the size of such assessment factors, a number of uncertainties have to be considered. These include intra and interlaboratory variation of toxicity data, intra- and interspecies variations, short- to long-term toxicity extrapolation, laboratory data to field impact extrapolation, as well as the possibility of mixture effects arising from multiple exposures in the field (Ahlers et al. 2006; Fent 2003; Verdonck et al. 2008). The different assessment factors depending on the extent of uncertainty are shown in Tab. 1.1.

Tab. 1.1: Assessment factors used in environmental risk assessment to calculate the predicted no-effect concentration (PNEC) cf. Girling et al (2000).

Available ecotoxicological tests	Assessment factor
At least one short-term L(E)C_{50} value from each of three tropic levels of the base-set (fish, *Daphnia*, and algae)	1000
One long-term NOEC (either fish or *Daphnia*)	100
Two long-term NOECs from species representing two trophic levels (fish and/or *Daphnia* and/or algae)	50
Long-term NOECs from at least three species (normally fish, *Daphnia*, and algae) representing three trophic levels	10

In exposure assessment, local (PEC_{local}) and regional predicted environmental concentrations ($PEC_{regional}$) are calculated. The $PEC_{regional}$ acts as the background concentration for the local assessment. Subsequently, computer programs calculate releases into the environment of chemicals based on the volume produced or imported, the use pattern and the physicochemical properties of chemical under concern. These release estimates are then translated into PECs for each environmental compartment (air, water, sediment, soil) based on the transport and fate of the substance (Fent 2003; Verdonck et al. 2008).

The risk characterization includes the quantitative comparison of the PEC with the PNEC. If the risk characterization ratio (RCR), or PEC/PNEC ratio, is larger or equal to 1, it indicates that there is a potential risk of adverse effects occurring. A high quotient is likely to indicate a high level of risk (Girling et al. 2000). If the RCR is larger or equal to 1, a refinement of the assessment is performed with an outcome of a more precise estimate of the risk (Murin et al. 1997). A RCR smaller than 1 signifies no need for further information and/or testing (Fent 2003; Verdonck et al. 2008).

1.2 Why tests on fish?

Fish are of particular importance for man, since they have always been an important food source for humans and, for large portions of the population, they represent a major source of protein (Nagel & Isberner 1998). In fact, aquaculture is one of the most rapidly expanding food industries (DeTolla et al. 1995). On the other hand, the aquatic environment is a sink for many chemicals (Schwarzenbach et al. 2006), as illustrated by the occasionally high pollution levels and frequencies of chemical spills, and fish have frequently been the targets of overt chemical pollution, e.g. the Sandoz accident in 1986 at Schweizerhalle, Switzerland (Güttinger & Stumm 1990). Thus, since fish are the only primary aquatic vertebrate, they

receive particular attention as monitor system in the surveillance of aquatic ecosystems and in the protection of natural waters from pollutants (Nagel & Isberner 1998). In addition, fish have been used as sentinels for the quality of waters that serve as sources for human drinking water.

In conventional ecotoxicity testing strategies, fish has been regarded as an indispensable component of integrated toxicity testing strategies for the aquatic environment. Current guidelines at the OECD level are a good example of this with fish-targeted guidelines covering acute toxicity (OECD 203, OECD 1992a), early life-stage toxicity (OECD 210, OECD 1992b), short term toxicity test on embryo and sac-fry stages (OECD 212, OECD 1998), and juvenile growth test (OECD 215, OECD 2000b).

In particular, fish acute toxicity tests play an important role in environmental risk assessment and hazard classification, because they allow for first estimates of the relative toxicity of various chemicals in various species (Wedekind et al. 2007). They have become widely required for the aquatic hazard assessment of new and existing synthetic substances as well as for use in the evaluation of complex effluents (Hutchinson et al. 2003). Thus, in the "base set" of data requirements for all substances which are produced or imported in quantities of one ton or more per year, the acute toxicity for freshwater fish (96h LC_{50}; OECD 203, 1992a) is a mandatory component beside the acute toxicity for daphnids (48h EC_{50}; OECD 202, 2004b) and the growth inhibition test with freshwater algae (growth rate: 72h ErC_{50} and/or biomass: 72h EbC_{50}; OECD 201, 2006).

However, in the attempt to evaluate adverse effects of environmental chemicals to fish, it is important to realize that "fish" as an archetype does not exists (Nagel & Isberner 1998). The standardization of a test protocol like the acute fish test with only a few recommended species automatically leads to a number of problems when the tests results are used to predict the possible influence of a substance on a given fish population (Wedekind et al. 2007).

The test species that are recommended by the OECD guidelines are rainbow trout (*Oncorhynchus mykiss*), fathead minnow (*Pimephales promelas*), bluegill (*Lepomis macrochirus*), zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), guppy (*Poecilia reticulata*), and common carp (*Cyprinus carpio*). This recommendation helps to achieve comparable results and thus saves experiments and resources (i.e. they increase practicability). However, it may be argued that none of the recommended species is native to Western Europe. Hence, they may not be species representative of European aquatic systems and their fish fauna. Moreover, it is not clear whether these species can be seen as representative for the fish fauna even in the regions where they naturally occur (Wedekind et al. 2007). Finally, no

single species is universally sensitive to all chemicals (Isomaa & Lilius 1995). Thus, single species LC_{50} data are questionable with respect to accuracy and, in more general terms, to toxicological relevance (Braunbeck et al. 2005), and it seems largely accepted that there is no most susceptible species that could be used in a possibly conservative testing approach (Fent 2003). The retreat to the single, most sensitive species toxicity test is to ignore any knowledge of the complexity of ecosystems (Isomaa & Lilius 1995). Therefore, the susceptibility and tolerance of most fish populations may be hard to predict from single standard fish acute toxicity tests. Multiple tests on various species are necessary to get an appropriate estimate of the range of susceptibilities to chemical substances.

On the other hand, considerations of animal welfare have increasingly questioned ecotoxicity testing with fish and supported efforts to develop various alternatives and/or refinements based on, e.g. primary and permanent fish cell cultures as well as early developmental stages of fish embryos. Another approach to reduce the number of fish used for toxicological purposes is the use of quantitative structure-activity relationships (QSARs) especially for the prediction of the inherent bioaccumulation potential of chemical substances. For ecotoxicity assessment, however, QSAR procedures may not be appropriate.

1.3 Alternatives to fish testing

Since the implementation of the Animal Welfare Guideline 86/609/EC in 1986 (EC 1986), the development and validation of alternatives to animal testing is strongly promoted by the EU institutions (Lilienblum et al. 2008). Since 2004, on the basis of the 7th amendment to the Cosmetic Directive (EC 2003; 2007a), animal testing has been banned for finished cosmetic products, and in March 2009 the ban of animal testing will be extended to ingredients of cosmetic products (EC 2008). Likewise, REACH explicitly promotes non-animal testing and gives preference to alternative methods for animal testing wherever possible (EC 2001; Lilienblum et al. 2008).

Alternative test methods are generally regarded as technologies that incorporate aspects of replacement, reduction, or refinement of animal use (Stokes & Marafante 1998). In the frame of safety testing and safety assessment, the definition of alternative methods includes “testing methods” (e.g. *in vitro*, *ex vivo* or reduced/refined methods *in vivo*) as well as “non-testing methods” such as the use of expert systems. Under REACH, non-testing methods also include the adequate use of existing data of a substance or other considerations such as the chemical category or chemical analogue (read-across) approach that may contribute to reduce or avoid testing *in vivo* (Lilienblum et al. 2008).

In particular, the acute fish test with mortality as an exclusive endpoint should be replaced by alternative methods, since an increasing number of researchers have suggested that some form of pain perception, similar to what is present in mammals, may be present in bony fish (teleosts), and adult fish exposed to acute toxic concentrations of chemicals may at least be suspected to suffer severe distress and pain (Braunbeck et al. 2005; Chandroo et al. 2004; Huntingford et al. 2006; Nagel 2002; Sneddon et al. 2003). This is not compatible with the most current animal welfare legislation in Europe.

1.3.1 *In vitro* tests

In vitro assays with permanent fish cell lines have been used in ecotoxicology for screening, for toxicity ranking of chemicals, chemical mixtures, environmental samples and in Toxicity Identification Evaluations (T.I.E.) during the last 30 years, and they have been suggested as an alternative to acute lethality tests with fish (Castano et al. 2000; Isomaa & Lilius 1995; Schirmer 2006). Moreover, a good correlation can be found between different cell lines, endpoints and different laboratories. The basis for relating *in vitro* cytotoxicity to acute lethality *in vivo* is that lethal doses of chemicals may kill the organism through toxicity at the cellular level. If *in vivo* lethality arises from interference by the toxicant with basal cell structures and processes, *in vitro* basal cytotoxicity assays should be good predictors of *in vivo* toxicity (Clemedson & Ekwall 1999; Ekwall 1999; Segner 2004). However, beside their potential to replace or reduce animals in toxicity tests, vertebrate cell cultures have several advantages compared to whole animal tests. Large numbers of potentially toxic substances can be screened rather quickly in multi-well plates, which can be analyzed rapidly. Little amounts of test substances are needed, and thus less toxic waste is produced. Cells may also help to identify the mechanisms underlying a toxic response. If, for a particular purpose, a suitable continuous cell line can be used, a donor animal is never needed again, which is in agreement with animal welfare (Schirmer 2006).

However, Segner (2004) reported that the absolute sensitivity of *in vitro* cytotoxicity assays with fish cells is one to three orders of magnitude lower compared to the acute lethality test using fish. Assay sensitivity is an important argument in ecotoxicological hazard assessment, since toxic thresholds are used to define environmental quality standards (Castaño et al. 2003; Segner 2004). While *in vitro* assays seem to underestimate the toxic potentials of chemicals in fish, they potentially allow detecting environmental contaminant concentrations that may be harmful to fish. However, *in vitro* systems with fish cell lines could be used, for example, in tiered testing approaches including different test systems for priority estimation of test compounds and environmental samples (Segner 2004). Likewise, toxicity characterization of chemicals and environmental samples could be investigated with

fish cell lines (Castaño et al. 2003): Fish cells *in vitro* provide a bioanalytical tool for the assessment of differentiated effects, and the possibility of screening in a rapid and cost-effective way for a wide range of endpoints (for example, cytotoxicity, genotoxicity, dioxin-like activity, endocrine disruptor activity). Fish cells can be used to develop relevant ecotoxicological endpoints (for example, the prediction of genotoxic effects in terms of genetic diversity). For studying the toxic mode of action, *in vitro* tests with fish cell lines represent an useful tool as well (Castaño et al. 2003).

1.3.2 Quality structure-activity relationship (QSAR)

Quality structure-activity relationship (QSAR) modeling is based on correlations between the chemical structure of a compound and its biological effects (Hengstler et al. 2006). They come about through the interaction of biology, chemistry and statistics, and the successful interconnection of these three subjects has permitted the development of structure-activity relationships as a sub-discipline in toxicology (Fig. 1.3; McKinney et al. 2000). The function of an ecotoxicological QSAR is to characterize chemical toxicity and to predict toxic potency. In the field of aquatic toxicology, first-generation QSARs have developed as scientifically tools for predicting the acute toxicity of chemicals when little or no empirical data are available (Russom et al. 1997). Such predictions are typically for short-term exposure in an aquatic environment. To achieve this, knowledge of the information on which models are based, both toxicologically and chemically, is essential (Schultz & Cronin 2003). In part, the success in establishing these QSARs is dependent upon well-defined and quantifiable toxicity endpoints of superior quality (Russom et al. 1997; Schultz & Cronin 2003). Traditionally, the selection of structural analogues or QSARs has been based on the assumption that compounds from the same ‘‘chemical class’’ should behave in a toxicologically similar manner. Although this working hypothesis seems reasonable, the identification of chemical classes is problematic, and research completed over the past several years has challenged the notion that similarities in modes of toxic action are necessarily related to typical chemical classification schemes. As a consequence,

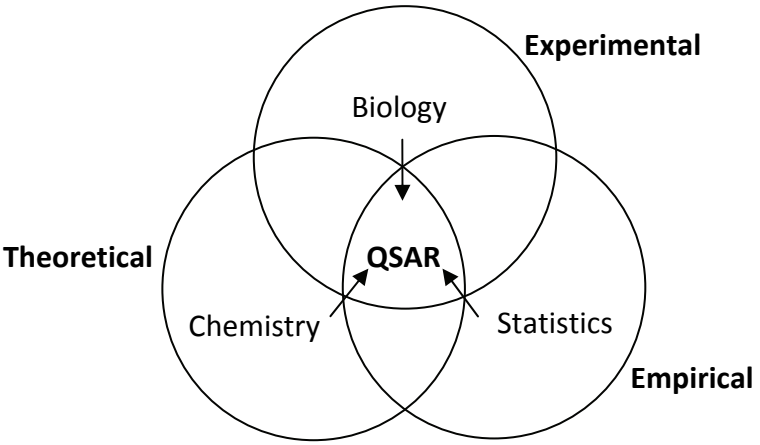

Fig. 1.3: QSARs resides at the intersections of biology, chemistry and statistics; cf. McKinney at al. (2000).

QSAR development and application have been evolving from a chemical class perspective to one that is more consistent with assumptions regarding modes of toxic action (Russom et al. 1997). Thus, the extent to which QSARs can be used as alternatives to fish testing depends on the state of knowledge of mechanisms of toxicity and the characterization of their domain of applicability (ECETOC 2004). At present, this approach can therefore only be used to make certain limited predictions of toxicity within certain groups of compounds known to share a common mode of action. For the prediction of fish toxicity for a wider range of chemicals with unknown mechanism of action, however, there is a general agreement that QSAR models are still at an early stage of development and that it is necessary to include more data to refine these models (ECETOC 2004).

1.3.3 Fish embryo tests

Another alternative to replace respectively refine the acute fish test is to use fish embryos instead of adult fish. According to Huntingford et al. (2006), the longer the life span of a given animal species and the more sophisticated its general behavior, the greater is its need for complex mental processes similar to those which in humans generate the conscious experience of suffering. *Vice versa*, it may be concluded that embryos, due to their shorter life span, may have not yet developed the same complex mental processes and may not have pain perception identical to adult fish. As a consequence, with respect to animal welfare, tests with fish embryos should be preferred to test with adult fish.

On the other hand, from a review on approx. 150 toxicological studies using different life-stages of fish, McKim (1977) arrived at the conclusion that in at least 80 % of the cases long-term toxicity could be predicted by results from studies with early life-stages. This conclusion was later corroborated by other studies, e.g. by Chorus (1987) or Woltering (1984).

Therefore, the fish embryo toxicity test (FET) appears to be a very promising tool to replace the acute fish test also for chemical testing (Braunbeck et al. 2005; Nagel 2002). Since in the current version of the test protocol the test duration is limited to two or three days, and since in legal terms, based on the current UK Animal Procedures Act (UK 1986) that covers fish solely, the FET is classified as a non-animal test, because the protection of immature forms of fish starts first when they become capable of independent feeding.

The FET has a lot of advantages: (1) Since a single mature female zebrafish lays 50 - 200 eggs per day (Braunbeck et al. 2005), under laboratory conditions several thousand embryos can easily be produced daily and used for parallel experimental treatments (Scholz et al. 2008). Thus, a large numbers of substances can be tested in parallel. (2) The test is conducted in 24-well microtiter plates. Hence, the test only requires very low amounts of test substances,

which is particularly important when only limited volumes of test solutions are available. (3) In its present version, the test duration of the embryo test is limited to two or three days, i.e. it is less time-consuming than the conventional *in vivo* acute fish test. (4) Sublethal endpoints can easily be implemented. (5) As mentioned above, embryos may have not yet developed the same pain perception like adults. (6) Compared to the fish cytotoxicity test, the FET is more sensitive (Lange et al. 1995). (7) Additionally, Braunbeck et al. (2005) provided data confirming that an optimized test protocol can equally be applied to the early embryonic stages of other OECD species such as the fathead minnow *(Pimephales promelas*) and the Japanese medaka (*Oryzias latipes*).

However, one of the most important features for a test to be accepted as an alternative to a conventional test is the correlation between the alternative procedure and the test to be replaced. Although the correlation between the acute fish test and the fish embryo test is basically very good (Braunbeck et al. 2005), there is certainly a number of substances, for which the embryo test is significantly more or less sensitive (Braunbeck et al. 2005). Several reasons might be speculated to account for such outliers: (1) differential uptake, accumulation and metabolization of the test compounds in embryonic and adult stages; (2) protection of the embryo by the chorion; (3) restricted availability of the test substances due to the static nature of the standard fish embryo test.

1.4 The fish embryo toxicity test (FET)

1.4.1 Zebrafish (*Danio rerio*)

The zebrafish (*Danio rerio*, Hamilton-Buchanan 1822) is a small benthopelagic cyprinid fish. The fish originates from the Ganges River system, Burma, the Malakka peninsula and Sumatra (Eaton & Farley 1974; Talwar & Jhingran 1991). The mean adult length varies between 3 and 5 cm. In both soft and hard waters, zebrafish grow quickly at 26 °C. Already after five days post fertilization, at the time of complete yolk consumption and start of external feeding, organogenesis of major organs is completed, and within three months the zebrafish reaches maturity (Scholz et al. 2008). The species is easily obtainable, inexpensive, readily maintainable and, under appropriate conditions, yields a large number of non-adherent, fully transparent eggs (Laale 1977). The eggs can be obtained all-seasonally and the transparent chorion enables the easy observation of development (Kimmel et al. 1995; Laale 1977). Thus, the zebrafish has become a major model in neurobiology and toxicology as well as in general molecular and developmental biology (Hill et al. 2005; Kimmel et al. 1995; Laale 1977; Langheinrich 2003; Lele & Krone 1996; Nüsslein-Vollhard 1994; Westerfield 2000).

Under spawning conditions, males can easily be distinguished from females by their more slender body shape and an orange to reddish tint in the silvery bands along the body (Fig. 1.4). Lack of distinctive secondary sex characteristics makes it difficult to distinguish between immature males and females. However, particularly during spawn maturity, due to the large number of eggs produced, females can be recognized by their swollen bellies. One female spawns between 50 and 200 eggs on a daily basis.

Fig 1.4: Zebrafish (*Danio rerio*) females (upper individual) can easily be differentiated from males (lower individual) by their extended bellies and the lack of reddish tint (Photo: Erik Leist).

1.4.2 Embryonic development of zebrafish

The embryonic development of zebrafish has been described in detail in several studies (Hisaoka & Battle 1958; Kimmel et al. 1995; Laale 1977). The zebrafish egg is telolecithal, cleavage is meroblastic and discoidal. Kimmel et al. (1995) describe seven broad periods of embryogenesis: the zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching period (Tab. 1.2). Selected major stages of zebrafish development are given in Figs. 1.5 and 1.6.

Tab. 1.2: Stages of embryonic development of the zebrafish (Danio rerio) at 26 ± 1 °C (Nagel 2002)

Time	Stage	Characterization after Kimmel et al. (2005)
0	Fertilization	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) division: 8-cell-stage
1.5		4. (vertical and parallel to the second) 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; blastoderm thins and interface between periblast 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 segmented or metameric
20		Muscular twitches; sacculus; tail well extended
22		Side to side flexures; otoliths
24	Pharyngula period	Phylotypic stage, spontaneous movements, 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 visible
36		Tail pigmentation; strong circulation; single aortic arch pair, early motility; heart beating
72 - 96	Hatching period	Heart-beat regular; yolk extension beginning to taper; dorsal and ventral pigmentation stripes meet at tail; segmental blood vessels detectable: thickened sacculus with two chambers visible; foregut development; neuromasts

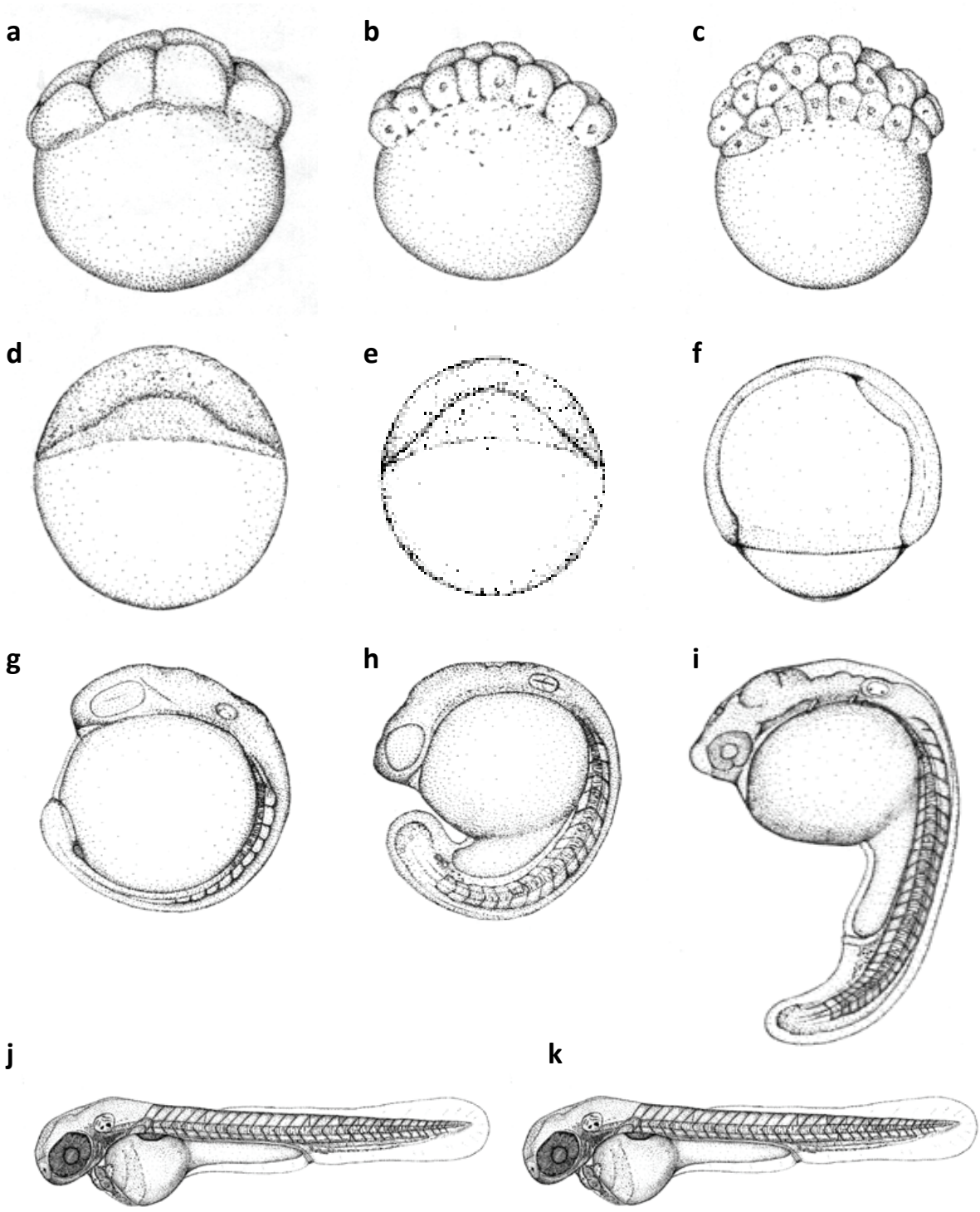

Fig. 1.5: Selected stages of the embryonic development of *Danio rerio* - illustrations without chorion (Kimmel et al. 1995):

a 8-cell stage (1.25 h) **b** 16-cell stage (1.5 h) **c** 32-cell stage (1.75 h)
d beginning epiboly (4.3 h) **e** 30 % epiboly stage (6 h) **f** 75 % epiboly stage (8 h)
g embryo at an age of 14 h **h** embryo at an age of 18 h **i** embryo at an age of 22 h
j embryo at an age of 48 h **k** embryo at an age of 72 h

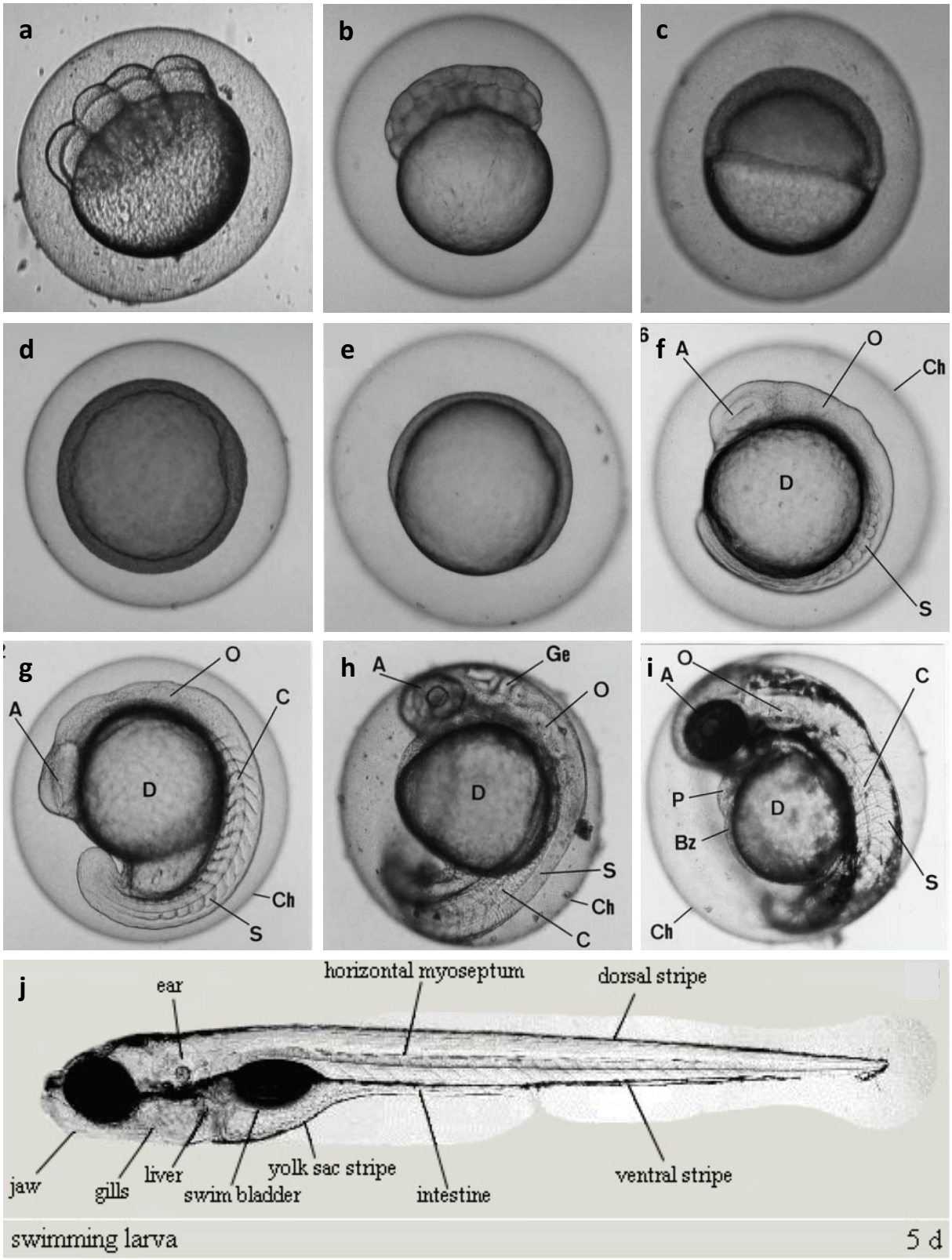

Fig. 1.6: Selected stages of the embryonic development of *Danio rerio*. Photos (a - i): Rudolf (2000); Photo (j): Haffter et al. (1996)

a 8-cell stage (1.25 h) **b** 32-cell stage (1.75 h) **c, d** 50 % epiboly stage (6 h)
e 75 % epiboly stage (8 h) **f** embryo at an age of 16 h **g** embryo at an age of 18 h
h embryo at an age of 24 h **i** embryo at an age of 48 h **j** embryo at an age of 120 h

A: eye anlage/eye; Bz: blood cells; C: chorda; Ch: chorion; D: yolk sac; Ge: brain anlage; O: ear bud; P: pericardium; S: somites

1.4.3 Principles of the fish embryo toxicity test (FET)

The FET has become a mandatory component in routine whole effluent testing in Germany since 2005 (DIN 38415-T6; DIN 2001). At an international level, the whole effluent test with zebrafish (*Danio rerio*) embryos has also been standardized since 2007 (ISO 2007), and a modified version has been submitted by the German Federal Environment Agency as a draft guideline for an alternative to chemical testing with intact fish (Braunbeck & Lammer 2006).

In general, fertilized zebrafish eggs are used to investigate lethal and sublethal effects of different chemicals. Since already malformations in the very early developmental stages can lead to tremendous effects (Sander & Baumann 1983), exposure should start as soon as possible after fertilization. Also Gellert & Heinrichsdorff (2001) reported that not later than 1 h after spawning the eggs already became relatively insensitive to the tested complex chemical mixture. Fig. 1.7 describes the selection of fertilized eggs directly after spawning including exposure of the embryos directly after hatch.

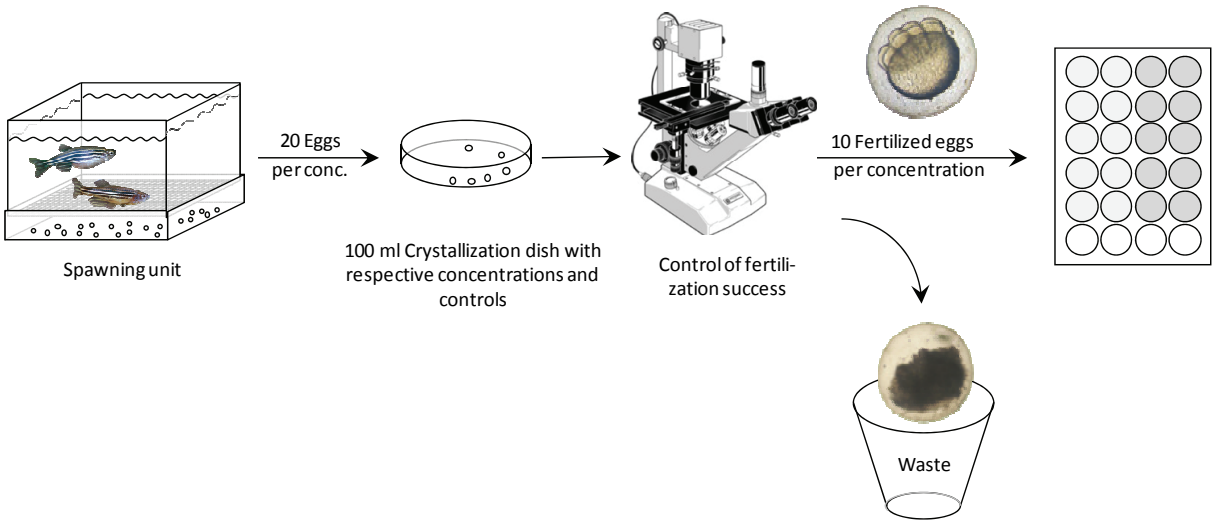

Fig. 1.7: Scheme of the FET test procedure (from left to right): collection of the eggs, pre-exposure of the eggs immediately after fertilization in crystallization dishes, selection of the fertilized eggs with an inverted microscope or binocular and distribution of the fertilized eggs in the prepared 24-well microtiter plates.

The test is carried out in 24-well microtiter plates. Due to their transparent bottom, they have the advantage that they can easily be used with an inverse microscope and only 2 ml test substance per well are needed for the investigation. However, polystyrene is an amorphous, clear and breakable all-plastic material, and its surface is highly hydrophobic (Koutsopoulos et al. 2007; Palmgren et al. 2006). There are several studies (Dahlström et al. 2004; Knorr & Gätzschmann 1966; Koutsopoulos et al. 2007; Palmgren et al. 2006) indicating that lipophilic and positively charged drugs and chemicals show strong interactions with negatively charged polystyrene and, therefore, tend to adsorb to the surface of the

polystyrene plates *via* electrostatic binding and hydrophobic interactions (Dahlström et al. 2004; Palmgren et al. 2006). But the limited electrostatic binding sites of the polystyrene plates can easily be saturated.

In the current version of the test protocol, the embryos are exposed to two or three days. Since independent, external feeding starts at approximately 96 h, according to the UK Animal Procedures Act (UK 1986) the protection of immature forms of fish starts at that point of time. However, the embryos hatch at approximately 72 h. From a physiological and toxicological point of view, this is a critical stage during embryogenesis (Van Leeuwen et al. 1985). Hence, it should be included in the testing procedure. Due to the transparency of the chorion, the development of the embryo inside the chorion can easily be observed, and since the zebrafish has a very short reproduction cycle, the test is very fast (Scholz et al. 2008). The sublethal and lethal endpoints (Tab. 1.3) can easily be detected and recorded after 24, 48, 72 and 96 h.

Defined are four lethal endpoints which are coagulation of eggs, non-development of somites, non-detachment of the tail and absence of heartbeat. If these effects are observed, the embryos won't survive the next hours or days and are therefore defined as dead. In contrast, the sublethal and teratogenic endpoints include effects which might provide extra information about sublethal or teratogenic modes of action of the sample tested (Küster & Altenburger 2007). The detection of sublethal and teratogenic endpoints is a major advantage compared to the 96 h acute fish test, since there only mortality is recorded and no information about the mode of action of tested chemicals can be obtained.

Tab. 1.3: Lethal and sublethal endpoints for evaluating the toxicity and teratogenicity of chemicals on zebrafish embryos as listed by Nagel (2002)

	Exposure time				
	8 h	**24 h**	**48 h**	**96 h**	**108/120 h**
Lethal endpoints *					
Coagulation	•	•	•	•	
Tail not detached		•	•	•	
No somite formation		•	•	•	
No heart-beat			•	•	
Lack of hatching					•
Sublethal developmental endpoints					
Completion of gastrula	•				
Formation of somites		•			
Development of eyes		•	•	•	
Spontaneous movement		•	•	•	
Heartbeat / blood circulation			•	•	
Heartbeat frequency			•	•	

	Pigmentation		•	•	
	Formation of edemata		•	•	
Endpoints of teratogenicity					
	Malformation of the head	•	•	•	
	Malformation of sacculi / otoliths	•	•	•	
	Malformation of tail	•	•	•	
	Malformation of heart	•	•	•	
	Modified chorda structure	•	•	•	
	Scoliosis	•	•	•	
	Rachitis	•	•	•	
	Yolk deformation	•	•	•	
	General growth retardation	•	•	•	
	Length of tail **				•

* After 48 h, the 4 endpoints were assessed to be lethal.

** There is the option to measure the length of tail after 120 h. In this case, the eggs are transferred into water without the test compound after 48 h. After natural hatching, the larval body becomes straight, and tail length can be determined precisely.

However, as already mentioned above, the test has limitations and “outliers” in comparison with the acute fish test can be observed (Braunbeck et al. 2005). Concerning the “outliers”, further investigations are necessary. However, since the FET should replace the 96 h fish test in chemical and risk assessment, the test would be part of a test battery including also the acute toxicity test for daphnids (OECD 202, 2004b) and the growth inhibition test with freshwater algae (OECD 201, 2006). The lowest EC_{50}/LC_{50} or NOEC out of the three single-species tests will be divided by an assessment factor to calculate the predicted no effect concentration (PNEC). However, Hoekzema et al. (2006) found out that in 88 % out of 507 compounds fish were not the most sensitive compared to the acute toxicity to daphnids and algae. The EC_{50}s for algae and *Daphnia* were lower than or equal to the LC_{20} for fish. For these 507 compounds, the lowest LC_{50} or EC_{50} was a non-vertebrate value, and application of the acute threshold test with fish would not reduce this value. Likewise, Weyers et al. (2000) reported that the algal growth inhibition test is the most sensitive (giving the lowest value) in 43.5 % of cases. The algal growth inhibition test alone triggered strictest classification in 22.9 % of all cases, whereas fish and *Daphnia* results together led to stricter classification in 17 %. Hence, if these results are taken into account, the FET can already be established in chemical and risk assessment, since, due to the good correlation of the FET and the 96 h acute fish test, the results of the chemical and risk assessment are highly likely to be the same.

1.5. Scope of the present thesis

In a preliminary statistical analysis of existing data for the FET by Ratte & Hammers-Wirtz (2003), 56 data pairs for acute fish and fish embryo toxicity data were included and resulted in an R^2 of 0.854 (Fig. 1.8). However, this analysis also included data from personal communications and acute fish test data from non-verified sources. Therefore, a thorough re-evaluation of both acute fish and fish embryo toxicity data was carried out in order to provide a sound basis for the validation of the FET. In order to detect the outliers, the data were correlated with the respective acute fish test data taken from the US EPA ECOTOX database (US EPA 2002) or the ECETOC Aquatic Toxicity (EAT) database. The purpose was to evaluate if the outliers could be eliminated by a larger database. If outliers could be detected, it had to be analyzed if the outliers could be related to specific classes of chemicals. Additionally, it had to be tested if the existing database was sufficient, or if data gaps could be found concerning chemical properties or modes of action.

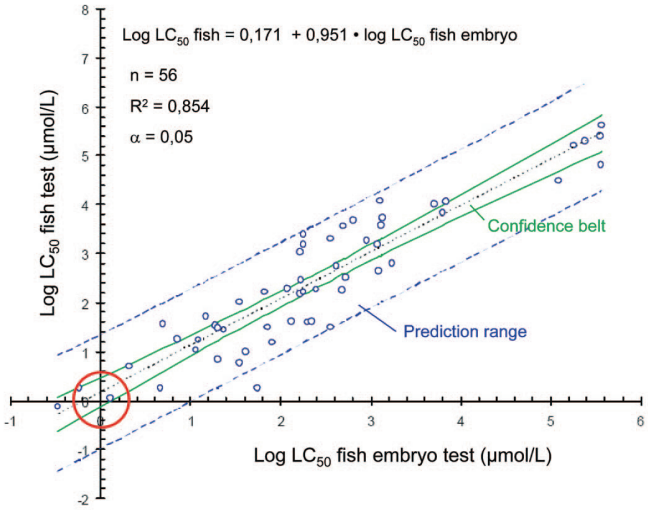

Fig. 1.8: Correlation between the embryo toxicity test with zebrafish (*Danio rerio*) and the 48 h acute fish LC50 test (various species; n = 56; Ratte & Hammers-Wirtz (2003)).

Although the existing correlation of Ratte & Hammers-Wirtz (2003) between the acute fish test and the fish embryo test is basically very good (Braunbeck et al. 2005), there are certainly a number of substances, for which the embryo test is significantly more or less sensitive (Braunbeck et al. 2005). There are different hypotheses for the difference in the FET and the 96 h acute fish test. One of these hypotheses should be tested in this investigation: if the restricted availability of the test substances due to the static nature of the standard fish embryo test is responsible for some of the outliers. To this end, a flow-through system was developed in order to improve the FET and to allow a permanent supply of fresh test substance in the flow-through system. A set of four outlier chemicals was tested in the flow-through FET in order to analyze if the improvement would eliminate the differences between the static FET and the 96 h acute fish test.

Chapter 2

Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test?

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2.1 Abstract

The fish acute toxicity test is a mandatory component in the base set of data requirements for ecotoxicity testing. The fish acute toxicity test is not compatible with most current animal welfare legislation because mortality is the primary endpoint and it is often hypothesized that fish suffer distress and perhaps pain. Animal alternative considerations have also been incorporated into new European REACH regulations through strong advocacy for the reduction of testing with live animals. One of the most promising alternative approaches to classical acute fish toxicity testing with live fish is the fish embryo toxicity (FET) test. The FET has been a mandatory component in routine whole effluent testing in Germany since 2005 and has already been standardized at the international level. In order to analyze the applicability of the FET also in chemical testing, a comparative re-evaluation of both fish and fish embryo toxicity data was carried out for a total of 143 substances, and statistical approaches were developed to evaluate the correlation between fish and fish embryo toxicity data. Results confirm that fish embryo tests are neither better nor worse than acute fish toxicity tests and provide strong scientific support for the FET as a surrogate for the acute fish toxicity test.

2.2 Introduction

The global production of chemicals has increased from 1 million t in 1930 to 400 million t in 2001 (EC 2001); approximately 100,000 different substances are registered in the EU market (Hengstler et al. 2006). The development of global chemical management programs is a reality with examples being the OECD HPV (High Production Volume) Program (OECD 2004a), the USEPA HPV Challenge Program (see http://www.epa.gov/HPV/), the Canadian categorization of the Domestic Substances List (CEPA 1999), and the European Union REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) initiative (EC 2007c). Prior to REACH (EC 2007c), European standard toxicity testing schemes distinguished between existing substances and new substances. All chemicals on the market before the publication of national chemicals acts were automatically termed existing substances and did not have to be tested according to the regulation of national legislation. Only for approx. 2700 so-called “new substances”, i.e. substances commercialized after publication of national chemicals regulations, with volumetric usage > 10 kg, the risk to human health and environment had to be tested and assessed; for higher volumes, more rigorous testing with a focus on long-term and chronic effects was required (EC 2001; Rogers 2003).

In fact, “existing substances” account for more than 99 % of substances on the market, and, since they were not subject to the same testing requirements (Bodar et al. 2003; Rogers 2003), there is a lack of toxicity and ecotoxicity data for about 95 % of the substances that are on the market. The number of existing substances reported in 1981 was 100,106 and the number of existing substances currently marketed in volumes > 1 ton is estimated to be 30,000 (EC 2001). Some 140 out of these have been identified as priority substances and are subject to comprehensive risk assessment carried out by EU member state authorities.

REACH, as a regulatory tool, was envisaged to revise the existing chemical policy and to harmonize chemical legislations in Europe (EC Directive 1907/2006, EC 2007c). REACH eliminates the distinction between existing and new substances and replaces more than 40 EU Directives and Regulations (Brown 2003; Petry et al. 2006). The major aim of REACH is to systematically evaluate the risk to human health and the environment of approximately 30,000 chemical substances produced, used or imported in quantities of 1 - 100 t/year (Combes et al. 2003; Lilienblum et al. 2008). Themed with “no data, no market”, only substances with an adequate database depending on their production volumes will be given authorization for commercialization.

In conventional ecotoxicity testing strategies, fish are an indispensable component of integrated toxicity testing strategies for the aquatic environment. Current OECD guidelines acknowledge this importance by covering acute toxicity (OECD 203, OECD 1992a), early life-stage toxicity (OECD 210, OECD 1992b), short term toxicity test on embryo and sac-fry stages (OECD 212, OECD 1998), and juvenile growth (OECD 215, OECD 2000b). The prominence of fish in ecotoxicity guidelines and subsequently environmental risk assessment has several reasons:

- The aquatic environment is a sink for many chemicals, as illustrated by the occasionally high pollution levels and frequencies of chemical spills;
- fish play a critical role in aquatic food webs by top-down and bottom-up regulation of nutrient and energy flow;
- fish have been used as sentinels for the quality of waters that serve as sources for human drinking water;
- accidental fish kills are visible to the public when they occur and sociologically indicate to the public on the need to protect natural waters from pollutants;
- fish are an important food resource for humans;
- fishing has a large recreational value in many cultures.

For individual substances, there are extensive regulatory requirements for information on fish acute toxicity to support both environmental risk assessment and hazard classification. A "base set" of data is required in the EU for all substances for which the marketing quantity exceeds 1 t/year per manufacturer (Annex VII.A of the Directive), and this "base set" includes acute toxicity for freshwater fish (96h LC_{50}; OECD 203, OECD 1992a), acute toxicity for daphnids (48h EC_{50}; OECD 202, OECD 2004b) and growth inhibition test on freshwater algae (preferably growth rate inhibition, 72h EC_{50}; OECD 201, OECD 2006).

In acute tests with their exclusive endpoint of mortality, fish have been hypothesized to suffer severe distress and pain (Braunbeck et al. 2005; Chandroo et al. 2004; Nagel 2002), which would be in conflict with current animal rights legislations in many regulatory jurisdictions. However, this subject area remains open to debate and research is on-going (Arlinghaus et al. 2007; Rose 2007). Considerations of animal welfare organizations have increasingly questioned the use of fish in ecotoxicity testing, and there is currently a strong public and political pressure to replace fish tests with alternatives. The reasons are not only ethical, but there are also scientific and cost drivers to embrace a more differentiated testing approach. Recent work has focused on incorporating the 3Rs (Reduction, Refinement and Replacement) concept (Russell & Burch 1959) into fish acute lethality testing (for reviews, see Walker et al. 1998, 1997). Favored approaches include the use of *in vitro* assays based on fish cells or cell lines (Babich & Borenfreund 1991; Castaño et al. 2003; Castaño et al. 1996; Denizeau 1998; Fent 2001; Segner 1998; 2004), as well as the use of fish embryos instead of free-feeding larvae, juvenile or adult fish (Braunbeck et al. 2005; Canaria et al. 1999; Friccius et al. 1995; Lange et al. 1995; Nagel 2002; Schulte & Nagel 1994). Most importantly, it should be noted that fish embryos and eleutheroembryos are not provided protection under various governmental definitions. In fact, at present, only the UK Animals Procedures Act (UK 1986) explicitly covers fish as laboratory animals, and even in the UK protection of immature forms of fish starts, when they become capable of independent feeding.

Animal alternative considerations have also been incorporated into new REACH regulations through strong advocacy for the reduction of testing with live animals, and, wherever possible, alternative methods are clearly preferred to conventional *in vivo* testing when validated approaches are available (Lilienblum et al. 2008). In fact, as early as 1986, Directive 86/609/EEC had stated that an alternative method, once it is "practicably and reasonably available", should replace the respective animal method (Louhimies 2002).

At present, the most promising alternative approach to classical acute fish toxicity testing with live fish is the fish embryo toxicity (FET) test. The FET is a mandatory component in routine whole effluent testing in Germany since 2005 (DIN 2001). The whole effluent test

with zebrafish (*Danio rerio*) embryos has been standardized at an international level (ISO 2007), and a modified version has been submitted by the German Federal Environment Agency (UBA) as a draft guideline for an alternative to chemical testing with intact fish (Braunbeck et al. 2005; Nagel 2002). Both embryos (eggs) and eleutheroembryos (the life interval between hatch and the onset of exogenous feeding) are considered alternatives in the context of European legislation (EFSA 2005).

Apart from parameters such as adequacy, reliability and reproducibility, both from a scientific and a regulatory point of view, one of the most important features for a test to be accepted as an alternative to a conventional test is the correlation between results obtained with the potential alternative and the test to be replaced. In a preliminary statistical analysis of existing data for the FET by Ratte and Hammers-Wirtz (2003), 56 data pairs for acute fish and fish embryo toxicity data were included and resulted in an R^2 of 0.854. However, this analysis also included data from personal communications and acute fish test data from non-verified sources.

Three facets of the fish embryo test are reported here:

(1) First, a thorough re-evaluation of both acute fish and fish embryo toxicity data was carried out in order to provide a sound basis for the validation of the FET. Especially for conventional acute fish toxicity data, only verified sources such as the US EPA ECOTOX database (US EPA 2002) or the ECETOC Aquatic Toxicity (Solbe et al. 1998) database were used, thus certifying that only data generated under GLP or GLP-like conditions as well as data published after peer-review were used for subsequent correlation analyses.

(2) Secondly, in some instances, FET data were re-analyzed and, where deemed necessary, FET tests were re-conducted to either confirm or discard existing data (cf. Förster 2008). For the new confirmatory FET tests, this paper also describes the methods employed.

(3) Thirdly, statistical approaches to evaluate the correlation between fish (e.g. OECD 203) and fish embryo toxicity data are described which are useful for future validation exercises (Hartung et al. 2004).

The primary objective of this study is to assess the scientific supportability of the fish embryo test to be a surrogate for the OECD 203 acute fish toxicity test (or other guideline equivalent acute fish assays).

2.3. Material and methods

2.3.1 The fish embryo toxicity test (FET)

2.3.1.1 Fish maintenance

A breeding stock of zebrafish aged between 6 and 24 months is used for egg production. Spawners should be free from externally visible diseases and not treated with any pharmaceutical (acute or prophylactic) treatment for 6 months before spawning. Females and males are kept together in glass aquaria providing sufficient space for swimming (i.e. ≥ 1 L per fish). Standardized dilution water as specified in ISO 7346-1 and 7346-2 (ISO 1996; 294.0 mg/L $CaCl_2 \cdot 2\ H_2O$; 123.3 mg/L of $MgSO_4 \cdot 7\ H_2O$; 63.0 mg/L $NaHCO_3$; 5.5 mg/L KCl) or suitable drinking water with ≥ 60 % oxygen saturation is used for keeping and breeding. Temperature is maintained at 26 ± 1 °C, and fish are kept under a constant artificial dark/light cycle of 8/16 h. Constant filtering or permanent flow-through conditions guarantee that ammonia, nitrite, and nitrate are kept below detection limits (0 - 5, 0.025 - 1 and 0 - 140 mg/L, respectively). Fish are fed commercially available artificial diets (e.g. TetraMin™ flakes; Tetra, Melle, Germany) twice daily, occasionally supplemented with *Artemia nauplii* or small daphnids of appropriate size obtained from an uncontaminated source. Overfeeding should be strictly avoided to ensure optimal water quality; remaining food and feces should be removed daily.

2.3.1.2 Egg production

Under spawning conditions, male zebrafish can easily be distinguished from females by their more slender body shape and an orange to reddish tint in the silvery bands along the body. Due to the large number of eggs produced, females can be recognized by their swollen bellies. The day before a test, males and females in a ratio of 2:1 are placed in breeding chambers (Fig. 2.1; water conditions as above) immediately before the onset of darkness. Since breeding groups of zebrafish may occasionally fail to spawn, the parallel use of at least three breeding chambers is strongly recommended. Artificial plants serve as breeding stimulant and substrate. Mating, spawning and fertilization take place within 30 min after the onset of light in the morning.

Since zebrafish is known to feed upon its own offspring, the bottom of the spawning dishes or spawning tanks should be covered with a grid of stainless steel, thus allowing the eggs to be sampled without interference by the adults. In the authors´ laboratory, for collection of eggs, the bottom of the 3 L breeding tanks are replaced by a stainless steel grid with a mesh

size of 1.25 mm in order to prevent predation of eggs. The breeding tanks are placed on rectangular full-glass dishes of similar dimensions (Fig. 2.1).

As a spawning stimulus, artificial plants made of green plastic or glass should be fixed to the grid covering the spawning dishes (Fig. 2.1). About 30 - 60 minutes after spawning, the spawning dishes can be removed, and the eggs are transferred to a temperature-controlled dissecting microscope or binocular by means of a plastic mesh sieve or pipettes. After determination of the overall egg number, viable (i.e. fertilized) eggs can easily be identified by their transparency, at best by putting the spawning dish on a black pad and using transverse light under the binocular.

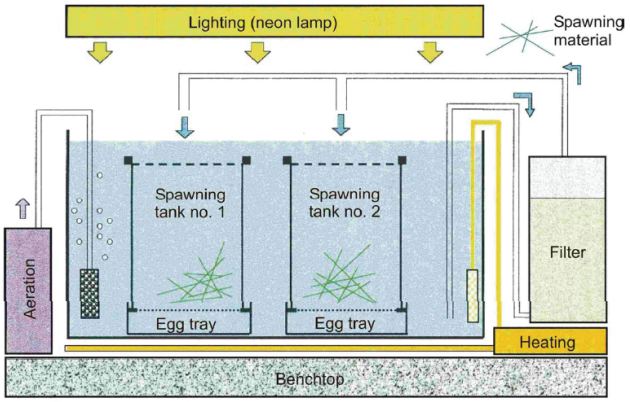

Fig. 2.1: Tanks setup used for breeding zebrafish (*Danio rerio*). Up to 10 tanks, the bottoms of which are replaced by a stainless steel grid, were placed on top of spawning dishes of similar dimensions. All spawning tanks were immersed into one bigger tank equipped with fully conditioned aquarium water. To collect the eggs after spawning, the dishes can easily be removed from the breeding facility (from Braunbeck et al. 2005).

Alternatively, eggs may be collected with larger spawning dishes covered with a stainless steel grid and placed at the bottom of the normal maintenance tanks. As a consequence of such a mass spawning procedures, however, it should be noted that the eggs recovered from a higher number of individuals are characterized by higher genetic diversity than those derived from well-defined spawning groups.

2.3.1.3 Initiation of the fish embryo toxicity test (FET)

The FET is initiated as soon as possible after fertilization of the eggs and not later than 3 h post-fertilization (128-cell stage). In order to start exposure with minimum delay, at least 20 randomly selected eggs per treatment group are transferred into 60 mm crystallization dishes containing 100 ml of the different test concentrations, the positive control or the negative control, respectively (Fig. 2.2).

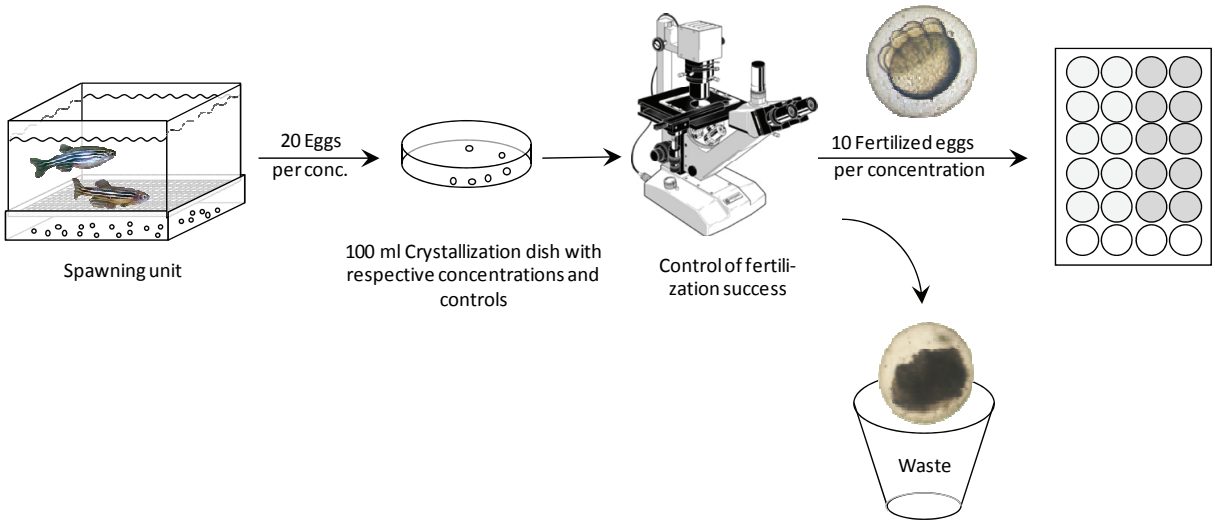

Fig. 2.2: Scheme of the FET test procedure (from left to right): collection of the eggs, pre-exposure of the eggs immediately after fertilization in crystallization dishes, selection of the fertilized eggs with an inverted microscope or binocular and distribution of the fertilized eggs in the prepared 24-well microtiter plates.

2.3.1.4 Selection of eggs

To identify fertilized eggs, an inverted microscope or a binocular with a minimum magnification of 25x should be used. Freshly spawned eggs are characterized by a fully transparent perivitelline space surrounded by the egg membrane and containing the yolk, and the germinal disc, which has already formed at the animal pole. After fertilization, the first cell division is initiated at 26 °C after about 15 min. Subsequently, the germinal disc is divided synchronously into 4, 8, 16 and 32 blastomers after 1 h, 1.25 h, 1.5 h and 1.75 h (Kimmel et al. 1995; 28.5 °C). From the 4-cell stage onwards, fertilized eggs can unambiguously be distinguished by their transparency from non-fertilized eggs. For the FET, only fertilized eggs between the 4- and 128-cell stages should be used. Eggs with overt anomalies (asymmetries, formation of vesicles) or damaged membranes should be discarded. Non-fertilized eggs can be identified by a lack of blastomer formation and, at later stages, by their non-transparency.

2.3.1.5 Test concentrations and controls

Chemicals should be tested in at least 5 concentrations prepared as dilutions with standard dilution water. The use of solvents or dispersants (solubilizing agents) should be avoided, but may be required in some cases in order to produce a suitably concentrated stock solution. In addition to the examples of suitable solvents given in OECD 215 (OECD 2000a),

dimethylsulfoxide (DMSO) might be useful. The final concentration of the solubilizing agent should not exceed 100 µl/L when required for preparing stock solutions and preferably be the same in all test vessels.

Dilution water in the absence of the chemical is used as a negative control. As a standard positive control, 3.7 mg/L 3,4-dichloroaniline should induce embryonic mortality > 10 % (DIN 38415-T6; ISO 15088); in the authors' laboratories, this concentration typically results in 20 - 80 % mortality. Each chemical is tested in at least two independent replicates (with ten eggs/embryos per test concentration and controls).

2.3.1.6 Distribution of eggs over 24-well plates

Following suspension in the test concentrations or any of the controls, 10 fertilized eggs are selected using a stereo microscope and transferred to 24-well plates filled with 2 ml freshly prepared test solutions and controls per well (Fig. 2.2). For the distribution of the 5 test concentrations and respective controls over the 24-well plates, see Fig. 2.3. The 24-well plates are then covered with self-adhesive foil and incubated at 26 °C ± 1.0 °C for 24, 48, 96 and 108 hrs.

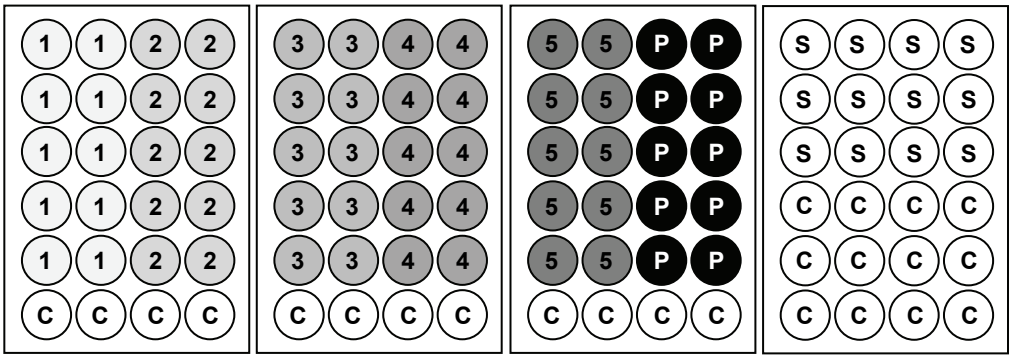

Fig. 2.3: Layout for the 24-well-plates of one replicate of a fish embryo test (FET): distribution of the different test concentrations and controls. The position of the 10 replicates of a given concentration (1 to 5) can be randomized; randomization, however, may become a serious source of pipetting errors. Negative controls (dilution water; C) are evenly distributed over the four plates of an experiment in order to identify plate-specific peculiarities. Whereas a positive control (e.g. 3,4-dichloraniline; P) is always obligatory, solvent controls are only run, if required.

2.3.1.7 Toxicological endpoints for the determination of chemical toxicity

The apical endpoints for the assessment of acute chemical toxicity are identical to those used for the determination of whole effluent toxicity (DIN 38415-T6; ISO 15088): coagulation

of the embryo, non-detachment of the tail, non-formation of somites and non-detection of the heartbeat. However, in contrast to whole effluent testing, these are recorded after 24,

Tab. 2.1: Lethal and sublethal endpoints in the Fish Embryo Test (FET) with the zebrafish (Danio rerio).

	Exposure time				
	8 h	**24 h**	**48 h**	**96 h**	**108/120 h**
Lethal endpoints *					
Coagulation	•	•	•	•	•
Tail not detached		•	•	•	•
No somite formation		•	•	•	•
No heart-beat			•	•	•
Lack of hatching					•
Sublethal developmental endpoints					
Completion of gastrula	•				
Formation of somites		•	•	•	•
Development of eyes		•	•	•	•
Spontaneous movement		•	•	•	•
Heartbeat / blood circulation			•	•	•
Heartbeat frequency			•	•	•
Pigmentation			•	•	•
Formation of edemata			•	•	•
Endpoints of teratogenicity					
Malformation of the head		•	•	•	•
Malformation of sacculi / otoliths		•	•	•	•
Malformation of tail		•	•	•	•
Malformation of heart		•	•	•	•
Modified chorda structure		•	•	•	•
Scoliosis		•	•	•	•
Rachitis		•	•	•	•
Yolk deformation		•	•	•	•
General growth retardation		•	•	•	•
Length of tail **					•

According to DIN 38415-T6 (DIN 2001) and ISO 15088 (ISO 2007), only the four lethal endpoints (coagulation, non-detachment of the tail, non-formation of somites and non-detection of the heartbeat) are recorded after 48 h. The test is deemed valid, if 90 % of the embryos in the negative control survive after 48 h.

* After 48 h, the 4 endpoints were assessed to be lethal.

** There is the option to measure the length of tail after 120 h. In this case, the eggs are transferred into water without the test compound after 48 h. After natural hatching, the larval body becomes straight, and tail length can be determined precisely.

48 and 96 h (Tab. 2.1). Since zebrafish embryos usually hatch after 72 h, non-hatching may be listed as a further lethal endpoint after 108 h. In addition to these core endpoints, which are regarded lethal, a number of endpoints may be recorded as evidence of sublethal toxicity or teratogenicity ("any other observation"; Tab. 2.1).

2.3.1.8 Validity criteria

For a test to be considered to fulfill the performance requirements, the following conditions should apply:

(1) The fertility rate of the parent generation should be ≥ 70%.

(2) The dissolved oxygen concentration should be ≥ 60 % of the air saturation value at the beginning of the test.

(3) The water temperature should be maintained at 26 ± 1 °C in test chambers at any time during the test.

(4) Overall survival of embryos in the negative control and, where relevant, in the solvent control should be ≥ 90% until hatch.

(5) When a solvent is used, it should not produce any adverse effects on the embryos as revealed by a solvent control.

(6) Mortality in the positive control should be > 10 %.

(7) Constant conditions should be maintained as far as possible throughout the test, and, if necessary, static renewal procedures should be used.

(8) There should be evidence that the concentration of the substance being tested has been satisfactorily maintained, and preferably it should be at least 80 % of the nominal concentration throughout the test. If the deviation from the nominal concentration is > 20%, results should be based on the mean measured concentrations.

2.3.2 Data collection and selection

2.3.2.1 Fish embryo toxicity (FET) data

Data for the fish embryo toxicity tests included in the present database were collected from experiments carried out between 1979 and 2008 with zebrafish (*Danio rerio*), fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*) and a few other fish species (e.g.

Clarias gariepinus; for references, see Tab. 2.3). In order to guarantee compliance and comparability of older data sets with more recent embryo toxicity tests, data sets had to fulfill the following criteria:

(1) Only tests, for which material and methods were clearly defined, were accepted. For the correlations, only LC_{50} values were taken that were calculated on the basis of DIN 38415-T6, ISO 15088 or comparable test methods;

(2) in order to guarantee an early onset of exposure, only exposures starting at latest at stage 11 (high blastula) were accepted. As a rule, fertilized eggs were exposed from 2 to 4 h after fertilization;

(3) all tests were conducted under static conditions; exposure systems based on microinjection techniques were discarded;

(4) normal exposure duration for zebrafish data sets was 48 - 96 h, i.e. only real embryo tests were accepted;

(5) tests with mixtures or different congeners were discarded, even if effects could be related a primary contaminant;

(6) data published in the scientific literature were only accepted, if they had undergone peer-review.

(7) MSc and PhD theses were only accepted from the laboratory of Roland Nagel (Technical University of Dresden), as well as theses from the authors´ group, because all of these had been conducted according to the German standard DIN regulation 38416-T6.

Confirmation of exposure concentrations is not yet common in fish embryo tests. In a single lab where both nominal and measured data are available for a set of compounds having varying hydrophobicities and modes of action, analytical verification was found on average to alter LC_{50}s by approximately 9 % (Rawlings and Belanger, unpublished data). For the fish tests, it should be noted that – whenever available – measured concentrations were used and listed in the tables of this review; in cases that no analytical data were available, nominal concentrations were used instead.

2.3.2.2 Acute fish toxicity data

The data for the acute fish toxicity test were primarily taken from the US EPA ECOTOX database (US EPA 2002: http://cfpub.epa.gov/ecotox/) and the ECETOC Aquatic Toxicity

(EAT) database (Hutchinson et al. 2003; Solbe et al. 1998). The databases were checked for entries for the major OECD fish species, zebrafish, fathead minnow, medaka, rainbow trout (*Oncorhynchus mykiss*) and bluegill sunfish (*Lepomis macrochirus*). Data were analyzed for the type of exposure (flow-through, static and semi-static) and whether concentrations were analytically confirmed or not (i.e. verified or nominal). Datasets extracted from the ECOTOX or the EAT databases with remarks such as “eggs”, “larvae”, “juvenile”, “young-of-the-year”, etc. were not included in the summarization for comparison to the FET. In order to obtain the maximum number of data pairs, all adult data were collected, independently of the duration of the exposure, although, as described below, ultimately comparisons were restricted to studies not exceeding 96 h.

For two chemicals (benzoic acid and dodecyl linear alkyl benzene sulfonate) no toxicity data could be found for the five major OECD fish species; in this case, data for other fish species (mosquito fish (*Gambusia affinis*) and golden ide (*Leuciscus idus melanotus*)) were available in the US EPA ECOTOX database and taken for correlation analyses to increase the number of data pairs. Data for dodecylbenzene sulfonate were ultimately supplemented with new zebrafish FET studies from (Rawlings & Belanger, submitted for publication).

2.3.3 Data management and statistical analysis

Acute fish and embryo toxicity data were collected in EXCEL spreadsheets. Due to the diversity of information, a series of decisions to harmonize, include or exclude studies was undertaken. Key aspects of this process were:

(1) All toxicity data were converted to the same units (µg/L). In a limited number of cases, toxicity was expressed in mol/L, since molecular weight data could not be retrieved to perform conversions. These studies were excluded from subsequent statistical analyses.

(2) All fish toxicity studies shorter than 24 or longer than 96 h were not used. If 96 h fish acute toxicity data were available, these were given priority followed by 72 h, 48 h, and 24 h studies.

(3) LC_{50}s were occasionally found that were given as ranges. In such cases, the geometric mean of the upper and lower bounds of the range were used, if the ratio was less than 3. These values were used only if other data were unavailable for the compound.

(4) Studies were included that employed static, static renewal and flow-through exposure techniques with and without analytical verification applied. These were evaluated separately and combined in order to understand the influence of exposure designs on FET *versus* acute fish toxicity relationships.

(5) Priority for fish acute toxicity data was given to the previously identified five taxa that dominate the literature: zebrafish, fathead minnow, rainbow trout, bluegill, and medaka. If FET data existed for a chemical which could not be paired with one of these five species, a search for an additional OECD test species was conducted and used.

(6) Toxicity data for Cd, Cu, and Zn, which are known to have toxicities modified by water hardness, were adjusted to a common level of 100 mg/L total hardness using US EPA water hardness correction regressions (US EPA 1980; 1987; 2000). Whereas it has become well-established that the biotic ligand model offers a mechanistic means forward for such analyses (Reiley 2007), it is not universal yet for all metals, and work relevant to embryo testing is not yet available.

The objective was to maximize the amount of high quality studies with paired FET and acute fish toxicity data. Ideally, all 5 species would be represented, and the best available data per species would be used. Analytically appropriate determinations for the type of chemical and exposure system were taken into account. Multiple studies per species per compound were summarized as geometric means.

In conventional regression modeling, only the measured response is assumed to be subject to random variation. However, in these data, the measured responses in both the acute fish toxicity test and the FET are results of experimental testing, and are subject to random variation. The statistical treatment of this issue is called errors-in-variables models, or measurement error models (Fuller 1987). Ordinary regression applied to data subject to measurement error under-estimates the slope of the regression, proportionate to the level of measurement error in the variable used as the predictor. In these data, the variability in the FET EC_{50}, derived from chemicals for which experimental replication is present in the database, is about the same as the experiment-to-experiment variability in the fish acute test LC_{50}. Orthogonal regression (Jackson 1991) is used to fit the linear relationship between the two experimental methods, adjusted for measurement error. Orthogonal regression minimizes the sum total of squared residual distances perpendicular to the line itself, *versus* ordinary regression, which minimizes the sum total of squared residual distances in the vertical dimension only. Although care must be taken with the application of orthogonal regression to an errors-in-variables problem (Carroll & Ruppert 1996), it is a useful tool

where it is not otherwise contraindicated. Fish embryo and fish acute toxicity data in this paper are generally chosen for the x and y axes, respectively, except for inter-species comparisons of fish acute toxicity data where all permutations of the 5 OECD species are explored. Slope, intercept, 95 % confidence intervals and correlation coefficients were computed using the statistical package R (Becker et al. 1988).

2.4 Results

2.4.1 Data collection

2.4.1.1 Fish embryo toxicity (FET) data

In total, 203 embryo toxicity tests were recorded for this review; 4 tests were conducted with fathead minnow, 3 tests with Japanese medaka and 5 tests with the African catfish (*Clarias gariepinus*; Tab. 2.2). It is worth noting that a great deal of zebrafish embryo toxicity data were provided by the laboratory of Dr. Roland Nagel, Technical University of Dresden, Germany in PhD theses by Bachmann (2002), Brust (2001) and Schulte (1997), which were tested according to pre-versions of the current standardized fish embryo toxicity (FET) test protocol. In total, 142 different chemical substances have been tested with the fish embryo toxicity test, so far. Of those chemicals used in the FET, 132 have acute fish toxicity data as well.

Tab. 2.2: Overview of the representation of different fish species in the fish embryo toxicity (FET) *versus* acute fish toxicity comparisons.

Species	Tests				Compounds			
	Embryo All	Embryo High Quality	OECD All	OECD High Quality	Embryo All	Embryo High Quality	OECD All	OECD High Quality
Danio rerio	212	204	162	48	143	133	33	26
Pimephales promelas	4	4	664	337	4	4	59	57
Lepomis macrochirus	0	0	523	265	0	0	38	34
Oncorhynchus kisutch	0	0	620	253	0	0	46	45
Oryzias latipes	2	2	177	81	2	2	37	36

Tab. 2.3: Fish embryo and eleutheroembryo toxicity data compiled from available literature and selected newly developed data. All chemicals and studies cited here have one or more available acute aquatic toxicity studies on juvenile or adult fish consistent with the OECD 203 Technical Guideline (OECD 1992a).

Substance	CAS no.	Molec. weight	FET species	Embryo interval	Dura-tion (h)	EC_{50} (µg/L)	Reference
Acetone	67-64-1	58.08	*Danio rerio*	E	48	13091232	Bachmann 2002
Acetone	67-64-1	58.08	*Danio rerio*	E	48	10500	Maiwald 1997
Acetyl salicylic acid	50-78-2	180.16	*Danio rerio*	E	48	53327.40	Bachmann 2002
Acetyl salicylic acid	50-78-2	180.16	*Danio rerio*	ELE	120	48102.75	Bachmann 2002
Acrolein	107-02-8	56.06	*Danio rerio*	E	48	370	Schulte 1997
2-Aminoethanol	141-43-5	61.08	*Danio rerio*	ELE	96	3683124	Groth et al. 1993
4-Aminophenol	123-30-8	109.13	*Danio rerio*	E	48	470	Schulte 1997
Aniline	62-53-3	93.12	*Danio rerio*	ELE	96	866016	Groth et al. 1993
Aniline	62-53-3	93.12	*Danio rerio*	E	48	161200	Schulte 1997
Aniline	62-53-3	93.12	*Danio rerio*	E	48	147100	Schulte 1997
Atrazine	1912-24-9	215.69	*Danio rerio*	E	48	36800	Wiegand et al. 2000
Benzoic acid	65-85-0	122.12	*Danio rerio*	E	48	19539	Bachmann 2002
Benzoic acid	65-85-0	122.12	*Danio rerio*	E	48	20100	Schulte 1997
4-Bromoindole	52488-36-5	195.95	*Danio rerio*	E	48	5100	ISIS 2003
4-Bromoindole	52488-36-5	195.95	*Danio rerio*	E	48	4300	Kammann et al. 2006
5-Bromoindole	10075-50-0	196.05	*Danio rerio*	E	48	5500	ISIS 2003
5-Bromoindole	10075-50-0	196.05	*Danio rerio*	E	48	5310	Kammann et al. 2006
6-Bromoindole	52415-29-9	196.05	*Danio rerio*	E	48	5310	ISIS 2003
6-Bromoindole	52415-29-9	196.05	*Danio rerio*	E	48	9210	Kammann et al. 2006

Substance	CAS no.	Molec. weight	FET species	Embryo interval	Dura-tion (h)	EC_{50} (µg/L)	Reference
2-Bromophenol	95-56-7	173.01	*Danio rerio*	E	48	45120	Kammann et al. 2006
3-Bromophenol	591-20-8	173.01	*Danio rerio*	E	48	60660	Kammann et al. 2006
4-Bromophenol	106-41-2	173.01	*Danio rerio*	E	48	46320	Kammann et al. 2006
n-Butylamine	109-73-9	73.14	*Danio rerio*	E	48	35911	Brust 2001
sec-Butylamine	13952-84-6	73.14	*Danio rerio*	E	48	95155	Brust 2001
Butyldiglycol	112-34-5	162.23	*Danio rerio*	E	48	1281617	Bachmann 2002
Butyldiglycol	112-34-5	162.23	*Danio rerio*	ELE	120	1281617	Bachmann 2002
p-tert-Butylphenol	98-54-4	150.22	*Danio rerio*	E	48	1730	Maiwald 1997
Cadmium	7440-43-9	112.411	*Danio rerio*	E	28	18885	Cheng et al. 2000
Cadmium (chloride)	7440-43-9	112.411	*Clarias gariepinus*	ELE	120	670	Nguyen & Janssen 2001
Carbaryl	63-25-2	201.22	*Danio rerio*	E	48	4700	Schulte 1997
Chloroacetaldehyde	107-20-0	78.5	*Danio rerio*	ELE	96	3351	Groth et al. 1993
2-Chloroaniline	95-51-2	127.6	*Danio rerio*	E	48	28300	Schulte 1997
3-Chloroaniline	108-42-9	127.6	*Danio rerio*	E	48	21000	Schulte 1997
4-Chloroaniline	106-47-8	127.6	*Danio rerio*	E	48	21300	Schulte 1997
4-Chlorophenol	106-48-9	128.56	*Danio rerio*	E	48	45600	Schulte 1997
4-Chlorophenol	106-48-9	128.56	*Danio rerio*	E	48	42800	Stangl 1991
4-Chlorophenol	106-48-9	128.56	*Danio rerio*	E	72	40100	Stangl 1991
4-Chlorophenol	106-48-9	128.56	*Danio rerio*	ELE	96	34600	Stangl 1991
4-Chlorophenol	106-48-9	128.56	*Danio rerio*	E	48	37800	Ensenbach 1987
4-Chlorophenol	106-48-9	128.56	*Danio rerio*	E	24	40400	Förster 2008

Substance	CAS no.	Molec. weight	FET species	Embryo interval	Dura-tion (h)	EC_{50} (µg/L)	Reference
4-Chlorophenol	106-48-9	128.56	*Danio rerio*	E	48	43860	Förster 2008
4-Chlorophenol	106-48-9	128.56	*Danio rerio*	ELE	72	42140	Förster 2008
4-Chlorophenol	106-48-9	128.56	*Danio rerio*	ELE	108	32180	Förster 2008
Chlorpyrifos	2921-88-2	350.58	*Pimephales promelas*	ELE	96	122	Jarvinen et al. 1988
Chromium (Potassium dichromate)	7778-50-9	52	*Clarias gariepinus*	ELE	120	20100	Nguyen and Janssen 2001
Colcemide	477-30-5	371.4	*Danio rerio*	E	48	3417	Bachmann 2002
Colcemide	477-30-5	371.4	*Danio rerio*	ELE	120	3417	Bachmann 2002
Copper(II) sulfate pentahydrate	7758-99-8	249.7	*Danio rerio*	E	48	499	Bachmann 2002
Cyclohexanol	108-93-0	100.16	*Danio rerio*	ELE	96	1382208	Groth et al. 1993
Cycloheximide	66-81-9	281.35	*Danio rerio*	E	48	1463	Bachmann 2002
Cycloheximide	66-81-9	281.35	*Danio rerio*	ELE	120	1294	Bachmann 2002
Cyclohexylamine	108-91-8	99.18	*Danio rerio*	E	48	63376	Brust 2001
n-Decylamine	2016-57-1	157.3	*Danio rerio*	E	48	3146	Brust 2001
Diazinon	333-41-5	304.34	*Oryzias latipes*	E	24	31043	Hamm et al. 2001
Diazinon	333-41-5	304.34	*Oryzias latipes*	ELE	120	31347	Hamm et al. 2001
2,4-Dibromophenol	615-58-7	251.9	*Danio rerio*	E	48	7950	Kammann et al. 2006
2,6-Dibromophenol	608-33-3	251.9	*Danio rerio*	E	48	41770	Kammann et al. 2006
Dibutylamine	111-92-2	129.25	*Danio rerio*	E	48	40454	Brust 2001
2,4-Dichloroaniline	554-00-7	162.02	*Danio rerio*	E	48	21500	Schulte 1997

Substance	CAS no.	Molec. weight	FET species	Embryo interval	Dura-tion (h)	EC_{50} (µg/L)	Reference
3,4-Dichloroaniline	95-76-1	162.02	*Danio rerio*	E	48	2400	Schulte 1997
3,4-Dichloroaniline	95-76-1	162.02	*Danio rerio*	E	48	1620	Lange et al. 1995
Dicyclohexylamine	101-83-7	181.32	*Danio rerio*	E	48	31187	Brust 2001
Diethylamine	109-89-7	73.14	*Danio rerio*	E	48	93254	Brust 2001
Diethylene glycol	111-46-6	106.14	*Danio rerio*	E	48	50809218	Bachmann 2002
Diethylene glycol dimethylether	111-96-6	134.18	*Danio rerio*	E	48	11056432	Bachmann 2002
N,N-Diethylmethylamine	616-39-7	87.16	*Danio rerio*	E	48	69993	Brust 2001
N,N-Diisopropylethylamine	7087-68-5	129.25	*Danio rerio*	E	48	104563	Brust 2001
Diisobutylamine	110-96-3	129.3	*Danio rerio*	E	48	47195	Brust 2001
Diisopropylamine	108-18-9	101.2	*Danio rerio*	E	48	91477	Brust 2001
N,N-Dimethylamine	124-40-3	45.09	*Danio rerio*	ELE	96	396792	Groth et al. 1993
N,N-Dimethylanilin	121-69-7	121.2	*Danio rerio*	E	24	54270	Förster 2008
N,N-Dimethylanilin	121-69-7	121.2	*Danio rerio*	E	48	54320	Förster 2008
N,N-Dimethylanilin	121-69-7	121.2	*Danio rerio*	ELE	72	54240	Förster 2008
N,N-Dimethylanilin	121-69-7	121.2	*Danio rerio*	ELE	108	54240	Förster 2008
N,N-Dimethylbutylamine	927-62-8	101.19	*Danio rerio*	E	48	50999	Brust 2001
N,N-Dimethylcyclohexylamine	98-94-2	127.23	*Danio rerio*	E	48	53055	Brust 2001
N,N-Dimethylethylamine	598-56-1	73.14	*Danio rerio*	E	48	82865	Brust 2001
N,N-Dimethylformamide	68-12-2	73.09	*Danio rerio*	E	48	10020639	Bachmann 2002
N,N-Dimethylformamide	68-12-2	73.09	*Danio rerio*	ELE	96	8843890	Groth et al. 1994
C8-10 N, N-dimethyl-N-(2-hydroxyethyl)-N-	80439-32-0	251.53	*Danio rerio*	E	48	391000	Rawlings & Belanger

Substance	CAS no.	Molec. weight	FET species	Embryo interval	Dura-tion (h)	EC_{50} (µg/L)	Reference
alkonium chloride							
C8-10 N, N-dimethyl-N-(2-hydroxyethyl)-N-alkonium chloride	80439-32-0	251.53	*Danio rerio*	ELE	96	325000	Rawlings and Belanger
Dimethylsulfoxide	67-68-5	78.13	*Danio rerio*	E	48	29100000	Maiwald 1997
4,6-Dinitro-o-cresol	534-52-1	198.14	*Danio rerio*	E	48	600	Förster 2008
4,6-Dinitro-o-cresol	534-52-1	198.14	*Danio rerio*	ELE	72	440	Förster 2008
4,6-Dinitro-o-cresol	534-52-1	198.14	*Danio rerio*	ELE	108	390	Förster 2008
2,4-Dinitrophenol	51-28-5	184.11	*Danio rerio*	E	48	900	Schulte 1997
Dipentylamine	2050-92-2	157.3	*Danio rerio*	E	48	42786	Brust 2001
Dipropylamine	142-84-7	101.19	*Danio rerio*	E	48	31167	Brust 2001
D-Mannitol	69-65-8	182.17	*Danio rerio*	E	48	77768	Bachman, 2002
Dodecyl linear alkyl benzene sulfonate	27176-87-0	324	*Danio rerio*	E	48	2700	Rawlings and Belanger
Dodecyl linear alkyl benzene sulfonate	27176-87-1	324	*Danio rerio*	ELE	96	3300	Rawlings and Belanger
Endrin	72-20-8	380.9	*Pimephales promelas*	ELE	96	0.7	Jarvinen et al. 1988
Ethanol	64-17-5	46.1	*Danio rerio*	E	48	11999830	Bachmann 2002
Ethanol	64-17-5	46.1	*Danio rerio*	E	48	11100000	Maiwald 1997
Ethyl acetate	141-78-6	88.106	*Danio rerio*	E	48	1859036	Bachmann 2002
Ethylenediamine	107-15-3	60.1	*Danio rerio*	E	48	396660	Bachmann 2002
1-Ethylpiperidine	766-09-6	113.2	*Danio rerio*	E	48	71318	Brust 2001
2-Ethylpiperidine	1484-80-6	113.2	*Danio rerio*	E	48	93958	Brust 2001
Fenvalerate	51630-58-1	419.9	*Pimephales*	ELE	96	0.85	Jarvinen et al. 1988

Substance	CAS no.	Molec. weight	FET species	Embryo interval	Dura-tion (h)	EC_{50} (µg/L)	Reference
			promelas				
Formamide	75-12-7	45.04	*Danio rerio*	ELE	96	9143120	Groth et al. 1994
α-D-Glucose	492-62-6	180.16	*Danio rerio*	E	48	77072448	Bachmann 2002
n-Heptylamine	111-68-2	115.22	*Danio rerio*	E	48	28459	Brust 2001
Hexamethyleneimine	111-49-9	99.18	*Danio rerio*	E	48	115346.34	Brust 2001
2,5-Hexanedion	110-13-4	114.14	*Danio rerio*	E	48	4668498	Bachmann 2002
n-Hexylamine	111-26-2	101.19	*Danio rerio*	E	48	42297	Brust 2001
High Solubility Alkyl sulfate	343978-24-5	353	*Pimephales promelas*	E	48	190	Rawlings and Belanger
Hydroquinone	123-31-9	110.11	*Danio rerio*	E	48	7900	Schulte 1997
Hydroxyurea	127-07-1	76.05	*Danio rerio*	E	48	2311920	Bachmann 2002
Hydroxyurea	127-07-1	76.05	*Danio rerio*	ELE	120	1270035	Bachmann 2002
Isobutylamine	78-81-9	73.1	*Danio rerio*	E	48	92617.70	Brust 2001
Isoniazid	54-85-3	137.14	*Danio rerio*	E	48	42390	Bachmann 2002
Isopentylamine	107-85-7	87.16	*Danio rerio*	E	48	59098	Brust 2001
Isopropylamine	75-31-0	59.11	*Danio rerio*	E	96	5390832	Groth et al. 1993
Isopropylamine	75-31-0	59.11	*Danio rerio*	E	48	149607	Brust 2001
Lindane	58-89-9	290.83	*Danio rerio*	E	48	800	Schulte 1997
Malathion	121-75-5	330.4	*Danio rerio*	E	48	6200	Schulte 1997
Malathion	121-75-5	330.4	*Danio rerio*	E	48	7600	Schulte 1997
Malathion	121-75-5	330.4	*Clarias gariepinus*	ELE	120	3420	Nguyen and Janssen 2001
Methanol	67-56-1	32.04	*Danio rerio*	E	48	22100000	Ensenbach 1987
Methoxyacetic acid	625-45-6	90.08	*Danio rerio*	E	48	53687	Bachmann 2002

Substance	CAS no.	Molec. weight	FET species	Embryo interval	Dura-tion (h)	EC_{50} (µg/L)	Reference
2-Methoxyethanol	109-86-4	76.09	*Danio rerio*	E	48	23831388	Bachmann 2002
2-Methoxyethanol	109-86-4	76.09	*Danio rerio*	ELE	120	17241994	Bachmann 2002
1-Methoxy-2-propanol	107-98-2	90.12	*Danio rerio*	E	48	16338756	Bachmann 2002
1-Methoxy-2-propanol	107-98-2	90.12	*Danio rerio*	ELE	120	13914528	Bachmann 2002
3-Methyl-1-butanol	123-51-3	88.17	*Danio rerio*	E	48	1075674	Bachmann 2002
3-Methyl-1-butanol	123-51-3	88.17	*Danio rerio*	ELE	120	1022772	Bachmann 2002
N-Methylamine	74-89-5	31.1	*Danio rerio*	ELE	96	712190	Groth et al. 1993
N-Methylanilin	100-61-8	107.2	*Danio rerio*	ELE	96	76	Groth et al. 1993
N-Methylanilin	100-61-8	107.2	*Danio rerio*	E	24	75000	Förster 2008
N-Methylanilin	100-61-8	107.2	*Danio rerio*	E	48	75000	Förster 2008
N-Methylanilin	100-61-8	107.2	*Danio rerio*	ELE	72	75000	Förster 2008
N-Methylanilin	100-61-8	107.2	*Danio rerio*	ELE	108	68310	Förster 2008
N-Methylformamide	123-39-7	59.07	*Danio rerio*	E	48	22315815	Bachmann 2002
N-Methylformamide	123-39-7	59.07	*Danio rerio*	ELE	120	17371840	Bachmann 2002
N-Methylformamide	123-39-7	59.07	*Danio rerio*	ELE	96	10986611	Groth et al. 1994
Methyl mercury (II) chloride	115-09-3	251.08	*Danio rerio*	E	48	144.62	Bachmann 2002
1-Methylpiperidine	626-67-5	99.18	*Danio rerio*	E	48	68332	Brust 2001
2-Methylpiperidine	109-05-7	99.18	*Danio rerio*	E	48	102350	Brust 2001
4-Methylpiperidine	626-58-4	99.18	*Danio rerio*	E	48	92928	Brust 2001
Morpholine	110-91-8	87.12	*Danio rerio*	E	48	601215	Brust 2001
2-Nitro-4'-hydroxydiphenylamine	54381-08-7	230.22	*Danio rerio*	E	48	540	Liu et al. 2007
2-Nitro-4'-	54381-08-7	230.22	*Danio rerio*	ELE	72	190	Liu et al. 2007

Substance	CAS no.	Molec. weight	FET species	Embryo interval	Dura-tion (h)	EC_{50} (µg/L)	Reference
hydroxydiphenylamine							
2-Nitroaniline	88-74-4	138.1	*Danio rerio*	E	48	21500	Schulte 1997
2-Nitroanisole (2-NA)	91-23-6	153.1	*Danio rerio*	E	48	30620	Lange et al. 1995
4-Nitrobenzoic acid	62-23-7	167.12	*Danio rerio*	E	48	29900	Schulte 1997
4-Nitrophenol	100-02-7	139.11	*Danio rerio*	E	48	4900	Schulte 1997
4-Nitrophenol	100-02-7	139.11	*Danio rerio*	E	48	43100	Schulte 1997
4-Nitrophenol	100-02-7	139.11	*Danio rerio*	E	48	57200	Ensenbach 1987
n-Nonylamine	112-20-9	143.27	*Danio rerio*	E	48	11462	Brust 2001
1-Octanol	111-87-5	130.23	*Danio rerio*	E	48	15628	Bachmann 2002
1-Octanol	111-87-5	130.23	*Danio rerio*	E	48	15400	Schulte 1997
n-Octylamine	111-86-4	129.25	*Danio rerio*	E	48	25461	Brust 2001
Penicillin G (sodium salt)	69-57-8	356.37	*Danio rerio*	E	48	5452461	Bachmann 2002
Pentachlorophenol	87-86-5	266.34	*Danio rerio*	E	48	90	Maiwald 1997
Pentachlorophenol	87-86-5	266.34	*Danio rerio*	E	48	1000	Stangl 1991
Pentachlorophenol	87-86-5	266.34	*Danio rerio*	ELE	72	650	Stangl 1991
Pentachlorophenol	87-86-5	266.34	*Danio rerio*	ELE	96	570	Stangl 1991
Pentachlorophenol	87-86-5	266.34	*Danio rerio*	E	48	380	Ensenbach 1987
Pentachlorophenol	87-86-5	266.34	*Clarias gariepinus*	ELE	120	550	Nguyen and Janssen 2001
n-Pentylamine	110-58-7	87.16	*Danio rerio*	E	48	30856	Brust 2001
4-tert-Pentylphenol	80-46-6	164.24	*Danio rerio*	E	24	3500	Baumann 2008
4-tert-Pentylphenol	80-46-6	164.24	*Danio rerio*	E	48	3500	Baumann 2008
4-tert-Pentylphenol	80-46-6	164.24	*Danio rerio*	ELE	72	3500	Baumann 2008

Substance	CAS no.	Molec. weight	FET species	Embryo interval	Dura-tion (h)	EC_{50} (µg/L)	Reference
Phenol	108-95-2	94.11	*Danio rerio*	E	48	49100	Schulte 1997
Phenol	108-95-2	94.11	*Danio rerio*	E	48	49800	Ensenbach 1987
Phenol	108-95-2	94.11	*Danio rerio*	E	48	115000	Rawlings and Belanger
Phenol	108-95-2	94.11	*Danio rerio*	ELE	96	210000	Rawlings and Belanger
Piperidine	110-89-4	85.2	*Danio rerio*	E	48	110504	Brust 2001
Prochloraz	67747-09-5	376.67	*Danio rerio*	E	24	2900	Baumann 2008
Prochloraz	67747-09-5	376.67	*Danio rerio*	E	48	2900	Baumann 2008
Prochloraz	67747-09-5	376.67	*Danio rerio*	ELE	72	2900	Baumann 2008
2-Propanol	67-63-0	60.1	*Danio rerio*	E	48	10138870	Bachmann 2002
2-Propanol	67-63-0	60.1	*Danio rerio*	ELE	120	8618340	Bachmann 2002
n-Propylamine	107-10-8	59.11	*Danio rerio*	E	48	79150	Brust 2001
Quinone	106-51-4	108.1	*Danio rerio*	E	48	472	Groth et al. 1993
Retinoic acid	302-79-4	300.44	*Danio rerio*	E	48	1090	Bachmann 2002
all-trans-Retinol	68-26-8	286.46	*Danio rerio*	E	48	610	Bachmann 2002
Saccharin sodium salt hydrate	82385-42-0	205.17	*Danio rerio*	E	48	20947857	Bachmann 2002
Salicylic acid	69-72-7	138.12	*Danio rerio*	E	48	24585	Bachmann 2002
Salicylic acid	69-72-7	138.12	*Danio rerio*	E	48	22790	Bachmann 2002
Sodium chloride	7647-14-5	58.44	*Danio rerio*	E	48	12009420	Lange et al. 1995
Tetrachloroethylene	127-18-4	165.83	*Oryzias latipes*	ELE	96	26800	Spencer et al. 2002
2,4,6-Tribromophenol	118-79-6	330.8	*Danio rerio*	E	48	4420	Kammann et al. 2006

Substance	CAS no.	Molec. weight	FET species	Embryo interval	Dura-tion (h)	EC_{50} (µg/L)	Reference
Tributylamine	102-82-9	185.36	*Danio rerio*	E	48	301210	Brust 2001
Triclocarban	101-20-2	315.59	*Danio rerio*	ELE	96	26	Rawlings and Belanger
Triclosan	3380-34-5	289.54	*Danio rerio*	E	48	360	Rawlings and Belanger
Triclosan	3380-34-5	289.54	*Danio rerio*	ELE	96	280	Rawlings and Belanger
Tridecyl mono-octyl ether	24938-91-8	552	*Danio rerio*	E	48	2000	Rawlings and Belanger
Tridecyl mono-octyl ether	24938-91-8	552	*Danio rerio*	ELE	96	870	Rawlings and Belanger
Triethylamine	121-44-8	101	*Danio rerio*	E	48	60398	Brust 2001
Triethylene glycol	112-27-6	150.2	*Danio rerio*	E	48	53900000	Maiwald 1997
Tripropylamine	102-69-2	143.27	*Danio rerio*	E	48	188833	Brust 2001
Urea	57-13-6	60.06	*Danio rerio*	E	48	22360338	Bachmann 2002
Urea	57-13-6	60.06	*Danio rerio*	E	48	22462440	Lange et al. 1995
Urea	57-13-6	60.06	*Danio rerio*	E	48	22900000	Schulte 1997
Valpromide	2430-27-5	143.23	*Danio rerio*	E	48	1131509	Bachmann 2002
Valpromide	2430-27-5	143.23	*Danio rerio*	ELE	120	1016926	Bachmann 2002
Valproic acid	99-66-1	144.21	*Danio rerio*	E	48	20189	Bachmann 2002
Zinc (Zinc sulfate heptahydrate)	7446-20-0	65.39	*Clarias gariepinus*	ELE	120	18700	Nguyen and Janssen 2001

2.4.1.2 Adult fish toxicity data

In total, 2146 entries were identified for 80 different chemicals tested with adult fish (data not shown): 664 tests for 59 different chemicals tested with fathead minnow; 620 tests for 46 chemicals tested with rainbow trout; 523 tests for 38 chemicals tested with bluegill sunfish; 177 tests for 37 different chemicals tested with Japanese medaka; 162 tests for 33 chemicals tested with zebrafish; 3 tests for 1 chemical with mosquito fish and 1 test for 1 chemical with golden ide.

2.4.2 Correlation between conventional acute fish toxicity data and fish embryo toxicity (FET) data

Eleutheroembryo and embryo studies generally provided highly similar results. Thirty-two chemicals with paired data were available. A correlation of 0.96 and a slope of 1.05 were obtained (Fig. 2.4). An isolated few examples are available which underscore the importance of evaluating eleutheroembryos as the interval immediately after hatch is more vulnerable to some chemicals than others (Léonard et al. 2005; Rawlings & Belanger, submitted for publication). Examples include relatively large molecules such as polymers and higher molecular weight surfactant. In general, however, this broader comparison supports inclusion of both embryo (egg) and eleutheroembryo data in comparisons to acute fish toxicity.

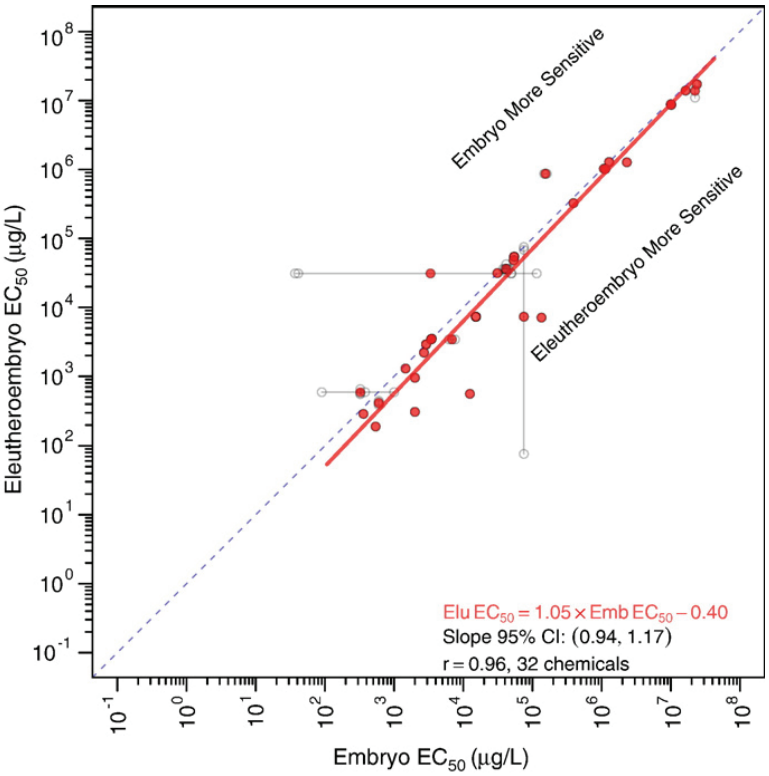

Fig. 2.4: Comparative toxicity of 32 chemicals to fish embryos and eleutheroembryos (all fish species combined). Geometric mean values are indicated by the solid circles, and the ranges (x or y direction) are indicated by the bars. The line of 1:1 correspondence is given dashed gray.

A series of comparisons were made using permutations of the data set listed in Table 2.3. Inclusion and exclusion of species (both embryo and acute fish), exposure durations (exclusively within acute fish studies), exposure types (static, static renewal, flow through), and analytical confirmation of exposure concentrations were explored. Thus, a large number of regressed relationships are possible, and only selected ones are presented here. A future manuscript will discuss statistical properties of the relationships in greater detail.

Tab. 2.4: Summary of several representative fish embryo toxicity (FET) *versus* acute fish toxicity relationships.

FET			Acute Fish Toxicity								95%	95%
Spec-ies	Me-thod	Dur-ation	Spec-ies	Me-thod	Dur-ation	Analy-tical	Chem-icals	Inter-cept	Slop e	R^2	LCL	UCL
All	All	All	All	All	All	Any	77	-0.36	1.07	0.90	0.95	1.20
All	All	All	All	All	96-hr	Any	73	-0.42	1.07	0.90	0.95	1.20
Danio	All	All	All	All	96-hr	Any	70	-0.47	1.09	0.87	0.95	1.30
Danio	All	All	*Danio*	All	96-hr	Any	21	-0.62	1.12	0.81	0.79	1.60

95 % LCL and UCL – 95 % lower and upper confidence limits

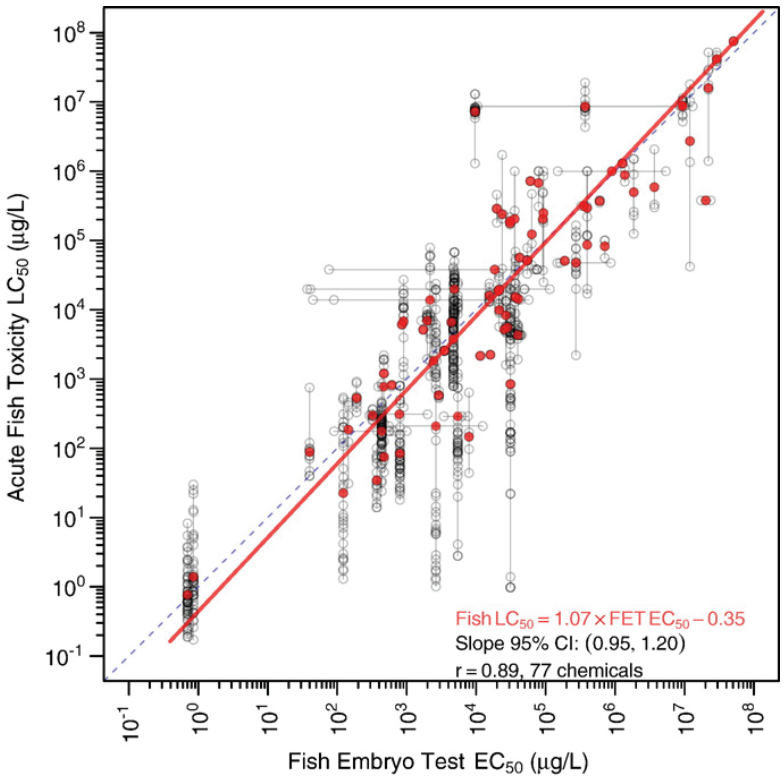

Fig. 2.5: Comparison of all available fish embryo toxicity and acute fish toxicity (independent of exposure duration) for 77 chemicals. Confidence intervals refer to the slope of the regression. Geometric mean values are indicated by the solid circles, and the ranges (x or y direction) are indicated by the bars. The line of 1:1 correspondence is given dashed gray. Data for acute fish toxicity were taken from the US EPA ECOTOX (US EPA 2002) or the ECETOC Aquatic Toxicity (Solbe et al. 1998) databases.

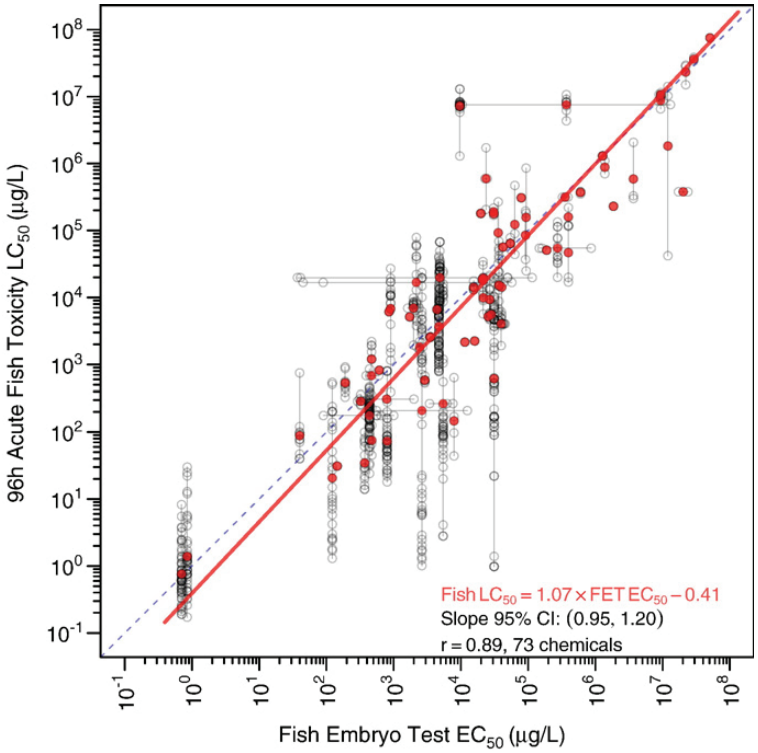

Fig. 2.6: Comparison of all FET values to acute fish toxicity (all species) in 96 h exposures for 73 chemicals. Geometric mean values are indicated by the solid circles, and the ranges (x or y direction) are indicated by the bars. The line of 1:1 correspondence is given dashed gray. Data for acute fish toxicity were taken from the US EPA ECOTOX (US EPA 2002) or the ECETOC Aquatic Toxicity (Solbe et al. 1998) databases.

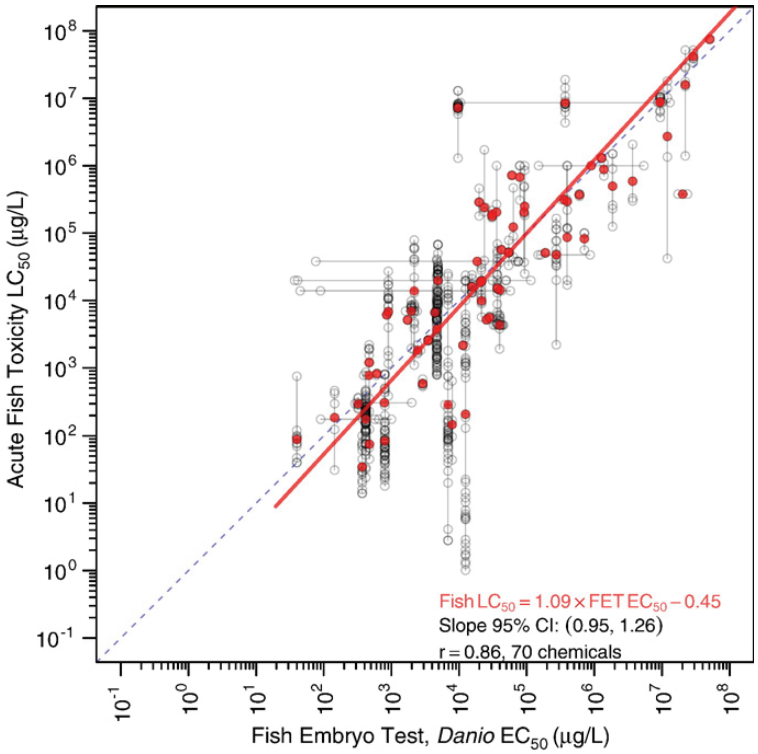

Fig. 2.7: Comparison of *Danio rerio* embryo toxicity to acute fish toxicity (all species) for 70 chemicals. Geometric mean values are indicated by the solid circles, and the ranges (x or y direction) are indicated by the bars. The line of 1:1 correspondence is given dashed gray. Data for acute fish toxicity were taken from the US EPA ECOTOX (US EPA 2002) or the ECETOC Aquatic Toxicity (Solbe et al. 1998) databases.

All fish embryo and eleutheroembryo toxicity data and all available acute fish toxicity data were compared on a total of 77 chemicals provided a correlation of 0.90 with a slope of 1.07 (Fig. 2.5, Tab. 2.4). The 95 % confidence intervals for the slope include the value of 1 and the regression is extremely close to the 1:1 correspondence line. When the data set is constrained to only 96 h acute fish toxicity data (n = 73 chemicals), the relationships are nearly identical indicating that the inclusion of additional time points (when unique) are not

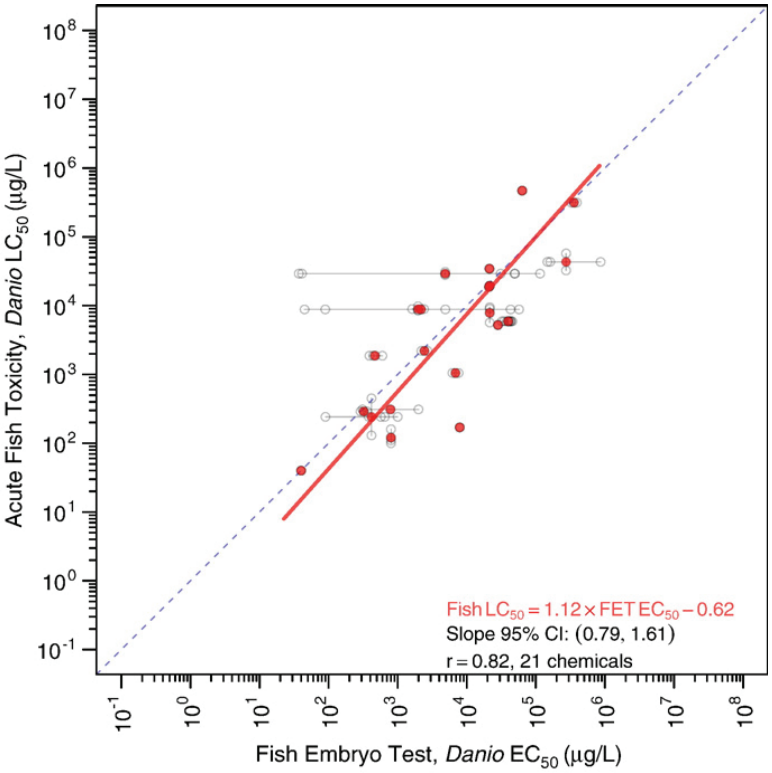

Fig. 2.8: Relationship between *Danio rerio* FET and *Danio rerio* fish toxicity (n = 21 chemicals). Geometric mean values are indicated by the solid circles, and the ranges (x or y direction) are indicated by the bars. The line of 1:1 correspondence is given dashed gray. Data for acute fish toxicity were taken from the US EPA ECOTOX (US EPA 2002) or the ECETOC Aquatic Toxicity (Solbe et al. 1998) databases.

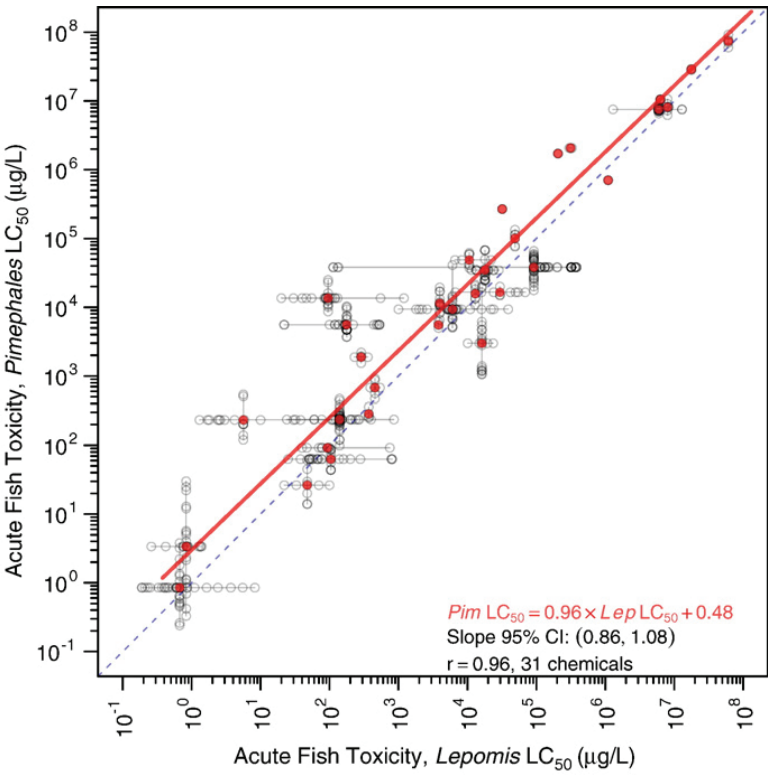

Fig. 2.9: Example of acute fish toxicity relationships. In this example, *Lepomis macrochirus* is compared to *Pimephales promelas* (n = 31 chemicals). Geometric mean values are indicated by the solid circles, and the ranges (x or y direction) are indicated by the bars. The line of 1:1 correspondence is given dashed gray. Data for acute fish toxicity were taken from the US EPA ECOTOX (US EPA 2002) or the ECETOC Aquatic Toxicity (Solbe et al. 1998) databases.

terribly influential (Fig. 2.6, Tab. 2.4). *Danio rerio* data comprise the bulk of the fish embryo and eleutheroembryo toxicity information (n = 70), and when these are compared to all acute fish toxicity (all species) resulted in a correlation of 0.87 and a regression slope of 1.09 (still with the slope of 1 bounded by the 95 % confidence interval; Fig. 2.7, Tab. 2.5). When both FET and acute fish toxicity are constrained to *Danio rerio* (n = 21), the regression still remains strong with a correlation of 0.81 and slope of 1.12. The confidence intervals widen

as the number of chemicals and tests covered are fewer than those discussed above (Fig. 2.8).

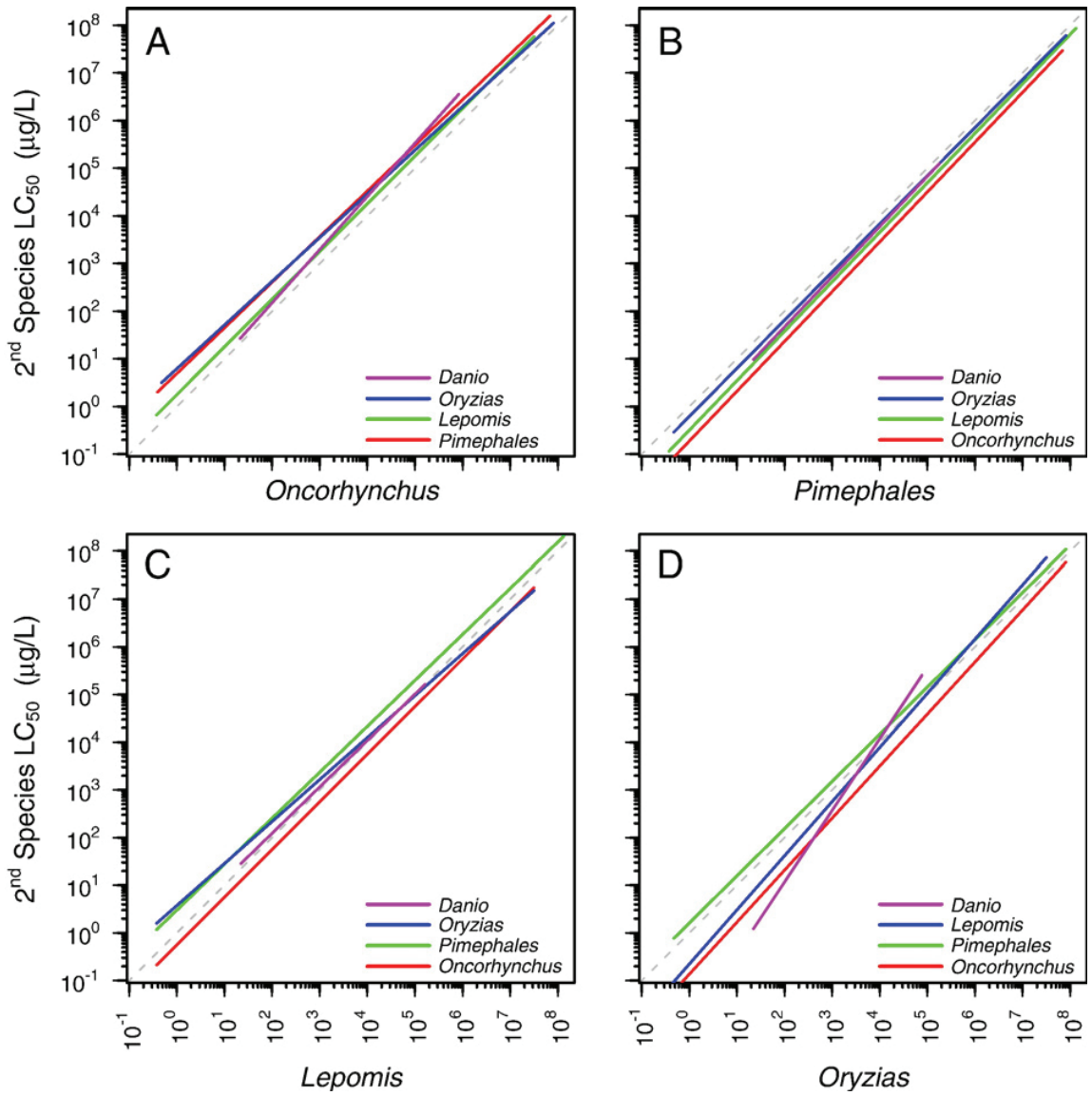

Fig. 2.10: Regression results for the 5 OECD fish acute toxicity test species used in the evaluation of fish embryo data. Data for acute fish toxicity were taken from the US EPA ECOTOX (US EPA 2002) or the ECETOC Aquatic Toxicity (Solbe et al. 1998) databases.

Acute fish toxicity data for all 5 species were compared to provide perspective on FET *versus* acute fish relationships. Because these studies are performed by well-accepted guidelines and any one of the species would provide data accepted for regulatory uses, these inter-species relationships could be considered to display the range of acceptability for fish embryo-acute toxicity. Fig. 2.9 provides details for bluegill *versus* fathead minnow as an example. The correlation, slope, and relative position to the 1:1 correspondence line are similar to regressions described earlier (cf. Tab. 2.5). All possible relationships for the species considered in this paper are given in Fig. 2.10. Inter-species correlations range from 0.719 to

0.946 (Tab. 2.5). Species pairs with greater numbers of chemical pair data have higher correlations.

Tab. 2.5: Correlations (R2) of acute fish toxicity values for 5 OECD test species.

	P. promelas	*L. macrochirus*	*O. latipes*	*D. rerio*
O. mykiss	0.946 (32)	0.944 (26)	0.960 (27)	0.866 (18)
P. promelas		0.956 (31)	0.942 (28)	0.825 (18)
L. macrochirus			0.899 (19)	0.915 (14)
O. latipes				0.719 (12)

All data were included in this analysis (all types of exposure [static, static-renewal, flow-through], with and without analytical confirmation). All correlations are statistically significant (number of studies in brackets).

2.5 Discussion

Ecotoxicity tests with fish have long been a subject of continual re-assessment with respect to species choice, exposure duration, exposure designs and the like (Norberg & Mount 1985; Sprague 1970; Woltering 1984). The present paper continues this evaluation by evaluating the suitability of fish embryos to quantitatively reflect toxicity to later life stages of fish. “Embryo” is the life interval that encompasses the period from fertilization to form a zygote to the time that the egg hatches. Further development of the embryo occurs outside the egg envelope during the interval known as the eleutheroembryo or free embryo (Balon 1975). In this interval, the embryo is free of the egg envelope, but dependent upon the yolk for food and is not feeding exogenously. Therefore, the first comparison of interest is whether embryos and eleutheroembryos are differentially sensitive to chemicals. The relationship between embryos and eleutheroembryos is very strong with a regression slope near 1 and R^2 of 0.96 (cf. Fig. 2.4). A small number of chemicals do appear to be less well predicted. Inspection of the data set suggests that eleutheroembryos may be more sensitive to selected polymers and higher molecular weight non-ionic surfactants. This intuitively makes sense as these compounds may not traverse the chorion as readily as compounds of smaller size dimensions. Overall, the strength of the relationship shows that few compounds have toxicities that differ by more than a factor of 5 to 10, and these life intervals can be considered unless additional information is available to address observed differences. More testing of an even greater diversity of chemicals may help in this regard.

Embryonic life stages are also highly predictive of later life stages, which are covered under the OECD 203 Test Guideline for acute fish toxicity (cf. Figs. 2.5 - 2.8). The relationships are so strong that all the regressions explored here are virtually equivalent including completely uncensored all fish embryo *versus* all available fish toxicity (slope of 1.07), all fish embryo *versus* all 96 h acute fish toxicity (slope of 1.07), *Danio* fish embryo only *versus* all available fish acute toxicity (slope of 1.09) and the most censored only Danio fish embryo *versus* only *Danio* fish acute toxicity (slope of 1.12). Slight deviations from a slope of 1.0 might raise concern that one or the other test would be over- or underpredictive at the extremes. Fish *versus* fish acute toxicity test comparisons provide a benchmark of previous acceptability of this relationship for scientific and regulatory purposes. Fig. 2.9 provides a detailed comparison of a representative regression of fathead minnow *versus* bluegill sunfish with a similar slope and R^2, if compared to those of fish embryo *versus* fish acute toxicity regressions. The idea that the "standard" fish species provide equivalent types of information for hazard assessment, classification and labeling and for risk assessment is well established. Recently Dyer et al. (2006, 2008) provided detailed analyses of inter-species correlation estimations (US EPA ICE, also at http://www.epa.gov/ceampubl/fchain/webice/index.htm) and regressions for use in hazard assessment. Much of the rainbow trout, fathead minnow and bluegill data used in the ICE program overlap with information used in this paper. It is not surprising that correlation and regression results between this paper and ICE are highly similar for the following species pairs (given as this paper, Tab. 2.5, and ICE, respectively): rainbow trout *versus* fathead minnow (0.946, 0.895), rainbow trout *versus* bluegill (0.95, 0.96), and bluegill *versus* fathead minnow (0.96, 0.96). This analysis supports the overall expectation for acceptable predictability between different fish species as well as between fish and fish embryo toxicity.

The establishment of a quantitative fish embryo toxicity (FET) *versus* acute fish toxicity relationship is necessary purely on a scientific basis in order to give justification for use of fish embryo toxicity testing in a regulatory context. On the basis of the data presented in this review, fish embryo toxicity testing can clearly provide equivalent data for priority setting, hazard screening, and may be used as the fish "input" into early tier risk assessment schemes. Of course, the use of fish embryos has other advantages: Testing with fish embryos is an advance in animal alternatives in the spirit of the 3 Rs of Russell and Burch (1959) - Replace, Reduce, and Refine. Additional tools are in development and deployment to assist the animal alternative needs in environmental toxicity testing such as the threshold approach (Hutchinson et al. 2003; OECD draft TG). Technical advantages of fish embryo testing are also evident including the need for small amounts of test substances, shorter time periods of exposure, and the need for only breeding stock and not a rear-out phase to

produce testable fish. These technical advantages will soon translate into reduced testing costs, which will encourage the generation of data more frequently than in the past. Lastly, sublethal endpoints can be easily implemented in this testing framework which may translate into understanding prospects for chronic responses, teratogenicity, or other indications of effect (Braunbeck et al. 2005; Nagel 2002). The use of fish embryos in wastewater effluent acute testing is already established (DIN 2001; ISO 2007) and complements the potential use for chemicals described here.

2.6 Conclusion

Fish embryo testing of chemicals has matured to the point that international standardization, method validation, and broadening of chemical coverage is rapidly occurring. This paper confirms that fish embryo tests are neither better nor worse than acute fish toxicity tests and offer a reasonable alternative to increased use of fish in the future. Still, research is needed to provide the technical basis for other species in addition to zebrafish embryos to be used in this testing scheme. Fathead minnow, medaka, and rainbow trout embryo research in particular may provide important new insights. However, the strength of existing acute fish interspecies relationships (OECD 203 type tests) would suggest the inter-embryo differences should not be very great.

Chapter 3

Development of a flow-through system for the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*)

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3.1 Abstract

The acute fish test is still a mandatory component in chemical hazard and risk assessment. However, one of the objectives of the new European chemical policy (REACH - Registration, Evaluation, Authorization and Restriction of Chemicals) is to promote non-animal testing. For whole effluent testing in Germany, the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) has been an accepted and mandatory replacement for the fish test since January 2005. For chemical testing, however, further optimization of the FET is required to improve the correlation between the acute fish test and the alternative FET. Since adsorption of the test chemical to surfaces may reduce available exposure concentrations, a flow-through system for the FET using modified commercially available polystyrene 24-well microtiter plates was developed, thus combining the advantages of the standard FET with those of continuous delivery of test substances. The advantages of the design presented include: small test footprint, availability of adequate volumes of test solution for subsequent chemical analysis, and sufficient flow to compensate for effects of non-specific adsorption within 24 h. The flow-through test system can also be utilized to conduct longer-term embryo larval fish tests, thus offering the possibility for teratogenicity testing.

3.2 Introduction

Currently, a set of ecotoxicological tests is required for hazard and risk assessment of chemicals as well as for the registration of new chemicals to obtain information about their toxic potential. Beside the acute toxicity test with daphnids (OECD TG 202; OECD 2004b) and the growth inhibition test with freshwater algae (OECD TG 201; OECD 2006), the acute fish test (OECD TG 203; OECD 1992a) is still a mandatory component in chemical toxicity assessment. However, within the framework of the new European chemicals policy (REACH), testing with vertebrate animals should - whenever possible - be reduced or even replaced by alternative methods (according to the concept of 3Rs by Russell & Burch 1959; EC 2001). In fact, the acute fish test as an animal test with mortality as the exclusive endpoint is not compatible with most current animal welfare legislation in Europe: An increasing number of researchers have suggested that some form of pain perception similar to what is present in mammals may be present in bony fish (teleosts), and adult fish exposed to acute toxic concentrations of chemicals may at least be suspected to suffer severe distress and pain (Braunbeck et al. 2005; Chandroo et al. 2004; Huntingford et al. 2006; Nagel 2002; Sneddon et al. 2003). According to Huntingford et al. (2006), the longer the life span of a given animal species and the more sophisticated its general behavior, the greater is its need for complex mental processes similar to those which generate the conscious experience of suffering in

humans. *Vice versa*, it may be concluded that embryos, due to their shorter life span, may have not yet developed the same complex mental processes and may have not pain perception identical to adult fish. As a consequence, with respect to animal welfare, the replacement of adult fish by embryonic stages may be regarded compatible with the 3R principles (Russel & Burch 1959), and tests with fish embryos should be preferred to tests with adult fish.

For whole effluent testing in Germany, the acute fish test has already been replaced by the fish embryo toxicity test (DIN 2001), which has become mandatory since January 2005. Based on the protocol for whole effluent testing, a proposal for a new guideline on fish embryo toxicity for chemical testing has been submitted to the OECD by the German Federal Environment Agency in 2006 (Braunbeck & Lammer 2006).

There are several arguments to also incorporate the fish embryo toxicity test (FET) into routine chemical toxicity assessment: (1) In legal terms, the FET is usually classified as a non-animal test. In fact, at present, only the UK Animals Procedures Act (UK 1986) explicitly covers fish as laboratory animals, and even in the UK protection of immature stages of fish only starts with the onset of independent feeding. (2) As mentioned above, embryos may not yet have developed pain perception comparable to adults. (3) The test only requires very low volumes of test solution, which is particularly important, when only limited amounts of test substance are available. (4) In its present version, the test duration of the embryo test is limited to two or three days, thus it is less time-consuming than the conventional *in vivo* acute fish test. (5) A variety of sublethal endpoints can easily be incorporated into the test protocol, thus providing an option for a teratogenicity test.

One of the most critical requirements for a test to be accepted as an alternative to a conventional test is the correlation between the alternative procedure and the test to be replaced (i.e. the predictivity of the alternative method). Although the correlation between the acute fish test and the FET is very good (Lammer et al. 2009a), there is a number of substances for which the embryo test is significantly more or less sensitive than the conventional test (Braunbeck et al. 2005). Several reasons might be speculated to account for such outliers: (1) differences in uptake, accumulation and metabolization of the test compounds between embryonic and adult stages, (2) protection of the embryo by the chorion, and (3) restricted availability of the test substances due to the static nature of the standard FET. In order to address the latter point, adaptations to the standard FET to allow testing under flow-through conditions were investigated.

Whereas the standard FET is conducted under static conditions in polystyrene 24-well microtiter plates, flow-through conditions and glass aquaria are preferred for the acute fish

toxicity test. Static acute tests represent the simplest way to assess toxicity; however, potentially inadequate availability of the test compounds due to adsorption, degradation or metabolization, accumulation of undesired metabolites, shortage of oxygen or uncontrolled changes in pH and other test conditions may severely restrict the suitability of static exposure. As a consequence, flow-through acute tests are particularly preferable especially for toxicants which have a high oxygen demand, are highly volatile and/or unstable in aqueous solution, or which are constantly removed in significant quantities by test organisms during exposure (Becker & Crass 1982). Thus, in particular for the testing of lipophilic substances, flow-through systems are usually considered ideal.

Since the FET is carried out in polystyrene 24-well microtiter plates, there is particular concern about adsorption. Both charged and lipophilic chemicals have been shown to bind to polystyrene surfaces through ionic and hydrophobic interactions (Palmgren et al. 2006; Dahlstöm et al. 2004). However, by using a flow-through system, the limited non-specific binding sites on polystyrene can usually be saturated within reasonable periods of time (Palmgren et al. 2006).

Another problem with the static FET can be the very small amount of test substance and media used during exposure, which make chemical analyses extremely difficult. By implementing a flow-through system, larger volumes of test media can be made available for confirmatory chemical analysis, providing greater security for the interpretation of toxicity results.

Thus, in order to further optimize the static FET, we developed a flow-through test system utilizing modified commercially available polystyrene 24-well microtiter plates. In order to investigate the applicability of the flow-through FET in chemical testing, 4-chlorophenol was tested as a model compound, since the results of the conventional static FET and the acute fish toxicity test for this compound vary by a factor of 10.

3.3 Material and Methods

3.3.1 Chemicals and materials

The epoxy resin components for Spurr's medium (Spurr 1969; embedding medium ERL-4221D, D.E.R. 736, nonenylsuccinic anhydride, dimethylaminoethanol) were obtained from Serva (Heidelberg, Germany). The polystyrene 24-well microtiter plates were provided by Renner (TTP; Dannstadt, Germany) and the 250 µm gauze was obtained from Verseidag (Geldern, Germany). Patent blue, the positive control 3,4-dichloroaniline as well as 4-chlorophenol were purchased in analytical quality from Sigma-Aldrich (Deisenhofen,

Germany). 4-Chlorophenol (CAS 106-48-9; MW 128.6 g/mol; log P_{ow} 2.39) was tested at concentrations: 4, 10, 25 and 62.5 mg/L.

The artificial water corresponded to reconstituted water according to ISO 7346/3 (ISO 1996; conductivity: 700 µS/L, NO_3^-: < 4.5 µg/L, NO_2^-: < 8 µg/L, NH_4^+: < 1.9 µg/L, PO_4^{3-}: < 50 µg/L, Fe^{2+} and Fe^{3+} not detectable; chemical oxygen demand (COD): < 4 mg/l), which was diluted 1:5 using double-distilled water. Before use the pH is adjusted to 7.8 ± 0.2.

3.3.2 Test system and experimental design of the flow-through

In order to achieve flow-through conditions, the 24-well microtiter plates were modified as follows: The rear space between the wells was completely filled with Spurr's resin (Spurr 1969). After polymerization at 70 °C for 24 h, holes were drilled in longitudinal direction across the entire plate to obtain four parallel flow channels consisting of six consecutive wells (Fig. 3.1 a). To identify the optimal drilling size for the ideal distribution within the flow-through channels, plates with 5 and 6 mm holes were drilled and tested. In order to prevent hatched embryos from swimming across the openings between the wells under conditions of prolonged test duration, 250 µm gauze moistened with double-distilled water was placed in each well (Fig. 3.1 b). For the flow-through FET the plates can also be sealed with self-adhesive foil (Nunc, Roskilde, Denmark) to minimize the loss of volatile substances.

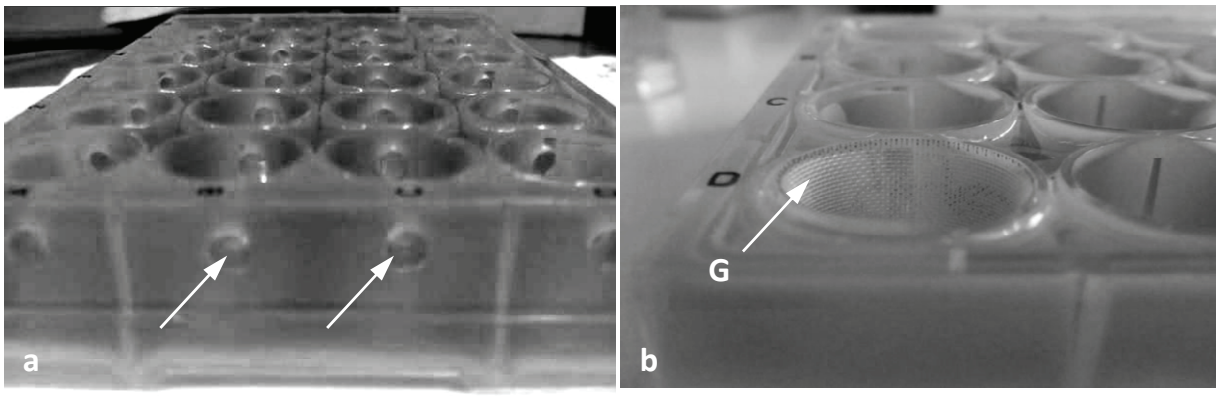

Fig. 3.1: a. Drilled polystyrene 24-well microtiter plate with flow channels (arrow). b. Gauze (G) inside in the first well to prevent hatched embryo from passing from one well to the next one.

Plates were placed in an incubator to maintain constant temperature conditions (26 ± 1 °C). Peristaltic pumps (Minipuls 3; Gilson, Limburg-Offheim, Germany) were used to ensure constant flow conditions. The outflow of the test solutions was collected inside the incubator (Fig. 3.2).

Fig. 3.2: Inside an incubator (26 ± 1 °C), the flow-through system is set up in three parallels with 24-well microtiter plates (top shelf), and, after the passage, the test solutions are collected in 500 ml glass bottles. All tubing consists of saturated Teflon materials.

3.3.3 Evaluation of the flow-through system

In order to visualize the flow behavior of solutions across the flow-through plates, commercially available blue ink (Prussian blue, Lamy, Heidelberg, Germany) was used at elevated flow rates of 20x water exchange per day. Prior to addition of the ink, double-distilled water was pumped across the plates for at least 12 h in order to soak all material. Then, 25 µl ink were added to selected wells to analyze the distribution of the ink inside the wells and along the flow-through channels.

In order to analyze whether the distribution pattern was also constant under normal flow-through conditions of a 5x water exchange per day, patent blue was used, since normal ink had proved non suitable for this type of analysis due to its higher density compared to distilled water. As in the ink experiment, double-distilled water was pumped across the flow-through plates for 12 h. Subsequently, the water flow was replaced by a constant flow of an aqueous 25 mg/L patent blue solution. In order to analyze if the water in the wells of the flow-through plate was homogenously replaced by the patent blue solution with a 5x water exchange per day, the wells were separately mixed at constant intervals to avoid single streams of patent blue solution inside the wells, and 100 µl per well of the flow-through

plates were removed so that the flow itself was not influenced significantly by an excessive change of the volume inside the wells. Patent blue concentrations inside the wells were determined photometrically by measurement of the absorption at 620 nm in a microtiter plate reader (GENios, Tecan, Crailsheim, Germany).

3.3.4 Fish maintenance and egg production

3.3.4.1 Fish maintenance

Brood stock of zebrafish aged between 6 and 24 months is used for egg production. Spawners were free of externally visible diseases and did not receive any pharmaceutical (acute or prophylactic) treatment for 6 months before spawning. Females and males were kept together in glass aquaria providing sufficient space for swimming (i.e. ≥ 1 L per fish). Standardized dilution water as specified in ISO 7346-1 and ISO 7346-2 (294.0 mg/L $CaCl_2 \cdot 2\ H_2O$; 123.3 mg/L $MgSO_4 \cdot 7\ H_2O$; 63.0 mg/L $NaHCO_3$; 5.5 mg/L KCl) or suitable drinking water with ≥ 80 % oxygen saturation was used for housing and breeding. Temperature was maintained at 26 ± 1 °C, and fish were kept under a constant artificial dark–light cycle of 16 and 8 hrs, respectively. Constant filtering or permanent flow-through conditions guaranteed that ammonia, nitrite, and nitrate were kept below detection limits. Fish were fed commercially available artificial diets (e.g. TetraMin™ flakes; Tetra, Melle, Germany) twice daily, occasionally supplemented with *Artemia nauplii* or small daphnids of appropriate size obtained from an uncontaminated source. Overfeeding was strictly avoided to ensure optimal water quality; remaining food and feces was removed daily.

3.3.4.2 Egg production

Under spawning conditions, male zebrafish can easily be distinguished from females by their more slender body shape and an orange to reddish tint in the silvery bands along the body. Due to the large number of eggs produced, females can be recognized by their swollen bellies. The day before a test, males and females in a ratio of 2:1 were placed in breeding chambers (water conditions as above) immediately before the onset of darkness. Artificial plants serve as breeding stimulant and substrate. Mating, spawning and fertilization take place within 30 min after the onset of light in the morning.

For collection of eggs, the bottom of the 3 L breeding tanks had been replaced by a 1.25 mm stainless steel grid in order to prevent predation of eggs. The breeding tanks were placed on rectangular full-glass dishes of similar dimensions. As a spawning stimulus, artificial plants made of green plastic were fixed to the grid covering the spawning dishes. About 30 - 60

minutes after spawning, the spawning dishes were removed, and the eggs were transferred to a temperature-controlled dissecting microscope. After determination of the overall egg number, viable (i.e. fertilized) eggs were selected for testing.

3.3.5 Static fish embryo toxicity test (FET) with 4-chlorophenol, epoxy resin and gauze

The static FET was conducted according to DIN 38415-T6 and ISO 15088 (DIN 2001; ISO 2007). A more detailed description can be found in Lammer et al. (2009a). In brief, embryo tests were initiated at latest after 3 h (~ 128 cell stage). In order to start exposure to 4-chlorophenol with minimum delay, at least 20 freshly spawned zebrafish eggs were selected per concentration and transferred into 60 mm crystallization dishes containing 100 ml of the different test concentrations, the positive control or the negative control, respectively. Subsequently, 10 fertilized eggs were selected for each test concentration and transferred to 24-well plates filled with 2 ml freshly prepared test solutions and controls or dilution water per well (for plate layout, see Lammer et al. 2009). For pre-saturation, 24-well plates were pre-treated with the respective concentrations 24 h prior to the exposure of the embryos. The 24-well plates were then covered with self-adhesive foil and incubated at 26 °C ± 1 °C.

In order not to miss any delayed effects, exposure was extended for 4-chlorophenol up to 120 h or even 144 h, respectively. The embryos were examined after 24, 48, 72 and 120 (144) h, respectively, and coagulation, non-detachment of the tail, non-formation of somites and non-detection of the heartbeat were recorded as the four key lethal endpoints. In general, the LC_{50} was determined graphically, by linear interpolation.

Artificial water was used as a negative control and 3,4-dichloroaniline (3.7 mg/L) served as a positive control. Embryo tests were classified as valid, if the mortality in the negative control was less than 10 % and the positive control gave a mortality of more than 10 %. For more details on the selection of the positive control and the identification of validity criteria, see Lammer et al. (2009a).

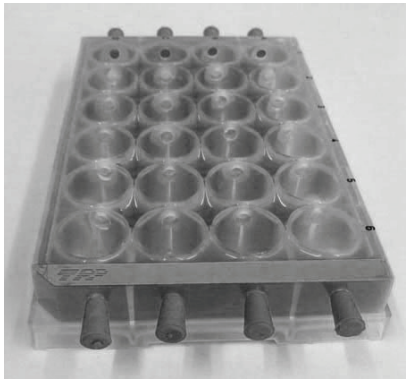

Fig. 3.3: Flow-through 24-well microtiter plate with ends sealed by rubber plugs.

In order to exclude potential toxicity of the epoxy resin and the gauze to zebrafish embryos, these components were also tested following the principles of DIN 38415-T6 and ISO 15088: The hardened epoxy resin was tested in direct contact with the embryos. For this end, 200 and 500 µl of the final epoxy resin mixture were filled into the wells of a 24-well microtiter plate. After

polymerization at 70 °C for 24 h, 2 ml of artificial water were filled into each well. In addition, a resin-prepared flow-through plate was sealed with rubber plugs (Fig. 3.3), and the wells were also filled with 2 ml artificial water to check the actual exposure scenario. In order to test potential embryo-toxic effects of the gauze, one stripe of gauze (5.3 cm length; 1.7 cm width) was placed into each well of a 24-well microtiter plate. The wells were filled with 2 ml artificial water, and one fertilized egg was placed in each well. Exposure duration and endpoint inspection were identical to the tests with 4-chlorophenol.

3.3.6 Flow-through fish embryo toxicity test (FET) with 4-chlorophenol

In order to investigate the applicability of the flow-through system for chemical testing, 4-chlorophenol was tested in the flow-through system according to 2.2. In order to saturate the flow-through system, the flow of the test solution was started 5 days prior to the exposure of the fish embryos.

For the flow-through fish embryo toxicity test, 20 freshly spawned zebrafish eggs were selected per concentration and transferred into 60 mm crystallization dishes containing 100 ml of the different test concentrations, the positive control solution or the dilution water (negative control), respectively. Since one flow-through plate was used for two concentrations, 12 fertilized eggs per concentration and controls were placed in the flow-through plates five days after pre-saturation of the flow-through system. The same exposure and examination scheme like in the static test was used. LC_{50} values were also determined graphically.

3.4 Results

3.4.1 Fish embryo toxicity tests (FET) with epoxy resin and gauze

Provided complete polymerization of the resin, no significant mortality could be observed in the tests with Spurr's epoxy resin and the gauze: In the resin-prepared flow-through plate, no mortality could be found after 24, 48 and 120 h. On the other hand, even in tests with plates filled with 200 and 500 µl of the non-polymerized epoxy resin mixture, less than 10 % mortality was observed after 24, 48 and 120 h. Likewise, in the negative control, mortality was less than 10 %. Mortalities in the positive controls (3.7 mg/L 3,4-dichloroaniline) were over 40 % after 24, 48 and 120 h, respectively. Hence, according to DIN 38415-T6 and ISO 15088, the tests were classified as valid. Since no significant mortalities were observed in the tests with epoxy resin and gauze, this test design had proved suitable for further fish embryo toxicity flow-through tests.

3.4.2 Analysis of the flow-through across the flow-through system

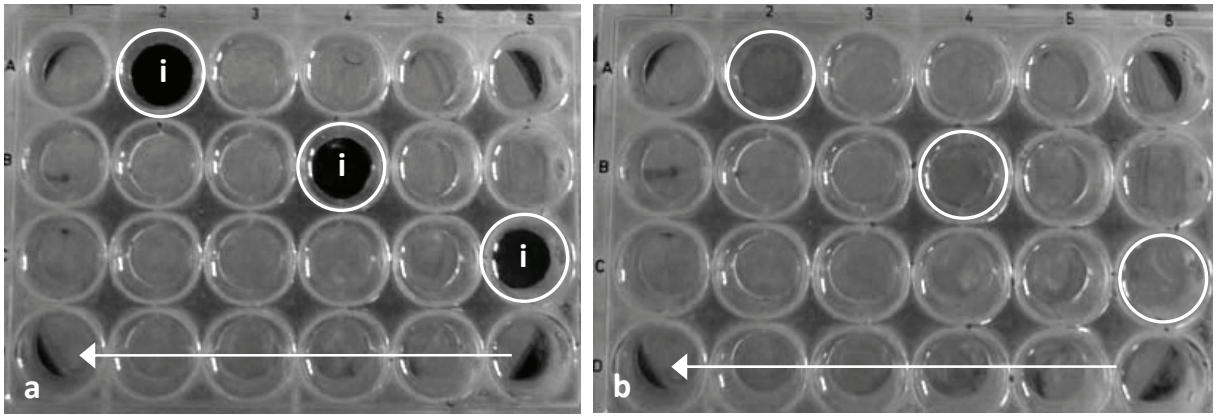

Fig. 3.4: Testing the transfer of solutions along the channels of the flow-through system by the use of ink. a. Start of test: Ink (i) inside the wells A2, B4 and C5. b. End of the test: The ink has been transported completely along the flow-through channels and is not visible in the wells anymore. Arrows indicate the direction of flow-through.

Fig. 3.4 illustrates the transfer and transport of Prussian blue ink within the flow-channels along consecutive wells. Fig. 3.4 a shows the modified flow-through well plate at the beginning of the test with ink (i) inside three selected wells (A2, B4 and C5). Fig. 3.4 b shows the plate after 26 h with a 20x water exchange per day. In all wells, the ink was evenly distributed and transported along the wells of the microtiter plate independently of the original position of the ink, thus documenting continuous flow-through along the entire flow-channels.

Due to the high density of ink compared to water, a different approach was required to analyze the flow-through system with lower flow rates (5x water exchange per day). Patent blue was used to check the system and to identify the optimal drilling size for a sufficiently rapid and constant flow-through of the solutions.

In Fig. 3.5, the patent blue concentrations of the single wells were plotted against time over 24 h with 5 and 6 mm drilling holes (black and white bars, respectively). Plates with either drilling sizes allowed a continuous flow-through, i.e. sufficient exchange of patent blue over 24 h with a 5x water exchange per day. However, the plates drilled with 6 mm holes proved more effective than those prepared with 5 mm hole size, since with the 6 mm drilling exchange was almost completed after 7 h, whereas the plates with 5 mm drilling size took approx. 24 h for a complete replacement by patent blue solution. The replacement of double-distilled water by the patent blue solution with 6 mm drilling holes was thus more than three times as fast than with 5 mm drilling holes. As a consequence, for conducting the FET under flow-through conditions, plates with 6 mm drilling holes should be preferred to ensure optimal saturation of the sample solutions.

Since the gauze did not modify the continuous flow, the system can also be utilized for the extended version of the fish embryo toxicity test (i.e. extending to beyond hatch).

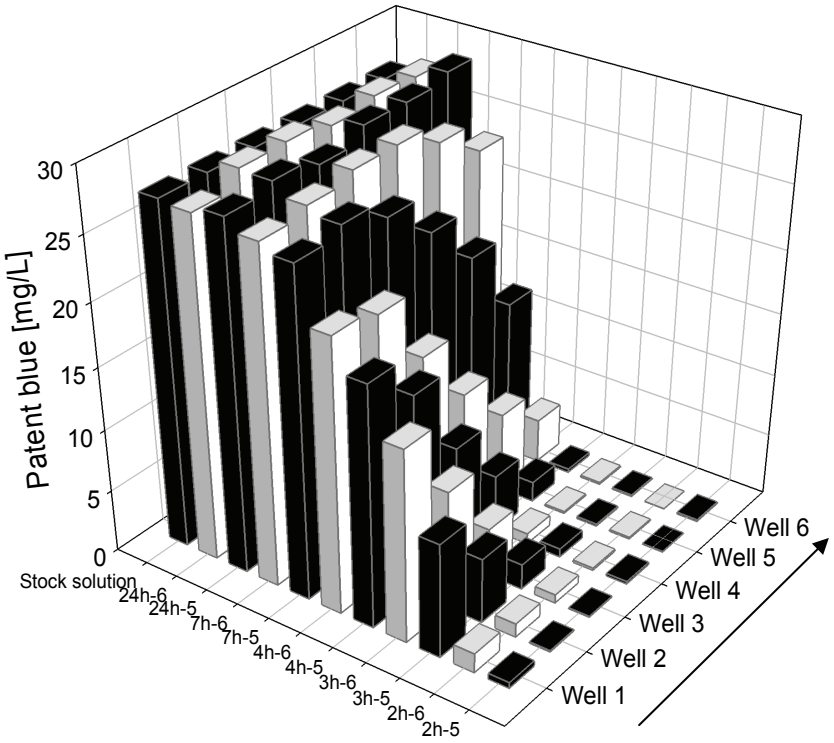

Fig. 3.5: Replacement of water by patent blue across the flow channels over a period of 24 h. ■ = 5 mm flow-through holes; □ = 6 mm flow-through holes. Arrow indicates direction of flow-through across the wells of the microtiter plate.

3.4.3 Static and flow-through fish embryo toxicity test (FET) with 4-chlorophenol

Fig. 3.6 shows the results of the static and the flow-through FET with 4-chlorophenol. The LC_{50} for the static FET could be determined at 45 mg/L after 24, 48, 72 and 120 h and at 40.7 after 144 h. The flow-through FET resulted in 42.8 mg/L after 24, 48 and 72 h and 40.4 mg/L after 120 and 144 h. Only lethal endpoints were recorded, since the test was restricted to the standard protocol of the FET (Lammer et al. 2009a) to check the applicability of the flow-through system for routine chemical testing.

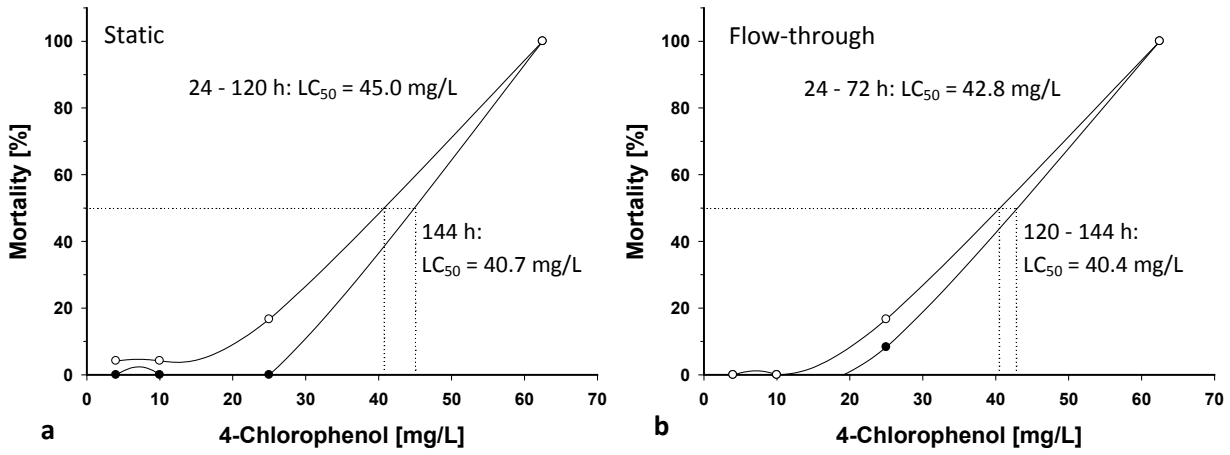

Fig. 3.6: Acute toxicity of 4-chlorophenol to zebrafish embryos in the static (a) and the flow-through (b) fish embryo toxicity test after 24 to 120 h.

3.5 Discussion

The flow-through version of the FET provides several advantages over the static system e.g. compensation of adsorption of the test substances to the plastic material of the microtiter plates, constant renewal of the test substance, prevention of oxygen deficiency, and the possibility for chemical analysis due to the higher substance volumes. Becker & Crass (1982) reported that, for acute tests, flow-through conditions should clearly be preferred especially for toxicants that have a high oxygen demand, are highly volatile, are unstable in aqueous solution, or are removed by metabolic activities of test organisms during exposure in significant quantities. Likewise, Birge et al. (1979) noted that flow-through test procedures provided more adequate regulation of toxicant exposure, giving good stabilization of concentrations even for moderately volatile and hydrophobic organic substances. In particular for the investigation of lipophilic substances, flow-through systems provide better results compared with the static system. However, it should be noted that only very few investigations have been conducted into direct comparisons between static and flow-through tests.

According to the current ISO protocol for the testing of whole effluents (ISO 2007) and the proposal for an OECD protocol for the testing of chemical substances (Braunbeck & Lammer 2006), the static version of the FET is conducted in 24-well microtiter plates made of polystyrene. Polystyrene is an amorphous, clear, brittle plastic, and its surface is highly hydrophobic (Koutsopoulos et al. 2007; Palmgren et al. 2006). There are several studies (Dahlström et al. 2004; Knorr & Gätzschmann 1966; Koutsopoulos et al. 2007; Palmgren et al. 2006) indicating, that lipophilic and positively charged drugs and chemicals may show strong interactions with negatively charged polystyrene and, therefore, tend to adsorb to

the surface of the polystyrene plates *via* electrostatic binding and hydrophobic interactions (Dahlström et al. 2004; Palmgren et al. 2006). As a consequence, due to adsorption in a static test, it is difficult to keep exposure concentrations constant. Hence, the embryo might be exposed to the incorrect test concentrations. In contrast, with a flow-through system, the limited electrostatic binding sites of the polystyrene plates can be rapidly saturated, and constant concentrations can be more easily maintained. Thus, flow-through conditions should provide more constant exposure concentrations during the entire test period.

A further major advantage of the flow-though system is that oxygen deficiency will not occur. Becker and Crass (1982) investigated different static and flow-through procedures for the determination of acute toxicity with the fathead minnow (*Pimephales promelas*) and water-soluble fractions of a liquid synfuel blend derived from coal. They showed unacceptable declines of dissolved oxygen levels in both static and static-renewal tests. In contrast, in the flow-through system, acceptable dissolved oxygen could be maintained. As a conclusion, Becker & Crass (1982) suggested that acute toxicity tests of complex synfuel blend originating from coal involving fish and water-soluble fractions should be conducted under flow-through conditions.

In general, Bang et al. (2004) found increasing oxygen consumption for zebrafish eggs from 4.54 to 8.34 nmol O_2/h in the period from 24 to 48 h after fertilization. Thereafter, the oxygen consumption rate remained more or less constant until hatching. The cumulated oxygen consumption for 24 to 75 h post fertilization ranges between 0.261 and 0.462 µmol O_2 per individual. Since the oxygen consumption of embryos, especially if they are still inside the chorion, takes place *via* passive processes depending on the oxygen gradient (Kranenbarg et al. 2003), adequate oxygen concentrations in the surrounding medium are essential for appropriate testing conditions. A flow-through system guarantees constant oxygen concentrations, whereas in static systems oxygen gradients may occur.

An additional problem in the static version of the FET is that chemical analyses are in most cases impossible due to the very small amounts of test substance inside the wells (2 ml). In contrast, the flow-through system automatically generates adequate amounts of the test solutions for chemical analysis; even after only 24 h of exposure with a 5 × daily exchange of the sample solution, the effluent from each row of wells results in a final volume of 90 ml.

As a first example, 4-chlorophenol was tested to examine the applicability of the flow-through fish embryo toxicity test in chemical testing. The substance was selected on the basis of the fact that its toxicity in the FET differs by a factor of 10 from that in conventional acute fish toxicity tests. The results of the static and the flow-through fish embryo toxicity tests with this particular compound are almost identical, and the flow-through test can,

therefore, be utilized in chemical testing. However, if compared to the 96 h acute fish test, the LC_{50} values of the FET indicate a significantly lower sensitivity of either embryo test system. Data for the 96 h acute fish test for the main OECD fish species could be found in the US EPA ECOTOX database (US EPA 2002): 3.8 mg/L for bluegill sunfish (*Lepomis macrochirus*); 1.9 mg/L for rainbow trout (*Oncorhynchus mykiss*); 4.9 mg/L for medaka (*Oryzias latipes*); 5.0 to 6.1 mg/L for fathead minnow (*Pimephales promelas*). For 4-chlorophenol, the static and the flow-through versions of the FET are approximately 10 fold less sensitive than the 96 h acute fish test. The reason for this lower sensitivity might be differential uptake and/or metabolization by embryonic and adult individuals.

Unfortunately, there are no data available in the literature about the specific adsorption behavior of 4-chlorophenol, but there is little reason to believe that unspecific binding sites for 4-chlorophenol should not be saturated during pre-incubation of the 24-well plates (cf. Palmgren et al., 2006). Since, however, one of the most important features for a test to be accepted as an alternative to a conventional test is the correlation between the alternative procedure and the test to be replaced, it is indispensible to further analyze the reasons for such chemical outliers, which show differential toxicity in adult and embryonic fish. The results with 4-chlorophenol cannot by themselves be extrapolated to substances with different physicochemcial and toxicological properties, and it is recommended that both more in-depth testing of the flow-through system with 4-chlorophenol as well as with other chemicals should be carried out to further validate this alternative test method.

3.6 Conclusion

The present investigation demonstrates that a flow-through version of the FET can be conducted with modified commercially available polystyrene 24-well microtiter plates. Well plates with 6 mm drilling holes provided optimal distribution of test substance in the flow-through channels. The replacement of double distilled water by patent blue solution was approx. three times faster with 6 mm than with 5 mm drilling holes. By using flow-through plates with 6 mm drilling holes, a rapid, consistent and complete distribution inside a given well as well as between wells along the flow-through channels is guaranteed during the entire test duration. Since hatched larvae are able to swim through 6 mm drilling holes, gauze should be used as a barrier to prevent escape, thus allowing extension of the flow-through embryo test to even examine larval endpoints within teratogenicity tests.

Since in a first test with 4-chlorophenol the static and the flow-through version resulted in almost identical LC_{50} values, the flow-through test can be utilized for chemical testing. However, more-in-depth testing is necessary to validate FET as an alternative method, since

the results of the acute fish test and the FET version may still vary by factor of up to 10 for specific chemicals (Lammer et al., 2009a).

The major disadvantage of the flow-through version of the FET is that flow-through plates are not yet commercially available, and that the preparation of the plates is a time-consuming and laborious procedure. Yet, given a more widespread use of the FET, flow-through microtiter plates will certainly be made available.

Chapter 4

Are flow-through conditions an improvement for the static fish embryo toxicity test (FET)? Tests with hydroquinone, 4-chlorophenol, α-endosulfan and pyraclostrobin

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4.1 Abstract

The new European chemical policy REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) will result in a dramatic increase in the number of animal experiments in the interest of protecting public health. However the health and safety benefits need to be balanced with the ethical considerations of animal testing, thus sound alternative tests must be implemented. Likewise, industries are interested in alternative testing methods that are less cost intensive and less time- and space-consuming. The fish embryo toxicity test (FET) is an accepted alternative to the acute fish toxicity test and has been used in Germany for regulatory whole effluent testing since 2005. However, for testing of chemicals, further optimization of the FET is required to improve the correlation between the acute fish toxicity test and the alternative fish embryo toxicity test. One cause for outliers in this correlation may be adsorption of the test chemicals to the polystyrene plates commonly used in the FET. To overcome this challenge, a flow-through version of the FET test has been designed. In order to validate the applicability of the flow-through FET to chemical testing, a set of outlier chemicals (hydroquinone, 4-chlorophenol, pyraclostrobin and α-endosulfan) encompassing a range of log P_{ow} has been tested. However, no significant difference could be observed between the results of the static and the flow-through FET. Thus for these chemicals, the differences between the results from the FET and the acute fish toxicity test could not be traced back to reduced bioavailability due to adsorption to the polystyrene plates. Further investigations are necessary to identify the causes for the poor correlation between FET and acute fish test results for some chemicals.

4.2 Introduction

In 2007, REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) has come into force to revise the existing chemicals policy and further harmonize chemical legislations in Europe. Since December 1st, 2008, preregistration for the so-called “phase-in substances” is complete and registration of tier 1 substances needs to be finished end of 2010. REACH has been designed to eliminate the distinction between existing and new substances and replaces more than 40 different EU Directives and Regulations (Brown 2003; Petry et al. 2006).

National and international regulations require that new chemicals are tested for ecotoxicity (Segner 2004). In particular, fish acute toxicity tests play an important role in environmental risk assessment and hazard classification, because they allow for first estimates of the relative toxicity of various chemicals in various species (Wedekind et al. 2007). The acute fish

toxicity test (OECD 1992a) is part of the basic test battery that is required for a large number of chemicals. As part of an environmental risk assessment the results of this test are used to evaluate the effects of chemicals on aquatic vertebrates in general. In a standard 96 h acute fish test (OECD 1992a) with five test concentrations and a control, a minimum of seven fish are exposed per concentration resulting in 42 fish used per test. In addition, the 96 h acute fish test is generally preceded by a range-finding test, which requires additional test fish. As a result, the total number of fish used for testing of one substance may be as high as up to more than 50 fish (Hoekzema et al. 2006). However, according to REACH, it may be necessary that the risk of approx. 30,000 substances that are produced, used or imported in quantities of 1 - 100 tons per year must be evaluated (Hengstler et al. 2006; Lilienblum et al. 2008; Petry et al. 2006); this may result in enormous numbers of fish used to meet this requirement. As vertebrates, fish are a subject of animal welfare legislation. There is increasing evidence that fish may have some form of pain perception similar to what is present in mammals and should therefore be considered as able to suffer from severe distress and pain (Braunbeck et al. 2005; Chandroo et al. 2004; Huntingford et al. 2006; Nagel 2002; Sneddon et al. 2003). For these reasons the development of a reliable alternative method is highly desirable. With regard to the requirements for the acceptance of such an alternative method, the normal variability of the results obtained with the acute fish toxicity test should be taken into account. A more-in-depth analysis of 96 h acute fish toxicity data reveals that acute LC_{50} data may vary by orders of magnitude not only between species, but also for the same species between laboratories (Braunbeck et al. 2005).

Since the implementation of the Animal Welfare Guideline 86/609/EC in 1986 (EC 1986), the development and validation of alternatives to animal testing is highly promoted by the EU institutions (Lilienblum et al. 2008). Additionally, since 2004, with the 7th amendment to the Cosmetic Directive (EC 2003; 2007a), animal testing is banned for finished cosmetic products, and in March 2009 the ban of animal testing for ingredients of cosmetic products comes into force (EC 2008). Likewise, REACH highly promotes non-animal testing and takes preference of alternative methods to animal testing as far as possible (EC 2001; Lilienblum et al. 2008).

In Germany, the fish embryo toxicity test (FET) has been a mandatory replacement for the acute fish toxicity test in regulatory whole effluent testing since 2005 (DIN 2001). The test method was standardized as an ISO guideline in 2007 (ISO 2007). Furthermore, Nagel (2002) and Braunbeck et al. (2005) documented that the FET test is a very promising tool to replace the acute fish toxicity test also for chemical testing. In the current version of the test protocol, the test duration is limited to two or three days. Thus, it is considered a non-animal test according to current legal definitions (UK 1986). In the experiments described in this

paper the exposure period was extended for up to 5 days to identify any delayed effects. According the current UK Animal Procedures Act (UK, 1986b), the test would keep its status as non-animal test even with this extension, since immature forms of fish become a subject of the animal welfare legislation only when they are capable of independent feeding.

One of the most important features for a test to be accepted as an alternative to a conventional test is the correlation between results obtained with the potential alternative and the test to be replaced. In order to investigate the possibility of replacing the acute fish test by the alternative fish embryo toxicity test in chemical assessment, Ratte & Hammers-Wirtz (2003) conducted a preliminary statistical analysis of existing data for the FET and corresponding data for the acute fish test. The analysis resulted in an R^2 of 0.854; yet, some outliers could be identified, which deviate by more than one order of magnitude. Several biological reasons might account for such outliers for example: differential uptake, accumulation and metabolism of the test compound in embryonic and adult stages as well as the protection of the embryo by the chorion and the lipophilicity of the test compound might be possible reasons. In addition, adsorption to the wells of the polystyrene microtiter plates used in the static FET test design may significantly limit availability of some substances.

As stated by Becker & Crass (1982), flow-through acute tests are preferable with toxicants that have a high oxygen demand, are highly volatile, are unstable in aqueous solution, or are removed by test organisms during exposure in significant quantities. Palmgren et al. (2006) demonstrated that lipophilic and positively charged basic drugs adsorb to negatively charged surfaces of polystyrene plastic, but the limited binding sites could be saturated. In order to further improve the static FET, flow-through plates for the fish embryo tests were developed in a feasibility study (Lammer et al. 2009b). Since the flow-through FET performed well for tests with certain dyes, the present study was designed to evaluate the applicability of the flow-through version of the FET for chemical testing and to determine if this test design could enhance the correlation with the acute fish toxicity test. For these objectives, four “outlier” chemicals (hydroquinone, 4-chlorophenol, pyraclostrobin and α-endosulfan) listed in the study from Ratte & Hammers-Wirtz (2003) with increasing log P_{ow} (0.59, 2.39, 3.99, 4.77, respectively) were investigated using the flow-through FET and the static FET.

Hydroquinone is a common water-soluble constituent of foods, and it is used in skin lightening preparations and hair dyes, as a reducing agent in photographic developers, as a stabilizer for certain monomer mixtures and as an intermediate in chemical synthesis. From an ecological point of view, it is of interest because of its widespread occurrence in nature (Devillers et al. 1990; Topping et al. 2007).

4-Chlorophenol is used as an intermediate for higher chlorophenols, dyes and drugs, the herbicide 2,4-dichlorophenoxyacetate, and for fungicides (IPCS 1989; Ucisik & Trapp 2008). It is also used as a disinfectant. Significant amounts of chlorophenols can be formed and subsequently released into the environment from the chlorine bleaching process in pulp and paper-mills, from the chlorination of waste-water and drinking-water, and from the incineration of municipal waste (IPCS 1989).

α-Endosulfan is a chlorinated hydrocarbon pesticide used extensively to control a wide range of insect pests and has been classified as very toxic to aquatic organisms. It is a fairly persistent insecticide with a half-life ranging from a few months to up to 2 years in soil and 1 - 6 months in water, depending upon climatic, anaerobic and pH conditions. It works as a GABA gated chloride channel antagonist, it is highly toxic to fish. In addition to its agricultural use, it has been used as a wood preservative and in the control of the tsetse fly (Gormley & Teather 2003; Naqvi & Vaishnavi 1993). Since α-endosulfan is about three times as toxic as the β-isomer, the former was used in this investigation.

Pyraclostrobin is a broad-spectrum strobilurin fungicide used to control major plant pathogens. Strobilurins act by inhibiting mitochondrial respiration in fungi. They bind at the Q_o-centre on cytochrome b and block electron transfer between cytochrome b and cytochrome c1. This disrupts the energy cycle within the fungus by halting the production of ATP (Bartlett et al. 2001).

4.3 Material and methods

4.3.1 Chemicals and materials

Hydroquinone, 4-chlorophenol and α-endosulfan were purchased by Sigma-Aldrich (Deisenhofen, Germany). Pyraclostrobin was kindly provided by BASF (Ludwigshafen, Germany). Hydroquinone (CAS 123-31-9; MW 110.1 g/mol; log P_{ow} 0.59) was tested in concentrations of 0.2, 0.7, 2.45 and 8.575 mg/L. 4-Chlorophenol (CAS 106-48-9; MW 128.6 g/mol; log P_{ow} 2.39) was tested in concentrations of 4, 10, 25 and 62.5 mg/L. α-Endosulfan (CAS 959-98-8; MW 406.93 g/mol; Log P_{ow} 4.77) was tested in concentrations of 10, 100 1000 and 3200 µg/L. Pyraclostrobin (CAS 175013-18-0; MW 387.82 g/mol; log P_{ow} 3.99) was tested in 0.011, 0.022, 0.11 and 0.22 mg/L. Ethanol (absolute purity; Sigma-Aldrich) in dilution of 1:1000 was used as a solvent for tests for α endosulfan. 3,4 Dichloroaniline was used as a positive control (3.7 mg/L; Sigma-Aldrich).

The artificial water corresponded to reconstituted water according to ISO 7346/3 (ISO 1996; conductivity: 700 µS/L, NO_3^-: < 4.5 µg/L, NO_2^-: < 8 µg/L, NH_4^+: < 1.9 µg/L, PO_4^{3-}: < 50 µg/L,

Fe^{2+} and Fe^{3+} not detectable; chemical oxygen demand (COD): < 4 mg/l), which was diluted 1:5 using double-distilled water. Prior to use, the pH is adjusted to 7.8 ± 0.2.

The polystyrene 24-well microtiter plates were provided by Renner (TTP; Dannstadt, Germany) and the gauze (250 µm) was obtained from Verseidag (Geldern, Germany). The epoxy resin components for Spurr's medium (Spurr 1969; embedding medium ERL-4221D, D.E.R. 736, nonenylsuccinic anhydride, dimethylaminoethanol) to build the flow-through plates were obtained from Serva (Heidelberg, Germany).

4.3.2 Test system and experimental design

4.3.2.1 Static fish embryo toxicity test (FET)

The static FET was conducted according to DIN 38415-T6 and ISO 15088 (DIN 2001; ISO 2007). A more in detailed description can be found in Lammer et al. (2009a). In brief, in order to start exposure with minimum delay, at least 20 freshly spawned zebrafish eggs were selected per concentration and transferred into 60 mm crystallization dishes containing 100 ml of the different test concentrations, the positive control or the negative control, respectively. Subsequently, 10 fertilized eggs were selected and individually transferred to the wells of a 24-well plate filled with 2 ml per well of freshly prepared test solutions and controls. The 24-well plates were then covered with self-adhesive foil and incubated at 26 °C ± 1 °C. All plates were pre-treated with the respective concentrations for 24 h prior to the exposure of the embryos.

In order to capture potentially delayed effects, exposure was extended beyond the standard 48 - 72 hours up to 120 h, 144 h or even 192 h (the latter for the test with α-endosulfan). Lethality (coagulation, non-detachment of the tail, non-formation of somites and non-detection of the heartbeat) as well as sublethal endpoints (e.g. spinal deformations, lack of pigmentation, edema) were recorded after 24, 48, 72, 120 and 144 h (α-endosulfan: also 168 and 192 h). In general, LC_{50}s were determined graphically. Artificial water was used as a negative control, and 3,4 dichloroaniline (3.7 mg/L) served as a positive control. The test was classified as valid, if the mortality in the negative control was less than 10 % and the positive control gave a mortality of more than 10 %. Additionally, for tests with α endosulfan, a solvent control with ethanol in a dilution of 1:1000 was tested.

4.3.2.2 Flow-through fish embryo toxicity test (FET)

Flow-through plates were prepared according to Lammer et al. (2009b): The rear space between the wells of a 24-well microtiter plate was filled with Spurr's resin (Spurr 1969). After polymerization at 70 °C for 24 h, 6 mm holes were drilled in longitudinal direction through the entire plate to obtain four parallel flow channels consisting of six consecutive wells each. In order to prevent the hatched embryos from swimming through the holes during the test, 250 µm gauze moistened with aqua bidest. was placed in each well. The plates were kept in an incubator to maintain constant temperature conditions (26 ± 1 °C). Peristaltic pumps (Minipuls 3; Gilson, Limburg-Offheim, Germany) were used to achieve constant flow conditions, and the outflow was collected inside the incubator. In order to saturate the system, the flow was initiated 5 days previous to the onset of exposure.

As in the static fish embryo toxicity test, 20 freshly spawned zebrafish eggs were selected per concentration and transferred into 60 mm crystallization dishes containing 100 ml of the different test concentrations, the positive control or the negative control, respectively. Since one flow-through plate was used for two concentrations, 12 fertilized eggs per concentration and controls were placed in the flow-through plates five days after pre-saturation of the flow-through system. The same exposure and examination scheme as in the static test was used. LC_{50}s were also determined graphically.

4.3.2.3 Chemical analysis

Except for pyraclostrobin, all tests were conducted with nominal concentrations. For pyraclostrobin chemical analysis, stock solutions as well as the outflow of the flow-through system were analyzed by means of reverse HPLC/MS (Tab. 4.1).

Tab. 4.1: Details of pyraclostrobin chemical analysis.

Analytical method	HPLC-method with MS-detection and external calibration
Column	YMC Pro C18 3µm 50 mm x 3 mm with guard-column of the same material
Mobile phase	A: Water / Formic Acid = 1000 / 1 B: Acetonitrile / Formic Acid = 1000 / 1
Injection volume	50 µl
Flow rate	0.7 ml/min
Column temperature	40 °C
MS-Detection ESI+	ESI+ M388 (Pyraclostrobin, Reg.No.304428)
Limit of Quantification LoQ	0.001 mg/L (Pyraclostrobin, Reg.No.304428)
Instrumentation	Agilent 1100 series: G1312A degasser G1312A pump

	G1313A autosampler
	G1316A column oven
	MS-Detector Agilent MSD G1946D
Data system for peak evaluation	Chemstation/Chemserver
Balance	An instrument capable of accuracy to within ±0.1mg

4.4 Results

4.4.1 Static and flow-through fish embryo test (FET) with hydroquinone

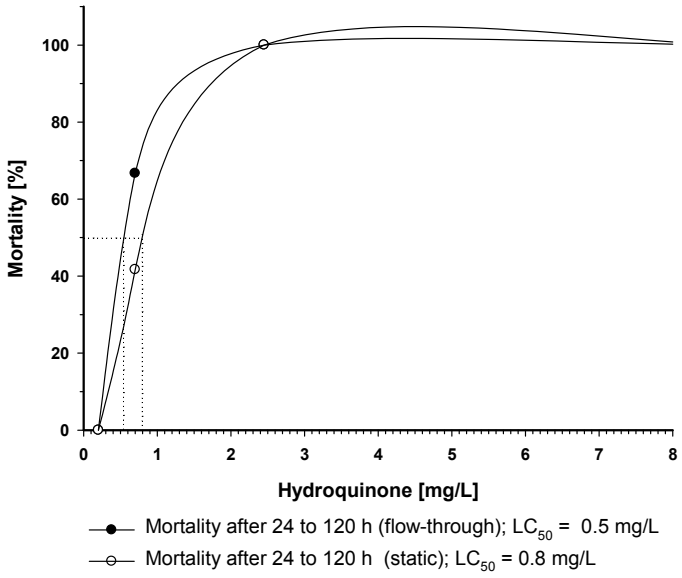

Fig. 4.1: Acute toxicity of hydroquinone to zebrafish embryos in the static and the flow-through fish embryo toxicity test (FET) after 24 to 120 h.

Both static and flow-through exposure to hydroquinone primarily caused coagulation of the embryos. After 24 h, all embryos in the two highest concentrations were coagulated; if the embryos did not coagulate by 24 h, they showed normal development. Since no sublethal effects or changes could be recorded after 24 h, hydroquinone only affected early developmental stages. The static FET gave an LC_{50} of 0.8 mg/L after 24, 48, 72 and 120 h (Fig. 4.1). For the flow-through FET, an LC_{50} of 0.5 mg/l was determined after 24, 48, 72 and 120 h , thus the flow-through design was only slightly more sensitive (Fig. 4.1) than the static FET.

4.4.2 Static and flow-through fish embryo toxicity test (FET) with 4-chlorophenol

The acute fish toxicity data have already been reported in Lammer et al. (2009b): The LC_{50}s determined in the static FET were 45 mg/L after 24, 48, 72 and 120 h, and at 40.7 after 144 h. The flow-through FET resulted in LC_{50} values 42.8 mg/L after 24, 48 and 72 h and 40.4 mg/L after 120 and 144 h. Thus, for the tests with 4-chlorophenol, the results the flow-through FET were not significantly different than the static FET.

Since in the first flow-through FET with 4-chlorophenol no improvement of the correlation to the acute fish test could be demonstrated, embryos were studied in more detail. In addition to lethal effects, embryos also showed conspicuous sublethal effects, particularly a dose-dependent reduction of pigmentation (Figs. 4.2, 4.3). Since reduced body pigmentation as an endpoint is quite difficult to quantify as a graded response, all embryos that showed clear differences to the negative control were regarded as affected. Within the range of test concentrations (4 to 62.5 mg/L), the EC_{50} for the static and the flow-through FET after 48 h could not be determined. Thus, the EC_{50}s for the static FET are < 4 mg/L after 48 h, 15.4 mg/L after 72 and 120 h and 10.5 mg/L after 144 h. The flow-through FET resulted in slightly more sensitive EC_{50}s: < 4 mg/L after 48 h, 8.9 mg/L after 72 and 120 h and 6.8 mg/L after 144 h. If compared to the LC_{50}s, the EC_{50}s for the endpoint "reduced pigmentation" were thus 4 to 10 times lower. This specific endpoint occurred only in the test with 4-chlorophenol and could not be detected with any other test chemical.

Fig. 4.2: Embryo exposed to 10 mg/L 4-chlorophenol after 48 h characterized by a lack of body and eye pigmentation.

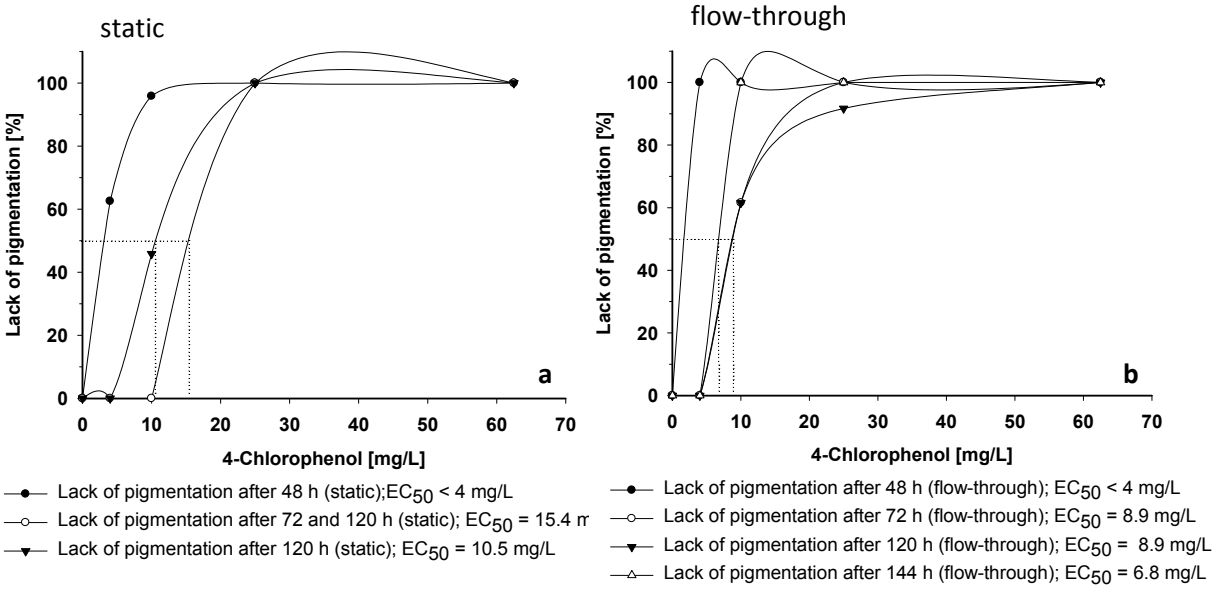

Fig. 4.3: Lack of pigmentation caused by 4-chlorophenol in zebrafish embryos in the static (a) and the flow-through (b) fish embryo toxicity test (FET) after 24 to 120 h.

4.4.3 Static and flow-through fish embryo toxicity test (FET) with α-endosulfan

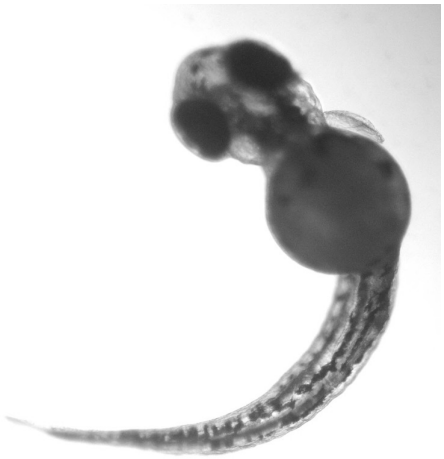

Fig. 4.4: Embryo exposed to 3200 µg/L α-endosulfan after 72 h with spinal deformations resulting in whole-body deformation.

For α-endosulfan, both the static and the flow-through FET were prolonged up to 192 h, since no significant mortality occurred up to 144 h (Tab. 4.2). Since less than 10 % mortality were recorded in the solvent and the negative control, the tests were regarded as valid even for 168 and 192 h. For the static FET, the LC_{50}s resulted in 2270 and 640 µg/L after 168 h and 192 h, respectively. For the flow-through FET, the LC_{50}s were determined at 2070 and 440 µg/L after 168 h and 192 h, respectively.

The most prominent sublethal endpoint could only be recognized after hatch: Deformations of the spinal cord resulting in horizontal flexion first became apparent after 72 h (Tab. 4.2, Fig. 4.4). This kind of abnormity could exclusively be detected with α-endosulfan. Only embryos that showed distinct flexion of the spinal column were regarded as affected. The EC_{50}s for the static FET resulted in 540, 350, 400, 500 and 360 µg/L after 72, 120, 144, 168 and 192 h, respectively. The flow-through FET resulted in similarly sensitive results. Compared to the LC_{50}s, the EC_{50} for spinal deformations was, therefore, considerably lower than the conventional acute LC_{50}.

Tab. 4.2: Summary of the results (µg/L) of the static and the flow-through fish embryo toxicity test (FET) with α-endosulfan. Exposure was prolonged, since no effects occurred until 168 h. (n.d. = not detectable)

	24 h	48 h	72 h	120 h	144 h	168 h	192 h
LC_{50} static FET	n.d.	n.d.	n.d.	n.d.	n.d.	2270	640
LC_{50} flow-through FET	n.d.	n.d.	n.d.	n.d.	n.d.	2070	440
EC_{50} (spinal deformations) static FET	-	-	540	350	400	500	360
EC_{50} (spinal deformations) flow-through FET	-	-	n.d	2980	500	400	400

4.4.4 Static and flow-through fish embryo toxicity test (FET) with pyraclostrobin

Since the results of the chemical analysis gave almost the same concentrations at the beginning and at the end of the test (data not shown), the test system was clearly saturated. Exposure to pyraclostrobin primarily resulted in lethal effects (Tab. 4.3). At the highest concentration (0.22 mg/L), the embryos were either coagulated after 24 h or they only reached the epibolic stage and stopped in further development (Fig. 4.5). The LC_{50}s for the static FET could be determined at 0.059 mg/L for all time points. The nominal LC_{50}s for the flow-through FET resulted in identical values. Based on chemical analysis, the LC_{50}s for the flow-through FET were approximately 0.04 mg/L at every time point (Tab. 4.3).

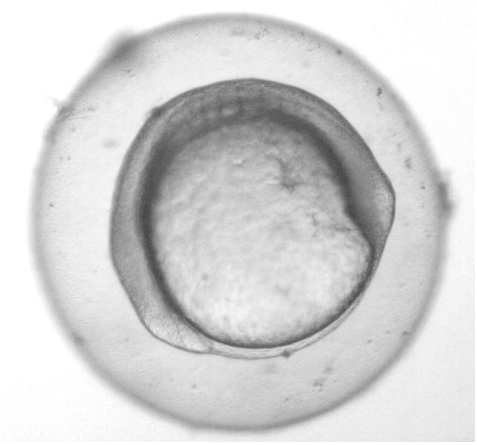

Fig. 4.5: Embryo exposed to 0.11 mg/L pyraclostrobin after 24 h. The embryo is arrested in the epibolic stage.

Tab. 4.3: Summary of the results (mg/L) of the static and the flow-through fish embryo toxicity test (FET) with pyraclostrobin.

	24 h	48 h	72 h	120 h	144 h
LC_{50} static FET (nominal)	0.059	0.059	0.059	0.059	0.059
LC_{50} flow-through FET (nominal)	0.059	0.059	0.063	0.059	0.059
LC_{50} flow-through FET (measured)	0.041	0.041	0.044	0.041	0.041

4.5 Discussion

4.5.1 Static and flow-through FET with hydroquinone

LC_{50} data for the 96 h acute fish test for the main OECD fish species were taken from US EPA ECOTOX database (US EPA 2002). They range from 0.044 mg/L to 0,097 mg/L for fathead minnow (*Pimephales promelas*) and 0.638 mg/L for rainbow trout (*Oncorhynchus mykiss*). For zebrafish, an LC_{50} of 0.17 mg/L was reported. The LC_{50} data for both the static and the acute FET (0.8 and 0.5 mg/L) are, therefore, very close to the range of the LC_{50} data of the acute fish test. There were no statistically significant differences between the static and the flow-through FET test responses. In contrast to the static FET data entered into the Ratte and Hammers-Wirtz database (2003; 6.1 and 7.9 mg/L), the present re-evaluation of both the static and the flow-through FET tests resulted in data approx. 10 time lower, and there

seems to be no more discrepancy between FET data for hydroquinone and the acute fish test.

In general, hydroquinone only influenced the very early developmental stages up to 24 h. If the embryos were not affected to that point of time, they showed normal development. This indicates a high cytotoxic potential of hydroquinone. These results are consistent with the findings by Devillers et al. (1990), who reported that the formation of highly reactive derivates, such as radicals and quinones, which are capable of binding covalently to and altering the structure of macromolecules, explains both the high cellular toxicity of hydroquinone and its genotoxic and immunotoxic properties. Also IPCS (1994) stated that hydroquinone reacts with many different biological components including macromolecules such as protein, DNA, tubulin, lipids, and low molecular weight molecules such as sulfhydryls and nucleotides. It is toxic to different cell types, has effects on cellular metabolism, and modulates enzyme activities. And since covalent binding and oxidative stress are mechanisms postulated to be associated with hydroquinone-induced toxicity (IPCS 1994), the coagulation of the embryos at very early developmental stages might be due to such effects. In addition, both oxidized hydroquinone species (*p*-benzosemiquinone radical and *p*-benzoquinone) and thiol-hydroquinone/quinone conjugates are believed to contribute to hydroquinone toxicity. Likewise, O'Brien (1991) reported that most benzoquinones are cytotoxic as a result of alkylation.

4.5.2 Static and flow-through FET with 4-chlorophenol

The following data could be retrieved from literature for the 96 h acute fish test (US EPA 2002): 3.8 mg/L for bluegill sunfish (*Lepomis macrochirus*); 1.91 mg/L for rainbow trout; 4.9 mg/L for medaka (*Oryzias latipes*); 6.11 mg/L and 5 mg/L for fathead minnow. Given the LC_{50}s of the static (45 and 40.7 mg/L; 24 - 120 h and 144 h) and the flow-through FET (42.8 and 40.4 mg/L; 24 - 72 h and 120 - 144 h), the FET is approximately 10fold less sensitive than the acute fish test. Again, no significant difference could be detected between the static and the flow-through FET, and the flow-through version did not decrease the difference between the embryo and the adult test. Since the LC_{50}s do not change dramatically after hatch, the chorion does not represent a barrier for 4-chlorophenol. However, the results of the FET confirm the data found in Ratte & Hammers-Wirtz (2003), who stated an LC_{50} of 45.6 mg/L after 48 h for the static FET. Similar results were found in another study in our laboratory (data not published): The LC_{50}s for the static FET resulted in 40.4 mg/L after 24 h, 43.9 mg/L after 48 h, 42.1 mg/L after 72 h and 32.2 mg/L after 108 h.

Beside lethal effects, a prominent lack of pigmentation was the most sensitive sublethal endpoint. With respect to lack of pigmentation, the flow-through FET resulted in slightly more sensitive EC_{50}s in a range similar to those of the LC_{50}s for acute fish toxicity. Since reduced pigmentation did not occur in combination with other endpoints, it is most likely due to a specific mode of action. However, hydroquinone is a degradation product of 4-chlorophenol (Devillers et al. 1990) and it is used in skin lightning preparations (Topping et al. 2007) because it generates depigmentation (Jimbow et al. 1974; Kasraee et al. 2003; Parvez et al. 2006). Thus the degradation product hydroquinone might have caused the reduced pigmentation. However, since lack of pigmentation did not occur after exposure to hydroquinone alone, a more complex mechanism may be responsible for this effect.

4.5.3 Static and flow-through FET with α-endosulfan

For α-endosulfan, no 96 h acute fish test data could be found for the main OECD fish species in the US EPA ECOTOX database. However, data for other fish species are available (US EPA 2002): 0.16 µg/L for snake-head catfish (*Channa punctata*) and 0.33 and 1 µg/L for rohu (*Labeo rohita*). For the static and the flow-through FET, no LC_{50} data could be calculated until 144 h. In order to check for delayed mortality, the FET was prolonged to 192 h. Even then, however, both the static and the flow-through FET LC_{50}s varied up to a factor of 14,000 from the 96 h acute fish test data. This is a tremendous difference, since α-endosulfan is highly toxic to adult fish (Naqvi & Vaishnavi 1993; Rao & Murty 1982).

After hatch, severe spinal deformation became apparent, but the EC_{50}s for the static and the flow-through FET only resulted in slightly more sensitive results compared to the lethal effects. Singh et al. (2007) also found teratogenic effects in embryos of pregnant Wistar rats, which were given endosulfan by oral exposure during the gestational days 6 - 20. They observed various fetal gross skeletal and visceral anomalies. Malformations such as wrist drop, curled tail and hematoma as well as skeletal defects including skull bones, ribs, sternebrae and vertebrae could be detected. The spines were curved and shorter than normal, and occasionally absence of sacral and caudal vertebrae was observed. However, the mechanism for endosulfan induced teratogenicity is still unknown (Singh et al. 2007). Nevertheless, even the EC_{50}s for spinal deformations did not reach the sensitivity of 96 h acute fish data. They still differ by minimum factors between 350 and 18,000.

However, embryonic LC_{50}s and EC_{50}s clearly decrease with test duration. This could be an indication that development-related changes in the metabolism eventually lead to more increased sensitivity. Differences in the metabolism between adults and embryos are thus the most likely explanation for the tremendous differences between results of the FET and

the acute fish test. Likewise Singh et al. (2007) reported that the elevation of specific enzyme activities such as NADPH-cytochrome C reductase, aminopyrine-*N*-demethylase, aniline hydroxylase and tyrosine amino-transferase during endosulfan exposure suggest a potential maternal P-450-dependent contribution to bioactivation and induction of teratogenicity. It may well be possible that the immature metabolism and enzyme activities in zebrafish embryos are responsible for differential bioactivation (cf. Busquet et al. 2008).

4.5.4 Static and flow-through FET with pyraclostrobin

No data for pyraclostrobin are available in the US EPA ECOTOX database. However, EC (2004) gave LC_{50}s of 0.006 mg/L for the active substance and 0.017 mg/L after 96 h for the technical product for rainbow trout after 96 h. Since in the present study the active substance was tested, both static and flow-through FET proved less sensitive than the acute fish test. After correction on the basis of analytically verified data, flow-through FET LC_{50}s vary from acute fish LC_{50}s by a factor of 6.8. No differences were evident between nominal LC_{50}s from the static and the flow-through FET. Thus, for pyraclostrobin the flow-through FET does not improve the correlation to acute fish toxicity data. Nevertheless, under flow-through conditions, at least larger amounts of test substance can be received, thus allowing for chemical analyses.

Since, according to chemical analysis, concentrations of the stock solutions were identical to those at the end of the test (outflow of the flow-through system), the flow-through system was capable of ensuring saturation of the well plates. The initial hypothesis that lipophilicity and hence availability of test substance during static testing procedures were responsible for differences between FET and acute fish test could, therefore, not be confirmed. Since lethal and sublethal effects did not increase after hatch, protection of the embryo by the chorion likely does not play a role in the differences between the FET and the acute fish test. As a consequence, for pyraclostrobin as well, higher toxicity in adult fish may be speculated to be due to differences in metabolism between embryos and adults.

4.5.5 Is the flow-through system an improvement for the static FET?

Various hypotheses have been postulated to explain why the FET does not react as sensitively to some chemicals as the 96 h acute fish test. Apart from differential uptake, accumulation and metabolization of the test compound in embryonic and adult stages and the protection of the embryo by the chorion, lipophilicity of the test compound and hence reduced availability during static test procedures have been discussed as potential reasons.

Several investigations (Dahlström et al. 2004; Knorr & Gätzschmann 1966; Koutsopoulos et al. 2007; Palmgren et al. 2006) reported that lipophilic and positively charged drugs and chemicals show strong interactions with negatively charged polystyrene and, therefore, tend to adsorb to the surface of the polystyrene plates *via* electrostatic binding and hydrophobic interactions; however, the limited binding sites could be saturated (Palmgren et al. 2006). By using a flow-through system for the FET, the problem of test substance adsorption in a static system could be overcome. However, at least for the compounds tested, significant differences in the results of the FET could not be documented under flow-through conditions. Since chemicals with both low and high log P_{ow} were used, but gave almost identical results in the static and the flow-through test designs, adsorption of the test substance in the FET is unlikely to account for the difference between FET and adult fish test results. Apparently, pre-saturation of the polystyrene 24-well plates for 24 h prior in the static test as applied in this study is sufficient to saturate the binding sites of the polystyrene and to compensate for unspecific adsorption. In fact, as shown in the present communication, 24 h pre-saturation may even be as effective as true flow-through conditions.

However, by providing sufficient amounts of test concentrations for chemical analysis, the flow-through system offers a big advantage over the static test. Even after only 24 h of exposure with a 5fold exchange of the sample solution per day, the effluent from each row of wells results in a final volume of 90 ml, whereas the static test only provides 20 ml per concentration, if 10 embryos are used per concentration (DIN 2001; ISO 2007).

4.6 Conclusion

Since adsorption could be eliminated as a reason for the differences in the results between the FET and the 96 h acute fish test, the question remains why, for some chemicals, zebrafish embryos do not react as sensitively as adult zebrafish. Already Van Leeuwen et al. (1985) reported that the chorion appears to 'protect' the embryo from the uptake of toxicants, not by completely preventing but by slowing down the intrusion. For example, the intrusion of heavy metal ions might be seriously hindered by complexation with SH-groups, which are abundantly present in the egg membrane. In contrast, Van Leeuwen et al. (1985) reported that most lipophilic compounds could easily penetrate membranes. In fact, dechorionation experiments with zebrafish eggs exposed to 4-chlorophenol did not result in any higher sensitivity of the embryos (own results, unpublished).

The reason why the embryo does not react in the same manner compared to the adult fish can only be hypothesized: (1) The chorion may act as a barrier for some chemical and they

cannot enter the chorion. (2) Due to the different stages of development the embryo may not have developed the same metabolism and chemicals may not be bioactivated like in adult fish which may result in less toxicity of the chemicals. Further investigations are necessary to figure out if one of these reasons can explain the differences between the FET and the acute fish test.

Chapter 5

Towards an alternative for the acute fish LC_{50} test in chemical assessment: The fish embryo toxicity test goes multi-species - an update

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5.1 Abstract

After its standardization at the national level in Germany (DIN 38415-T6), the 48 h sewage testing assay with zebrafish (*Danio rerio*) embryos has been submitted for standardization to ISO. As an alternative to the conventional acute (96 h) fish test, a modified fish embryo test will be submitted to the OECD for chemical testing in late 2005. For this, a protocol originally designed for zebrafish was adapted to fit also the requirements of other OECD species, namely medaka (*Oryzias latipes*) and fathead minnow (*Pimephales promelas*). Results document that the transfer of the protocol is possible with only minor modifications. Data obtained from embryo tests with the three species are comparable. Statistical analysis of existing zebrafish embryo toxicity data resulted in the conclusions (1) that there is a reliable correlation between the fish embryo test and the acute fish test, (2) that the confidence belt of the regression line was relatively small, but that the prediction range was relatively wide. The regression thus seems appropriate to describe the relationship between acute fish and embryo LC_{50} with good confidence, but is less appropriate as a prediction model. Investigations into oxygen requirements of zebrafish embryos reveal that they adapt to a broad range of oxygen levels and survive at concentrations of 2 mg/l without malformations. Zebrafish embryos can thus be exposed in very small toxicant volumes (100 µl), which is of particular interest for the testing of metabolites. Dechorionation studies with 48 h old zebrafish embryos indicate that the barrier function of the chorion increases with the lipophilicity of the test compound. Finally, examples are given as to how additional endpoints can be incorporated into the fish embryo test protocol to extend its scope, e.g. to sediment toxicity assessment or genotoxicity and mutagenicity testing.

5.2 Introduction

There are extensive regulatory requirements for fish acute toxicity data on individual chemicals both for environmental risk assessment and hazard classification. In the European Union, data requirements for the notification of new substances are listed in the annexes to the Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labeling of dangerous substances (EC 1967). The quality and amount of data required primarily depend on the amount of the substance to be marketed (OECD 1992a). The “base set” data are required for all substances for which the marketing quantity exceeds one ton per year per manufacturer (Annex VII.A of the Directive). This “base set” includes acute toxicity for freshwater fish (96 h LC_{50}; OECD 203), acute toxicity for daphnids (48 h LC_{50}) and growth inhibition test on freshwater algae (growth rate: 72 h ECr_{50} and/or biomass: 72 h ECb_{50}). Upon submission by

industry, these base set data are entered into the New Chemicals Database (NCD) hosted by the European Chemicals Bureau at Ispra (Italy) in the form of summary notification dossiers. The data reported are used to decide on the classification and labeling as well as for hazard and risk assessment of the substance. The acute fish test has thus long been a mandatory component in initial toxicity testing; as a consequence, a considerable body of information has been accumulated for existing chemicals. A closer inspection of existing acute fish LC_{50} data, however, reveals differences in orders of magnitude not only between species, but also for the same species between laboratories (e.g. Juhnke & Lüdemann 1978). Single species LC_{50} data may thus be highly questionable with respect to accuracy and, in more general terms, to toxicological relevance. Furthermore, the significance for environmental risk assessment of the death of individuals after short-term exposure to high toxicant concentrations is low – except in cases of accidental spills (Nagel 2002). In addition to such scientific considerations, severe ethical concern has arisen, since there is little doubt that fish subjected to acutely toxic concentrations of chemicals suffer severe distress and pain, which is clearly not compatible with current animal welfare legislation. Nevertheless, in the last 15 years, only a small group of laboratories has focused on the development of alternatives to the acute fish test; major focus has been put on cytotoxicity tests (Castaño et al. 2003; Denizeau 1998; Fent 2001; Fent & Meier 1992; 1994; Segner & Braunbeck 1998) and fish embryo tests (Bachmann 2002; Birge et al. 1985; Call et al. 1987; Lange et al. 1995; Nagel 2002; Oberemm 2000; Schulte & Nagel 1994; Strmac & Braunbeck 2000). For the time being, there is some evidence that the fish embryo test is slightly more sensitive and more accurately mirrors toxicity to fish than cytotoxicity tests (Lange et al. 1995; Nagel 2002). It should be noted, however, that for particular substances, cytotoxicity tests may be more sensitive than fish embryo tests (e.g. Zabel & Peterson 1996). In 2001, a working group convened by the German Standardization Organization (DIN) designed a protocol for an alternative test based on zebrafish embryos as a replacement for the acute fish test in routine whole-effluent testing (DIN 2001). After an exposure period of 48 h after fertilization, the toxicological endpoints coagulation, failure to develop somites, lack of tail detachment from the yolk as well as lack of heartbeat are assessed (Table 5.1). Since zebrafish only hatch after approx. 72-96 h, this alternative method does not represent an animal test in legal terms. Since the 1st of January 2005, the so-called “zebrafish egg test” has become mandatory in Germany. In an attempt to further advance the fish embryo test, the German Federal Environment Agency has submitted a modified protocol for sewage testing to ISO and will submit another modified protocol for chemical testing to the OECD in late 2005, which will not only cover zebrafish, but also other common OECD fish species such as the fathead minnow (*Pimephales promelas*) and the Japanese medaka (*Oryzias latipes*).

Since there is a strong commitment to alternative methods in future chemical testing within the framework of REACH (Registration, Evaluation, Authorization and Restriction of Chemicals), the new European Union chemicals policy (EC 2001), the fish embryo test seems a very promising candidate for future toxicological routine testing. The present communication provides an update to the report by Nagel (2002) and details attempts to not only extend the principle of the zebrafish embryo test to other fish species, but also to incorporate additional endpoints in order to broaden the scope of the fish embryo test.

5.3 Animals, material and methods

5.3.1 Fish, fish maintenance and spawning procedure

Table 5.2 gives a summary of the fish species used for the present study as well as specific maintenance and exposure conditions. All fish used for breeding were taken from the fish stocks maintained at the Department of Zoology, University of Heidelberg, Germany. The zebrafish ("Westaquarium" strain) had originally been obtained from H.J. Pluta (Federal Environment Agency, Berlin, Germany), the fathead minnow stock was a gift from R. Länge (Schering AG, Berlin, Germany), and the wild type medaka were derived from stock from J. Wittbrodt (EMBL – European Molecular Biology Laboratory, Heidelberg, Germany).

Tab. 5.1: Endpoints recorded for the routine testing of sewage waters according to the German standard DIN 38415, part 6 (DIN 2001)

	4h	8h	12h	16h	24h	36h	48h
Egg coagulation	+	+	+	+	+	+	+
Development of somites				+	+	+	+
Tail detachment from yolk					+	+	+
Presence of heart beat						+	+

5.3.2 Zebrafish

The zebrafish (Danio rerio, Hamilton-Buchanan 1822; Fig. 5.1) is a small benthopelagic cyprinid fish originating from the Ganges River system, Burma, the Malakka peninsula and Sumatra (Eaton & Farley 1974; Talwar & Jhingran 1991) with a mean adult length between 3 and 5 cm. In both soft and hard waters, zebrafish grow quickly at 26 °C and complete their life-cycle within three months. The species is easily obtainable, inexpensive, readily maintainable and, under appropriate conditions, yields a large number of non-adherent, fully transparent eggs (Laale 1977). The zebrafish has become a major model in neurobiology and toxicology as well as in general molecular and developmental biology (Ekker & Akimenko 1991; Goolish et al. 1999; Hisaoka & Battle 1958; Kimmel et al. 1995; Laale 1977;

Lele & Krone 1996; Nüsslein-Vollhard 1994; Roosen-Runge 1938; Sander & Baumann 1983; Westerfield 2000; Wixon 2000). Care should be taken to select a strain with continuously high egg production and high fertilization rate. Under spawning conditions, males can easily be distinguished from females by their more slender body shape and an orange to reddish tint to the silvery bands along the body. Due to the large number of eggs produced, females can be recognized by their swollen bellies. One female spawns between 50 and 200 eggs on a daily basis. Egg production can be significantly stimulated by additional rations of natural food (*Artemia spec*.). The fish used were between 6 and 12 months old. The embryonic development of zebrafish was described in detail (Hisaoka & Battle 1958; Kimmel et al. 1995; Laale 1977; Roosen-Runge 1938; Thomas & Waterman 1978).

Fig. 5.1: Zebrafish (*Danio rerio*) females (upper individual) can easily be differentiated from males (lower individual) by their extended bellies and the lack of reddish tint along the silvery longitudinal

The zebrafish egg is telolecithal, cleavage is meroblastic and discoidal. Selected major stages of zebrafish development are given in Fig. 5.2; for more details, see Kimmel et al. (1995).

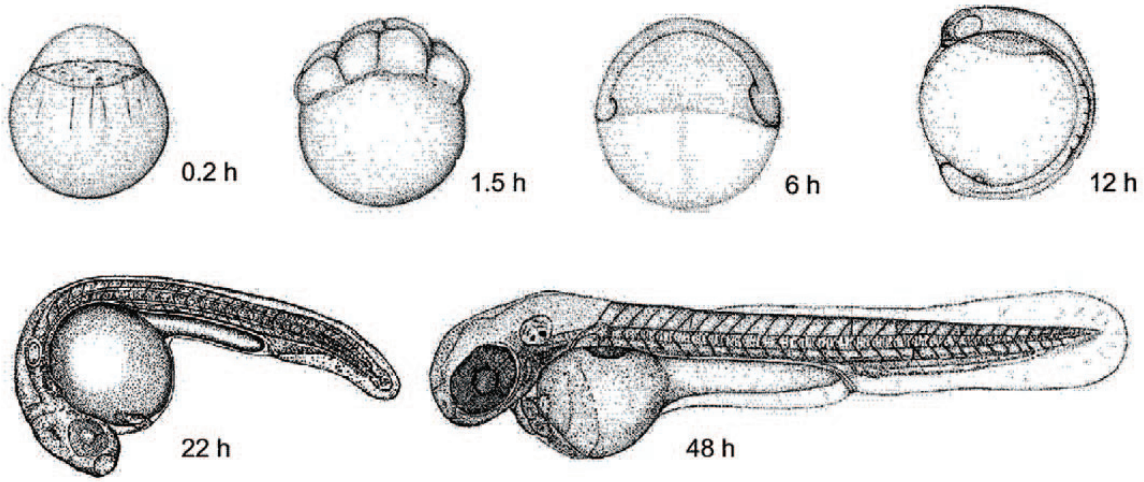

Fig. 5.2: Stages of zebrafish (*Danio rerio*) development, partly taken from Kimmel et al. (1995). For ease of differentiation, the 22 h embryo has been dechorionated. Note variable scaling of different stages. Top row stages from Kimmel et al. (1995).

Tab. 5.2: Fish species as well as maintenance, breeding and test conditions used in the comparative investigations

	Zebra fish *(Danio rerio)*	**Fathead min-now** *(Pimephales promelas)*	**Medaka** *(Oryzias latipes)*
Origin of species	India, Burma, Malakka, Sumatra	Temperate zones of central North America	Japan, China, South Korea
Sexual dimorphism	Females: protruding belly when carrying eggs Males: more slender, orange tint between blue longitudinal stripes (particularly evident at the anal fin)	Females: more plump when carrying eggs, ovipositor Non-spawning males: black spot on dorsal fin Spawning males: black coloration of head, dorsal nuptial pad and nuptial tubercles in spawning season, black bands along body sides	Females: generally more plump, carrying sticky eggs at anal fin Males: anal fin larger, papillary processes on posterior dorsal fin rays
Feeding regime	Dry food (TetraMin; Tetra, Melle, FRG) once daily; from three days before spawning, plus frozen adult brine shrimp (*Artemia spec.*) twice daily (*ad libitum*). To guarantee for optimal water quality, excess faeces was removed approx. one hour after feeding.		
Wet weight of adult fish	Females: 0.65 ± 0.13 g Males: 0.5 ± 0.1 g	Females: 1.5 ± 0.3 g Males: 2.5 ± 0.5 g	Females: 0.35 ± 0.07 g Males: 0.35 ± 0.07 g
Illumination	Fluorescent bulbs (wide spectrum); 10-20 µE/M^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels); 14 h light, 10 h dark		
Water quality	26.0 ± 0.5 °C, O_2 ≥ 8.5 mg/l, hardness: 20°dH (~ 213 mg/l $CaCO_3$), NO_3^-: ≤ 48mg/l, NH_3 and NO_2^-: < 0.01mg/l, pH = 7.8 ± 0.2	16.5 ± 1.5 °C, O_2 ≥ 8.5mg/l, hardness: 20°dH (~ 213 mg/l $CaCO_3$), NO_3^-: ≤ 48mg/l, NH_3 and NO_2^-: < 0.01mg/l, pH = 7.8 ± 0.2	24.0 ± 0.5 °C, O_2 ≥ 8.5mg/l, hardness: 20°dH (~ 213 mg/l $CaCO_3$), NO_3^-: ≤ 48mg/l, NH_3 and NO_2^-: < 0.01mg/l, pH = 7.8 ± 0.2
Further water quality criteria	Particulate matter < 20 mg/l, total organic carbon < 2 mg/l, unionized ammonia < 1 µg/l, residual chlorine < 10 µg/l, total organophosphorus pesticides < 50 ng/l, total organochlorine pesticides plus polychlorinated biphenyls < 50 ng/l, total organic chlorine < 25 ng/l		
Tank size for maintenance	180 l (max. 200 individuals)	180 l (max. 80 individuals)	50 l (max. 60 individuals)
Water purification	Permanent (internal filter)	Permanent (internal filter)	External (air-driven)
Male to female ratio for breeding	4:2	2:4	15:15
Breeding tanks	4 l tanks equipped with steel grid bottom and plant dummy as spawning stimulant; external heating mats (cf. Fig. 3)	30 l tanks with black glass walls maintained at 24°C and equipped with 2 clay tiles divided into two halves as spawning substrate	30 l tanks with black glass walls equipped with several plant dummies or *Ceratophyllum* spec. as substrate for spawning
Egg structure and appearance	Stable chorion, highly transparent, non-sticky, diameter: ~0.8 mm	Chorion only hardens in multicellular stage, transparent, sticks to surfaces, diameter < 1 mm	Stable chorion with spiny hooks (adheres to anal fin of female), moderately transparent, diameter < 1 mm
Embryo development at 25°C	18 h: Development of somites 21 h: Tail detachment 26 h: Heart-beat visible 28 h: Blood circulation 72 h: Hatching	22 h: Development of somites 25 h: Tail detachment 27 h: Heart-beat visible 30 h: Blood circulation 160 h: Hatching	28 h: Development of somites 28 h: Tail detachment 30 h: Heart-beat visible 32 h: Blood circulation 120 h: Hatching
Test type	Flow-through, 26°C, 24-well plates (2 ml per cavity)	Flow-through, 25°C, 24-well plates (2 ml per cavity)	Flow-through, 26°C, 24-well plates (2 ml per cavity)
Major toxicological endpoints at 25°C	24 h: Tail development, somite development 48 h: Heart-beat visible	28 h: Tail development, somite development 3 d: Blood circulation 3 d: Blood circulation 4 d: Blood circulation	30 h: Somite development 78 h: Blood circulation 7 d: Blood circulation 10 d: Blood circulation 14 d: Blood circulation

The maintenance and breeding of zebrafish has been described in detail by Nagel (2002). As modifications, green plastic wire materials were used as spawning stimulants instead of glass plants (Fig. 5.3).

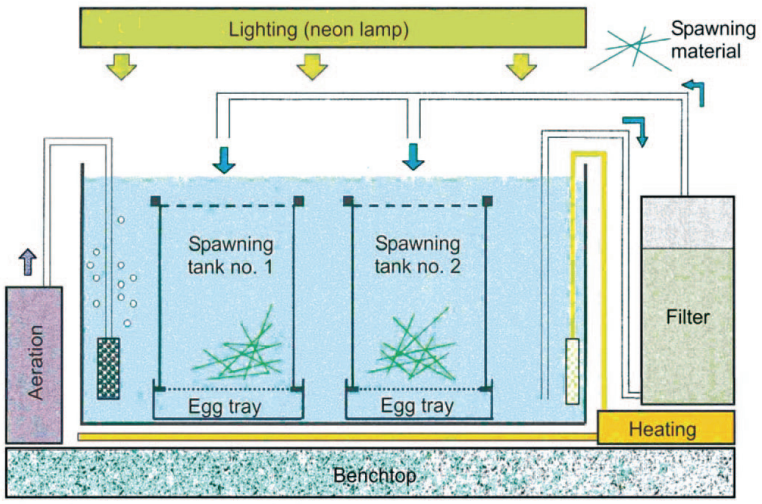

Fig. 5.3: Setup of the tanks used for breeding zebrafish (*Danio rerio*). Up to 10 tanks, the bottoms of which are replaced by a stainless steel grid, were placed on top of spawning dishes of similar dimensions. All spawning tanks were immersed into one bigger tank equipped with fully conditioned aquarium water. To collect the eggs after spawning, the dishes can easily be removed from the breeding facility.

The bottom of the 3 L breeding tanks had been replaced by a stainless steel grid with a mesh size of 1.25 mm; the breeding tanks were placed on rectangular full-glass dishes of similar dimensions as the tanks themselves. To collect the eggs after spawning, the dishes were removed from the breeding facility and placed under a temperature controlled dissecting microscope for counting and sorting of viable (i.e. fertilized) eggs. Alternatively, the eggs may be collected by means of larger trays placed below the normal maintenance tanks; however, it should be taken into consideration that the eggs recovered from a higher number of individuals in this mass spawning procedure are characterized by a higher genetic diversity than those derived from small spawning groups.

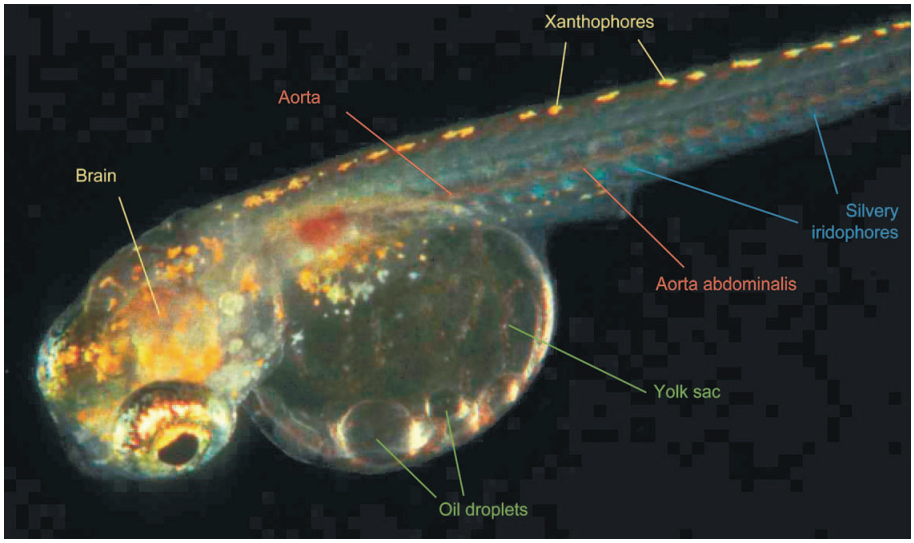

Fig. 5.4: Following dechorionation details of eye and brain formation, elements of the blood circulation and different pigment cells can easily be discerned in a 48 h old zebrafish (*Danio rerio*) embryo.

5.3.3 Fathead minnow

The fathead minnow (*Pimephales promelas*, Rafinesque, 1820) is another demersal cyprinid species originating from the temperate waters of central North America (Page & Burr 1991); it inhabits muddy pools of headwaters, creeks and small rivers. Among the three primary OECD species, the largest toxicological database is most likely that for the fathead minnow (Ankley et al. 2001; Gray et al. 2002; Keddy et al. 1995; Miracle et al. 2003; Sinks & Schultz 2001). Whereas females grow up to 1.5 ± 0.3 g, males may reach 2.5 ± 0.5 g in weight. Water quality parameters are essentially identical to those described for zebrafish; for water details of maintenance, see Table 5.2. In order to prevent permanent stress, parental fathead minnow were maintained at temperatures of approx. 16.5 ± 1.5 °C. Prior to spawning, the temperature was gradually raised to 24 °C at a rate of 1 - 2 °C/day, and fish were given increasing rations of live food (Tab. 5.2). During spawning, dominant males are not only considerably larger than females, but are also characterized by a pronounced black coloration of their heads, a thick dorsal nuptial pad and nuptial tubercles, as well as conspicuous vertical black bands along body sides. Spawning females can easily be identified by an ovipositor. As for zebrafish, the fish used in the present study were 6 to 12 months of age and were stimulated to spawn by extra rations of brine shrimp. For spawning, fathead minnow were kept in small groups of two males and four females in 30 L tanks. Since fathead minnow are quite sensitive to disturbance during spawning, the spawning tanks should be kept under quiet conditions. As soon as a red clay tile divided into two halves was offered to each spawning group as a spawning ground, male fathead minnow built up individual territories with the tiles as their centers. During the spawning period, the tiles were inspected for eggs at intervals of at latest 60 minutes post fertilization. Usually, 100 - 250 eggs are laid per spawning act. Since the chorion only hardens at the first multicellular stages, the transparent, sticky eggs were given about two hours before they were removed and directly transferred to 6- or 24-well plates by means of flexible forceps. The development of fathead minnow is basically similar to that of zebrafish.

5.3.4 Japanese medaka

Similar to zebrafish, the medaka (*Oryzias latipes*, Temminck & Schlegel, 1846) has become a favorite model in developmental biology and molecular genetics (Furutani-Seikia & Wittbrodt 2004; Winn 2002; Wittbrodt et al. 2001) as well as ecotoxicology (Arcand-Hoy & Benson 1998; DeKoven et al. 1992; Hatanaka et al. 1982; Ishikawa et al. 1984; Seki et al. 2003; Seki et al. 2002). Medaka belongs to the ricefish (*Adrianichthyidae*, Huber 1996; Ishizaki 1994) within the order of *Beloniformes* and originates from Japan, China, Vietnam

and South Korea (Jordan & Snyder 1906; Shima & Mitani 2004). The biology of medaka is well documented (Hyodo-Taguchi & Egami 1985; Kirchen & West 1976; Yamamoto 1975), and its requirements with respect to maintenance are comparable to those of zebrafish; in fact, both species can easily be raised side by side in one aquatic system (Furutani-Seikia & Wittbrodt 2004). Most of the standard experimental procedures can be applied to both species with slight modifications, including the observation of embryos, gynogenesis, sperm freezing and in vitro fertilization, cell transplantation, as well as RNA and DNA injection, in situ hybridization using riboprobes and immunohistochemistry. Once dechorionated, handling of the softer Medaka embryos requires some practice. Medaka embryos tolerate a wide temperature range (4 - 35 °C until the onset of heartbeat and 18 - 35 °C thereafter, compared to 25 - 33°C in the case of zebrafish, Westerfield 2000). Since the original habitat of the Japanese medaka is still water, care should be taken to avoid excessive turbulence. Water characteristics are basically identical to those described above for zebrafish. Up to 60 individuals of either sex were kept in 50 L tanks at an ambient temperature of 24 ± 0.5 °C (Tab. 5.2). During the spawning period, females (0.35 ± 0.07 g) can readily be identified by a generally more plump body shape and the sticky eggs attached to the anal fins, while male individuals (0.35 ± 0.07 g) are characterized by larger anal fins and the so-called papillary processes on posterior dorsal fin rays. For optimal stimulation of spawning, 5 to 12 month old medaka should best be kept under normal daylight conditions with extra rations of frozen adult brine shrimp (*Artemia spec*.). Moreover, spawning success can be further improved by means of natural plants (*Ceratophyllum spec.*) in the aquaria. The female medaka spawns between 20 and 40 eggs every day within an hour after the onset of light. Again, care should be taken to select a suitable strain (cf. Wakamatsu & Ozato 2002). Medaka eggs measure about 1 mm in diameter; they are transparent and characterized by an orange color and conspicuous spiny hooks, which allow firm adhesion to the anal fin of the female (cf. Fig. 5.7), but - to some degree - hinder the visibility of malformations during development. On the other hand, the attached egg clutch allows instant identification of reproductively active females. If required, attachment filaments on the chorion can be removed easily by rolling eggs on a piece of Whatman filter paper or by proteinase K digestion. Since the medaka chorion is quite stable, the eggs can be removed from the anal fin as soon as 30 min post fertilization and can be transferred to 6- or 24-well plates by means of a thin metal wire loop. Compared to zebrafish, the development of medaka is slightly slower; normally developing embryos only hatch after approx. 7 days of incubation. Developmental stages and corresponding morphological characteristics have been described in detail by (Iwamatsu 1994, 2004); an in-depth comparison of developmental staging with zebrafish has been compiled by Furutani-Seikia and Wittbrodt (2004).

5.3.5 Embryo toxicity test

The principle of the embryo assay has been described by Schulte and Nagel (1994) as well as Nagel (2002). Viable eggs of zebrafish, fathead minnow or medaka were exposed from at latest 30, 120 or 45 min after fertilization, respectively. To ensure exposure of the earliest stages of development, the eggs were rapidly transferred to an 18.5 cm diameter petri dish with one of the 4 - 5 dilutions of the test compounds. After at latest 30 minutes, the eggs were transferred by means of pipettes to 24-well microtiter plates and incubated without additional aeration at appropriate temperatures (Tab. 5.2) under a 12-hour light: dark regimen. For species comparison, the following compounds were tested without solvents in 3 - 7 independent replicates (microtiter plates): copper sulphate, sodium dodecyl sulphate (SDS), 3,4-dichloroaniline and 2,4-dinitrophenol. All toxicants were of p.a. quality and obtained from Sigma-Aldrich (Deisenhofen, Germany). The dilution water corresponded to reconstituted water according to ISO 7346/3 (ISO 1996; conductivity: 700 µS/L, NO_3^-: < 4.5 µg/L, NO_2^-: < 8 µg/L, NH_4^+: < 1.9 µg/L, PO_4^{3-}: < 50 µg/L, Fe^{2+} and Fe^{3+} not detectable; chemical oxygen demand (COD): < 4 mg/l), which was diluted 1:5 using double-distilled water. In order to prevent interaction between adjacent cavities in the 24-well plates, the plates were covered with self-adhesive foil (TTP; Renner, Dannstadt, Germany). Excessive adsorption of the chemicals to the wells of the microtiter plates was avoided by 24 h incubation of the toxicant prior to addition of the eggs. The major toxicological endpoints used for the species comparison are identical to those defined in the German DIN 38415-T6 (DIN 2001; Tab. 5.1) and are basically equivalent to those specified by McKim (1977) as sensitive “early parameters” (see also Chorus 1987, Woltering 1984). In addition to egg coagulation, non-development of somites, non-detachment of the tail and the lack of a heart-beat, the following endpoints were recorded as appropriate (Nagel 2002): completion of gastrulation, completion of somite formation, development of the eyes, spontaneous movement, presence of blood circulation in the dorsal aorta (cf. Fig. 5.4), degree of pigmentation, formation of (mainly cardiac and yolk sac) edemata, as well as malformations of the spinal cord (scoliosis, rachitis), the tail (bends), the heart, the yolk sac and general growth retardation. A test was classified as valid, if more than 90 % of the control embryos survived without malformation.

5.3.6 Determination of zebrafish embryo oxygen requirements and consumption

In order to determine the oxygen requirements and consumption by zebrafish embryos, eggs were incubated and exposed to 3,4-dichloroaniline and 2,4-dinitrophenol under low oxygen concentrations varying between 3 and 6 mg/l, which were prepared by selectively

fumigating the solutions with nitrogen. In addition, the 24-well microtiter plates were incubated within closed chambers under a pure nitrogen atmosphere. In a second series of experiments, zebrafish eggs (embryos) were maintained at densities between 10 and 60 individuals per ml medium under similar conditions. To elucidate the minimum space required by a zebrafish embryo, eggs were incubated in 96-well microtiter plates (TTP; Renner) filled with 100, 200 and 300 µl water or toxicant (3,4-dichloroaniline, 2,4-dinitrophenol) concentrations and compared to eggs incubated in a total volume of 2 ml in 24-well microtiter plates.

5.3.7 Dechorionation of zebrafish embryos

In order to investigate the role of the chorion as a barrier for the uptake of toxicants by zebrafish embryos, the chorion of 6 h old embryos (pre-exposed from 30 min post fertilization) was softened by incubation in a 2 mg/l pronase solution (protease from *Streptomyces griseus*, Westerfield, 2000; Sigma-Aldrich) with an activity of 4 units per mg in dilution water for 1 ± 0.5 min at 28.5 °C and mechanically disrupted by means of two very fine pairs of forceps or dissection (insect) needles. Control experiments were carried out to ensure that pronase treatment at the given concentrations did not have any effect on the further development of the embryos. Since the optimal incubation time depends on the developmental stage, the optimal duration should be checked in range finding experiments. Without enzymatic digestion, the mechanical stress resulted in destruction of the embryo. Disruption of the chorion without enzymatic softening of the egg shell turned out to be possible only in embryos older than 48 h. Dechorionated embryos were incubated in 24-well microtiter plates and exposed to potassium chromate (hydrophilic), 4-chloroaniline (moderately lipophilic) and lindane (lipophilic; all p.a. quality; Fluka-Sigma-Aldrich) and compared to non-dechorionated embryos exposed under similar conditions. In order to avoid excessive adsorption to the microtiter plates, the wells were incubated with toxicant 24 h prior to addition of the eggs.

5.4 Results and discussion

5.4.1 Correlation of zebrafish embryo toxicity to conventional acute fish toxicity

In an independent statistical analysis on behalf of the German Federal Environmental Agency (UBA), Ratte & Hammers-Wirtz (2003) analyzed the existing data from zebrafish embryo tests, most of which were carried out within various diploma and PhD theses at Dresden University as well as within a comparative laboratory study organized by Roland Nagel

(Dresden), with respect to their correlation to existing data from acute in vivo fish tests (Fig. 5.5). On the basis of data from tests with 56 substances, the authors arrived at the conclusion that there is reliable correlation between the fish embryo test and the acute fish test (R^2 = 0.854; alpha = 0.05). The confidence belt of the regression line was found to be relatively narrow, which was considered to be due to the large sample size of 56 (Fig. 5.5). In contrast, the prediction range was relatively wide (2.36 to 2.5 logarithmic units; Ratte & Hammers- Wirtz 2003). This corresponded to possible deviations by a factor of 229 and 320. As a consequence, the regression function seems appropriate to describe the average relationship between the acute fish test LC_{50} and the embryo test LC_{50} with good confidence, but less appropriate as a prediction model, since, for a single substance at a given value of embryo test LC_{50}, the prediction of the acute fish test LC_{50} may vary by a factor of up to 320 (and in 5 % of cases even more; Ratte & Hammers-Wirtz 2003). Since, however, in the initial steps of risk assessment, safety factors of up to 1,000 are common, a factor of even 320 would be covered. Moreover, it should be noted that for the correlation analysis the acute fish test LC_{50} values were taken as 48 h data from various sources including peer-reviewed publication, textbooks and data from the laboratory of Roland Nagel (Dresden University; 13 substances), but also from personal communications (8 substances). For all of these sources, it is difficult to assess the quality of the in vivo data. Only 24 out of the 56 chemicals were tested in zebrafish; all other data originated from tests with golden ide (*Leuciscus idus melanotus*; 18 substances), fathead minnow (10), bluegill sunfish (*Lepomis macrochirus*; 1) or even non-specified sources. Ratte & Hammers-Wirtz (2003), however, speculated that the majority of acute fish tests was conducted according to OECD 203 or DIN EN ISO 7346-1 (DIN 1998). In their statistical analysis, the authors arrived at the conclusion that even without the data from personal communications the correlation was good. For the analysis of a possible species-specificity, the number of data points was clearly too small. On the basis of 48 h in vivo data, the regression line for the correlation between acute fish and acute embryo toxicity starts almost perfectly at the origin of the axes, i.e. even very subtle fish toxicity will be mirrored by fish embryo toxicity. However, since the normal test duration of acute fish tests is 96 h, which may usually be expected to be lower than the corresponding 48 h values, the slope for the 96 h regression line may be expected to be smaller than shown in Fig. 5.5. Yet, with respect to the replacement of the acute fish test, the most important prerequisite is the stability of the correlation, not the slope. In their overall conclusion, Ratte an & Hammer-Wirtz (2003) maintained that, in spite of the prediction weakness, the average relationship between the acute fish and embryo toxicity would lead on average to the same risk assessments as with the acute fish tests. In contrast, for a given non-fish-tested substance, the assessment could be different by a factor of more than 300. Hence, it remains

to be discussed by the authorities in charge of risk assessment, how to weigh such uncertainty against the animal protection issue. The uncertainty would be reduced if targeted research were conducted on the species- and substance-class specific responses in both the embryo and acute fish test. In case the acute fish test should be replaced by the fish embryo test, Ratte & Hammer-Wirtz (2003) recommend using the embryo test LC_{50} data directly in the risk assessment, rather than first predicting the acute fish test LC_{50} using the regression model and then basing the risk assessment on the predicted acute fish test LC_{50}.

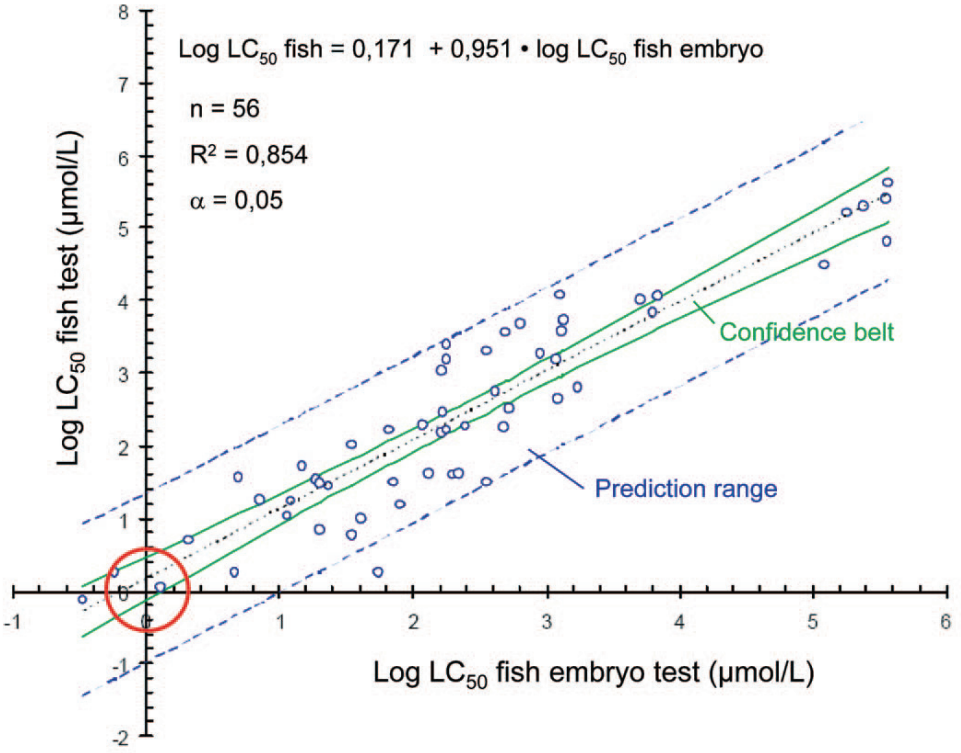

Fig. 5.5: Correlation between the zebrafish (*Danio rerio*) embryo test and the 48 h acute fish LC_{50} test (various species; n = 56). Note the facts that the slope of the regression is close to 1, and that the regression line almost perfectly crosses the origin of the axes, indicating that even lowest toxicities can be recorded. From Ratte & Hammers-Wirtz (2003).

5.4.2 Transferability of the fish embryo test to fish species other than the zebrafish

In a first set of comparative experiments, four substances (copper sulphate, sodium dodecyl sulphate, 3,4-dichloroaniline and 2,4-dinitro-phenol) were tested in parallel in zebrafish, fathead minnow and Japanese medaka embryos. Whereas in fathead minnow the observation of the core end-points (Tab. 5.1) did not pose any problems due to the good transparency of the egg shell (Fig. 5.6), the spiny hooks on the surface of the medaka chorion made the observations of embryonic malformation less clear (Fig. 5.7). Based on the

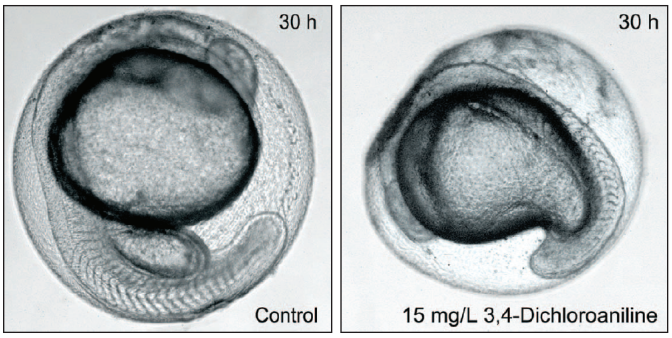

Fig. 5.6: Toxic effects of 3,4-dichloroaniline in fathead minnow (*Pimephales promelas*) embryos after 30 h of exposure are characterized by overall growth retardation, non-detachment of the tail, severe distortion of the yolk sac as well as conspicuous overall malformation of the embryo.

modifications to the zebrafish embryo test protocol outlined in Table 5.2, the difference in the results with the different species amounted to a maximum factor of approx. 10 only for sodium dodecyl sulphate, in that the medaka was significantly less sensitive than the other two species (Tab. 5.3). Except for 2,4-dinitrophenol, which proved most toxic in fathead minnow, all other substances were most effective in zebrafish embryos. According to the IUCLID (2000) database for existing chemicals of the European Chemicals Bureau, the 48 and 96 h LC_{50} values for sodium dodecyl sulphate in zebrafish are 8.81 and 7.97 - 20.1 mg/L, respectively (Fig. 5.8). For other fish species, 96 h LC_{50} values range from 3 mg/L in killifish (*Floridichthys carpio*) over 4.5 in bluegill sunfish, 4.6 - 8.6 mg/L in rainbow trout (*Oncorhynchus mykiss*), 4.8 - 22.5 mg/L in fathead minnow, 8.1 mg/L in American flagfish (*Jordanella floridae*), 10 - 46 mg/L in medaka, 13.5 - 18.3 mg/L in guppy (*Poecilia reticulata*), 23.7 - 34.9 mg/L in goldfish (*Carassius auratus*) to 60 mg/L in common carp (*Cyprinus carpio*). The example of sodium dodecyl sulphate illustrates the high variation of LC_{50} data not only found between species, but also within species (Fig. 5.8). In any case, the acute fish LC_{50} of sodium dodecyl sulphate are well represented by the data from the zebrafish embryo test (Tab. 5.3). The same conclusion holds true for 3,4-dichloroaniline, the acute fish 96 h LC_{50} data of which range between 2.6 and 13 mg/L, with 8.4 - 13 mg/L, 13 mg/L and 6.99 - 8.06 mg/L for zebrafish, medaka and fathead minnow, respectively (IUCLID 2000, Verschueren 1983). Acute fish toxicity LC_{50} values for 2,4-dinitrophenol average 0.75 - 1.55 mg/L. Thus, despite the limitations due to the still limited database of 4 substances, it seems basically possible to transfer the zebrafish embryo test protocol to the other major OECD

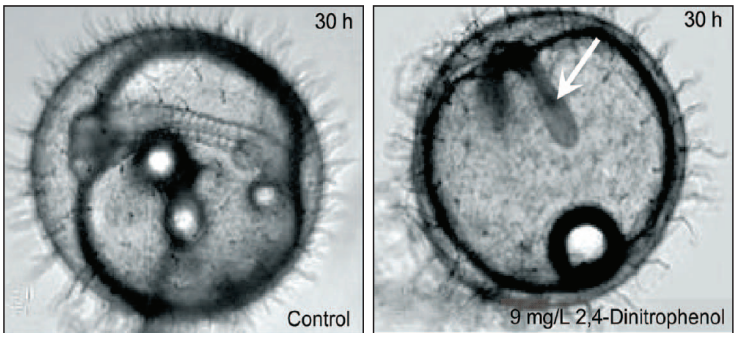

Fig. 5.7: Toxic effects of 2,4-dinitrophenol in Japanese medaka (*Oryzias latipes*) embryos after 30 h of exposure are mainly restricted to overall growth retardation. At arrow, note the clearly underdeveloped embryo.

species, the fathead minnow and the Japanese medaka.

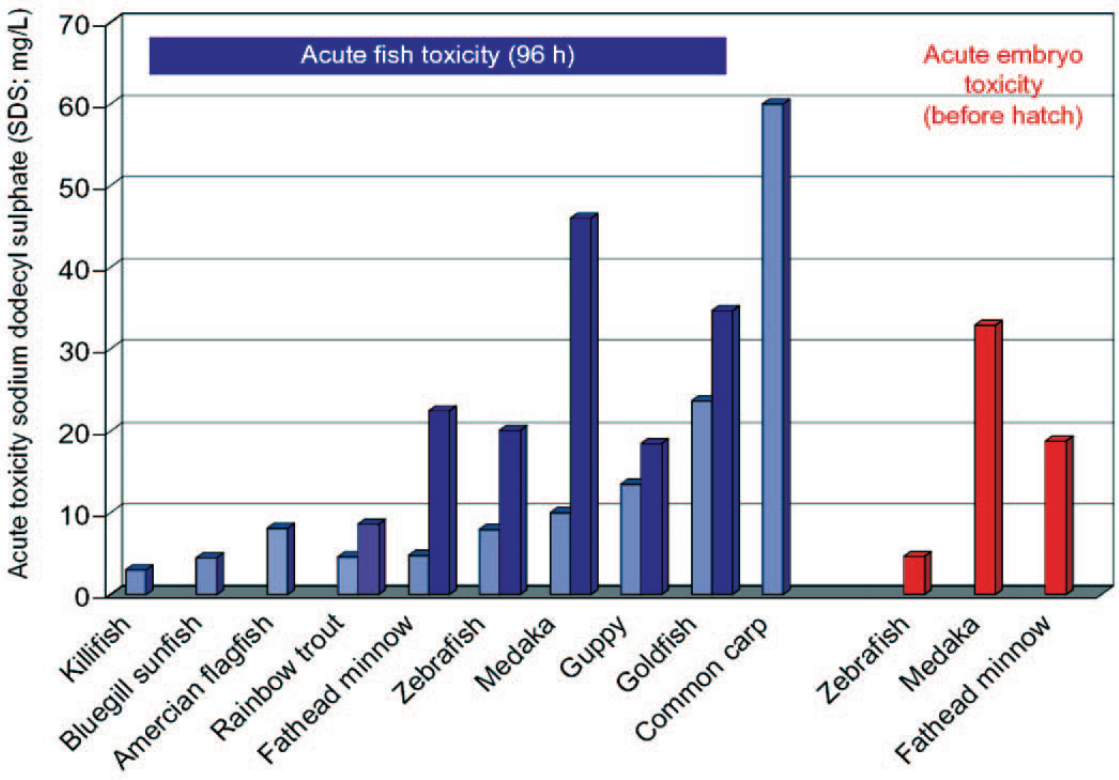

Fig. 5.8: Acute toxicity of sodium dodecyl sulphate (LC_{50}) to selected fish species *in vivo* as well as to embryos of zebrafish, medaka and fathead minnow *in ovo* after 48 h of exposure (red). For sources of *in vivo data*, see text.

Tab. 5.3: EC_{50} data for selected reference compounds from embryo tests with zebrafish (*Danio rerio*), Japanese medaka (*Oryzias latipes*) and fathead minnow (*Pimephales promelas*).

	Sodium dodecyl-sulfate (mg/l)	n	Copper-sulfate (mg/l)	n	3,4-Dichloro-aniline (mg/l)	n	2,4-Dinitro-phenol (mg/l)	n
Zebrafish (*Danio rerio*)								
24 h	3,96 ± 0,33	3	0,25 ± 0,08	4	3,28 ± 0,32	5	1,91 ± 0,33	4
48 h	4,68 ± 0,76	6	0,26 ± 0,07	6	3,44 ± 0,32	5	1,48 ± 0,19	4
Japanese medaka (*Oryzias latipes*)								
30 h	33,08 ± 3,54	7	2,95 ± 0,41	7	24,10 ± 11,88	6	6,42 ± 1,44	5
78 h	32,92 ± 2,79	7	2,22 ± 0,25	7	21,8 ± 2,97	7	5,24 ± 0,71	5
7 days	32,1 ± 3,08	7	1,60 ± 0,30	6	15,63 ± 1,07	7	5,42 ± 0,88	5
10 days	33,1 ± 1,41	3	1,13 ± 0,38	3	12,40 ± 3,25	3	4,53 ± 0,23	3
14 days	31,08 ± 2,97	4	0,68 ± 0,04	5	4,55 ± 1,01	5	3,88 ± 0,69	3
Fathead minnow (*Pimephales promelas*)								
28 h	22,68 ± 4,13	6	1,83 ± 0,29	5	17.1 ± 0,62	4	0,82 ± 0,35	6
56 h	18,82 ± 1,99	6	1,46 ± 0,48	5	8,08 ± 2,57	5	0,41 ± 0,10	4
3 days	15,37 ± 5,39	6	0,90 ± 0,01	5	5,93 ± 4,13	4	0,40 ± 0,15	4
4 days	13,83 ± 4,79	6	0,82 ± 0,08	5	4,35 ± 2,10	4	0,31 ± 0,09	4

5.4.3 Determination of zebrafish embryo oxygen requirements and consumption

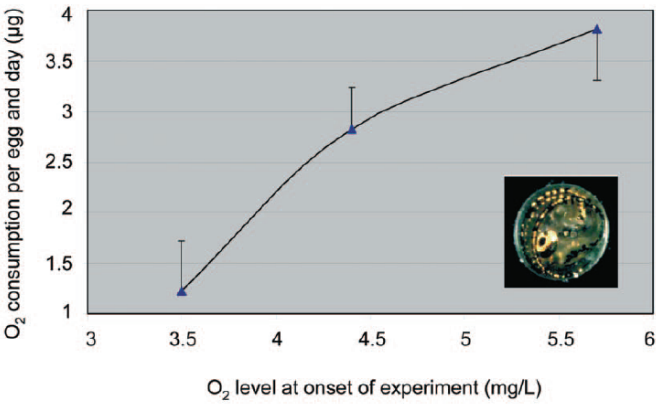

Fig. 5.9: Oxygen consumption of control zebrafish (*Danio rerio*) embryos in relation to the amount of oxygen provided in the medium. Apparently, zebrafish embryos are capable of adapting to low oxygen levels without any symptoms of developmental defects. Data are given as means from 4 independent experiments ± S.D.

In order to reduce the number of individual zebrafish embryos used for mechanical isolation of cells, the quantity of embryos to be homogenized per 2 ml PBS buffer was varied. Variation of DNA fragmentation negatively correlated with the number of embryos per preparation with a maximum deviation at 3 embryos per 2 slides (Fig. 5.5). The given correlation ($r = 0.88$, correlation coefficient according to Spearman) is not yet well understood, but might be explained by increasing mechanical stress, if cell titers decrease under a critical level during isolation. In more general terms, the mechanical stress induced during isolation might also result in direct disruption of the nucleus and chromosomal DNA in parallel to the release of cellular enzymes eventually leading to DNA alteration and fragmentation. Since the median tail moment varied only insignificantly between 1 and 4, and since, for reasons of animal care, the number of fish embryos should be kept to a minimum, eight embryos were selected as optimum for the standard protocol. Zebrafish embryos incubated at varying levels of oxygen in the medium were apparently well capable of adapting to low oxygen tension. The more oxygen was provided in the medium, the more oxygen the embryos consumed (Fig. 5.9). Even at oxygen concentrations as low as 2 mg/L, which should be expected to be lethal to adults of most other cyprinid fish species, zebrafish embryos did not show any symptom of malformation or even growth retardation (additional experiments; data not shown in Fig. 5.9). This observation is of particular importance for the routine testing of whole

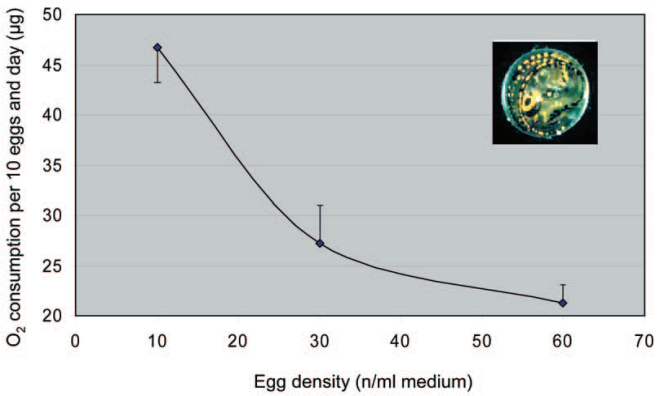

Fig. 5.10: Oxygen consumption of control zebrafish (*Danio rerio*) embryos in dependence of the stocking density in a given volume of medium. As expected from data presented in Fig. 9, the embryos adapt to low oxygen levels by reducing their oxygen consumption. Data are means from 5 independent experiments ± S.D.

effluents, since in sewage there may be severe oxygen depletion due to bacterial breakdown. As could be expected from the adaptive reduction of oxygen consumption under conditions of low oxygen levels, zebrafish embryos are also able to react to increasing stocking densities in a given volume of medium (Fig. 5.10). Exposure experiments with 3,4-dichloroaniline and 2,4-dinitrophenol did not show any change with respect to the exposure volume (Figs. 5.11, 5.12). As a consequence, zebrafish embryos cannot only be exposed in 24-well microtiter plates in a total volume of 2 ml medium, but also in even lower volumes of 300, 200 and even 100 µl within the cavities of 96-well microtiter plates. Thus, the fish embryo test provides a tool to test even smallest volumes of test substances, which may be of particular relevance to the testing of, for example, metabolites of pesticides. On the other hand, extremely lipophilic substances may require modification of the test protocol in that microtiter plates should be replaced by glassware. In such cases, the possibility to expose a higher number of embryos within one vessel to a small volume of test compound might also be a significant advantage.

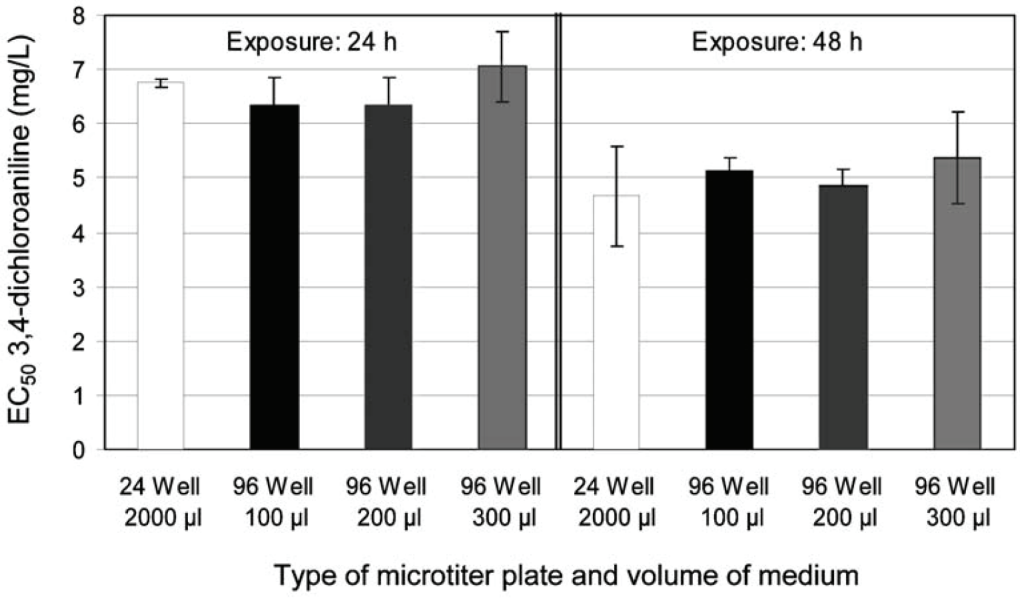

Fig. 5.11: Toxicity of 3,4-dichloroaniline to zebrafish (*Danio rerio*) embryos in various volumes of medium (2 ml in 24-well microtiter plates as well as 100, 200 and 300 µl in 96-well microtiter plates) after 24 h (left) and 48 h of exposure (right). The incubation volume does not seem to take any influence on the toxicity of 3,4-dichloroaniline.

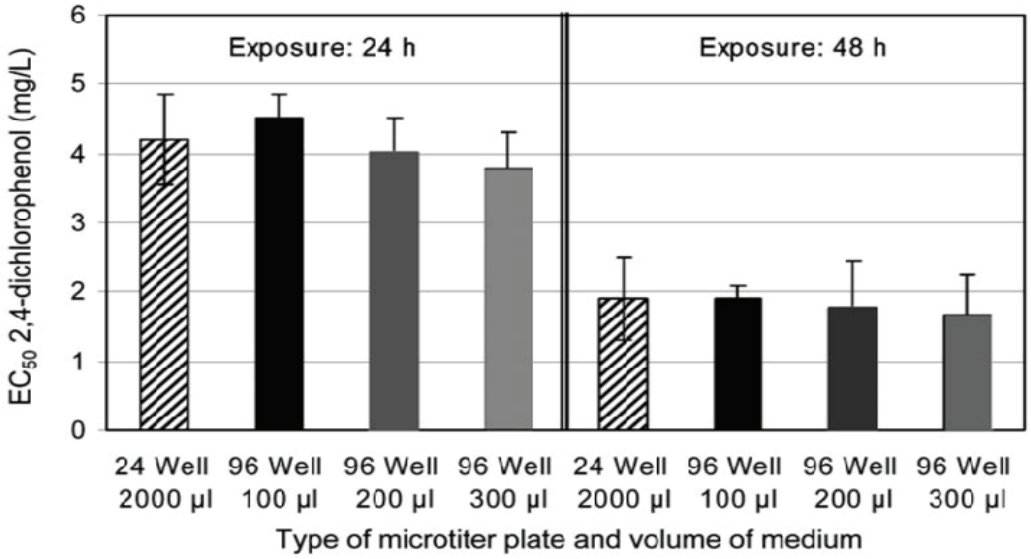

Fig. 5.12: Toxicity of 2,4-dinitrophenol to zebrafish (*Danio rerio*) embryos in various volumes of medium (2 ml in 24-well microtiter plates as well as 100, 200 and 300 µl in 96-well microtiter plates) after 24 h (left) and 48 h of exposure (right). Endpoints as listed by DIN standards. The incubation volume does not seem to influence the toxicity of 2,4-dinitrophenol.

5.4.4 The chorion of zebrafish embryos as a barrier for the uptake of chemicals

In an attempt to elucidate the effect of the chorion as a barrier, zebrafish embryos were exposed to various concentrations of potassium chromate, 4-chloroaniline, 3,4-dichloroaniline and lindane. Whereas exposure to the relatively hydrophilic potassium chromate did not result in any change of the core endpoints of the embryo toxicity test (details not shown), prolonged exposure of dechorionated embryos over 4 days (i.e. until hatching) produced severe disturbances to swimming equilibrium in hatched larvae (Fig. 5.13), thus indicating that the chorion did act at least as some form of barrier, even for hydrophilic substances. In contrast, for 4-chloroaniline, a significant increase in toxicity could already be recorded for the core endpoints of the fish embryo test as defined by the current DIN standards (Fig. 5.14; cf. Tab. 5.1). This increase in toxicity is even more pronounced for more lipophilic substances such as lindane: Whereas the EC_{50} value of lindane for normal embryos could be identified as 26.5 mg/L, the corresponding value for dechorionated embryos is 11.3 mg/L. Albeit significant, this difference in lindane embryo toxicity becomes relative when compared with the broad range of acute conventional (*in vivo*) fish toxicity from 2 mg/L in bluegill sunfish over 12 mg/L in fathead minnow, 14 mg/L in rain bow trout (*Oncorhynchus mykiss*) and 23 mg/L in golden ide to 26 mg/L in guppy (*Poecilia reticulata*; Verschueren 1983). Nevertheless, results indicate that the barrier function of the chorion

may increase with lipophilicity, a fact that should be taken into consideration in the interpretation of correlations between fish embryo tests and conventional acute fish tests.

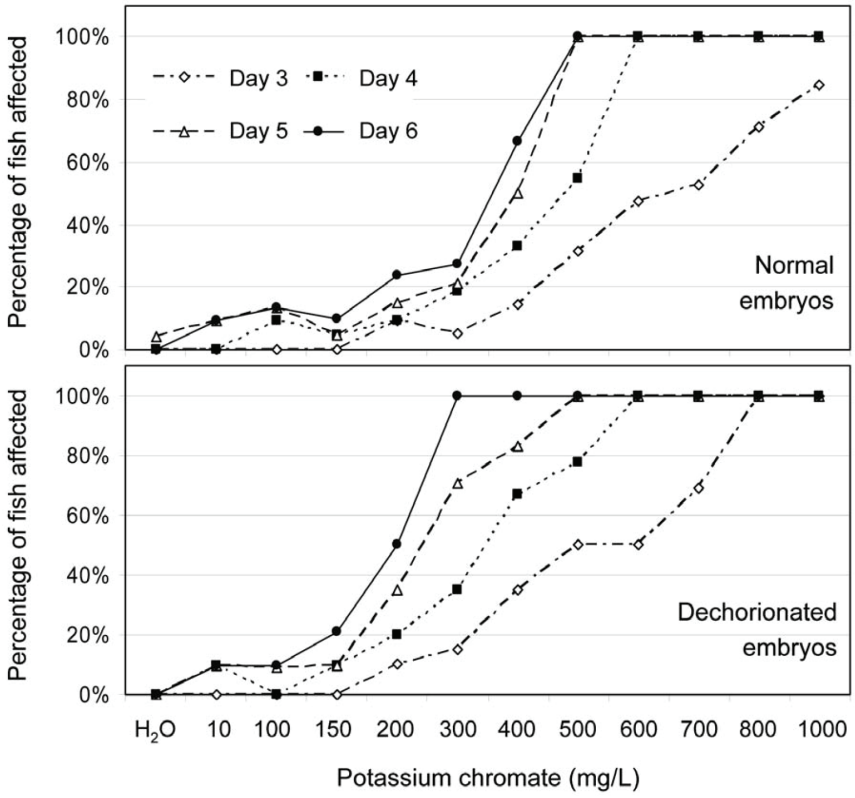

Fig. 5.13: Effects of potassium chromate on zebrafish (*Danio rerio*) embryos depending on the presence of a chorion (upper panel) or after dechorionation (lower panel) on the basis of disturbances of the swimming equilibrium after various periods of exposure. For potassium chromate, dechorionation does not affect acute embryo toxicity, but clearly shows sublethal effects.

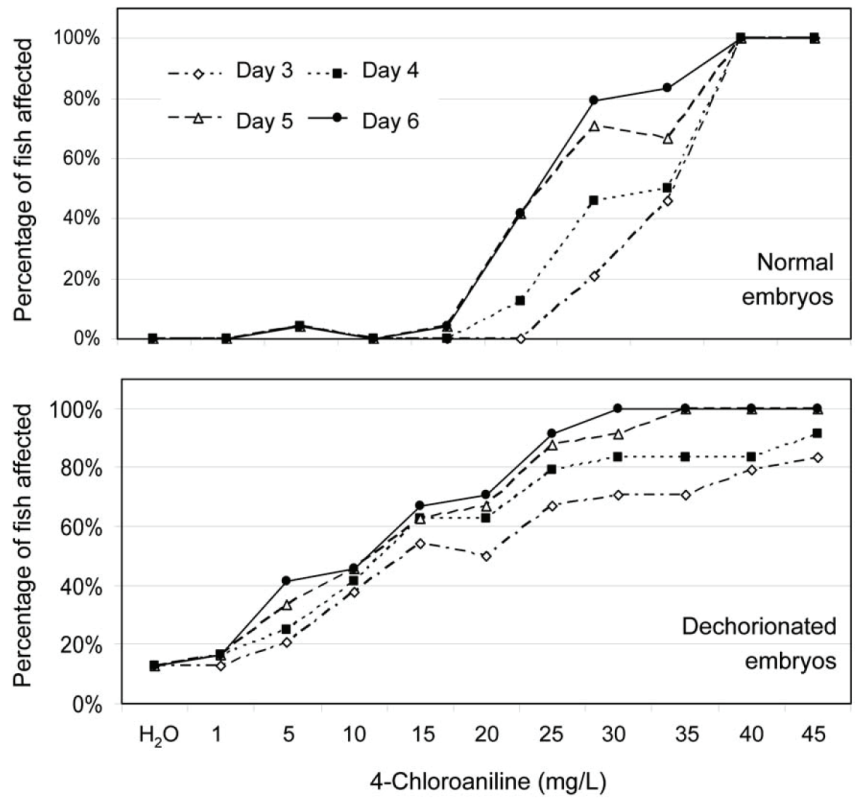

Fig. 5.14: Toxicity of 4-chloroaniline to zebrafish (*Danio rerio*) embryos depending on the presence of a chorion (upper panel) or after dechorionation (lower panel) based on acutely toxic effects as specified by DIN standards after various periods of exposure (cf. Tab. 5.1). For 4-chloroaniline, dechorionation results in an increase of acute embryo toxicity.

5.4.5 The fish embryo test - replacement or refinement of the acute fish test?

According to the widely accepted principle of the three Rs (Russell & Burch 1959), replacement, reduction and refinement are equally important strategies in the attempt to identify alternatives to animal experiments. In the European Community, the use of animals in scientific experiments has been regulated by Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes (EC 1986), according to which, fetuses and embryos are not defined as animals, and are, as such, not subject to animal welfare issues. As a consequence, from a purely juristic point of view, experiments with fish embryos are not regarded as animal experiments and might be classified as an alternative in the sense of replacement. However, there are ongoing discussions on how to define an "embryo" more precisely in the various vertebrate classes, and some consensus appears to be emerging that experiments with fetuses or embryos of mammals and birds should not be regarded as animal experiments, as long as they are allocated to the first half of gestation or incubation. For fish, however, the discussion is more complex, since there is not even consensus as to where the transition from an embryo to a larva should be set. Whereas in the majority of cases, hatching is thought to terminate embryonic life (as indicated by the solid red line in Fig. 5.15), there are views that the transition to external feeding, i.e. using up

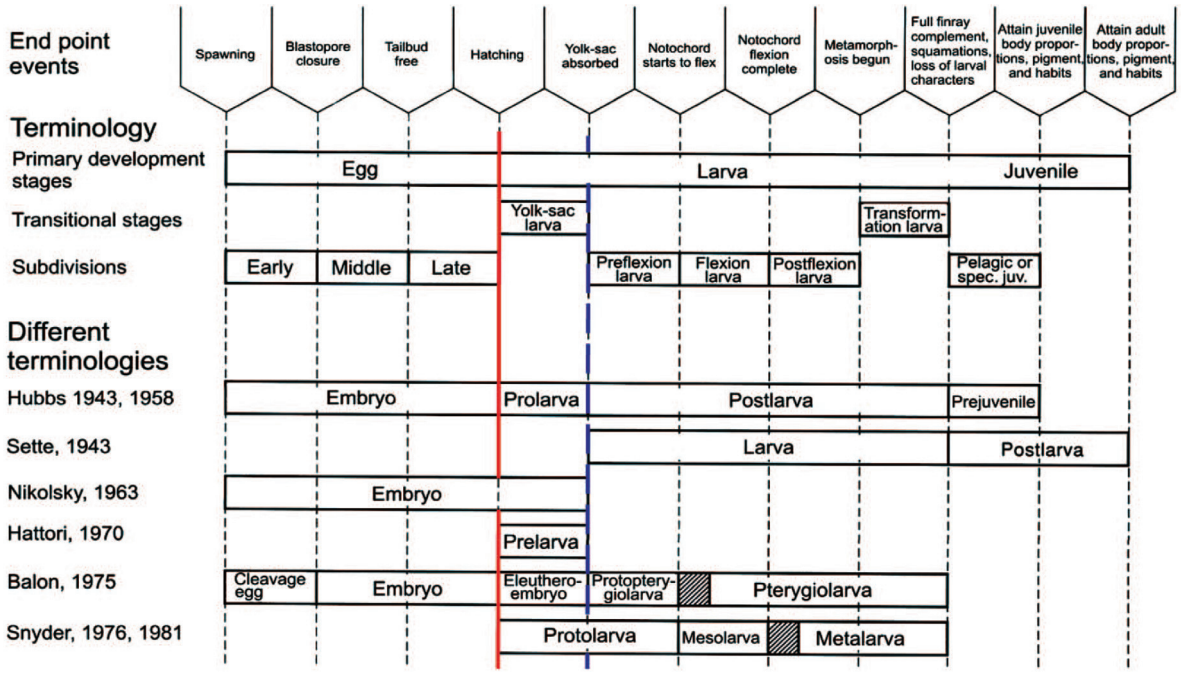

Fig. 5.15: Overview of different terminologies of a fish "embryo" and a fish "larva" (redrawn from Kendall et al., 1984). Whereas the solid red lines define the transition from embryo to larva at the point of hatching, the broken blue line takes the absorption of the yolk sac as the critical step to the larval stage.

the yolk-sac reserves, is the critical step to independence (broken blue line in Fig. 5.15). However, the alternative to the fish test as proposed by Nagel (2002) and the present communication, is, in any case, limited to the pre-hatching period and can, therefore be termed a genuine “embryo” test. Nevertheless, beyond the formal juristic view, albeit official consensus, there may still be ethical considerations as to whether or not a fish embryo does represent an animal. If a 2 day old zebrafish embryo is experimentally liberated from its chorion (“dechorionated”; Figs. 5.2, 5.4), this individual looks almost fully developed and is capable of both swimming and interacting with its environment in a way similar to a normally hatched 4 or 5 day old embryo. Thus, even an egg shell protected fish embryo bears both morphological characteristics and behavioral traits of an intact animal. As a consequence, the fish embryo test should be classified as a “refinement" rather than as a “replacement” of the conventional fish test.

5.5 Additional fields of application for the fish embryo test

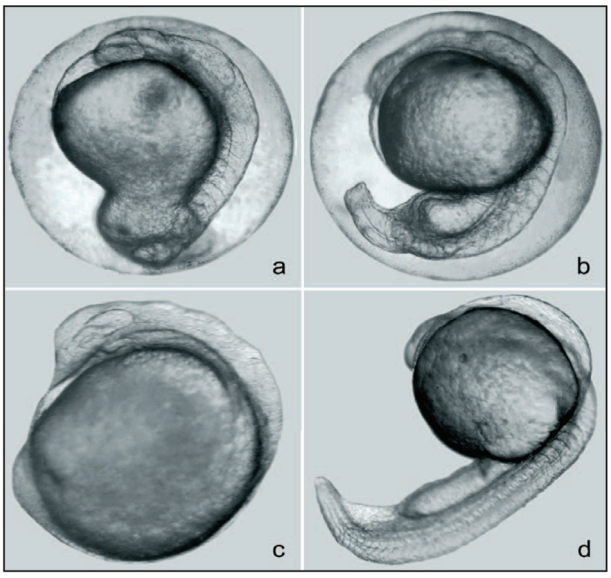

Fig. 5.16: Effects of 48 h exposure to organic extracts (a, b) or native samples (c, d) of Danube river sediments in zebrafish (*Danio rerio*) embryos (a, b) or dechorionated zebrafish embryos (c, d). In any case, the embryos are characterized by severe malformation of head, tail and yolk sac.

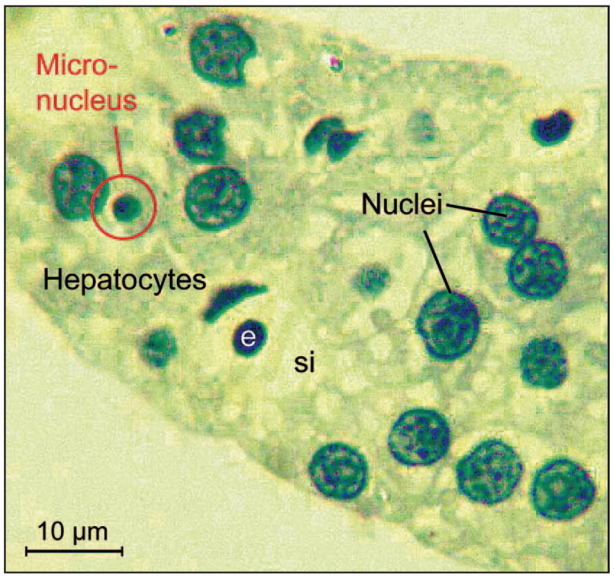

Fig. 5.17: Induction of micronuclei as indicators of genotoxicity in the liver of a 48 h old zebrafish (Danio rerio) embryo exposed in ovo to 190 µg/L 4-nitroquinoline-N-oxide. e - erythrocyte, si - sinusoid. Weigert’s ferric haemalaun stain.

Apart from its routine use in sewage and chemical testing, the fish embryo test may serve as a model system in other fields of ecotoxicology such as sediment toxicity assessment, genotoxicity and mutagenicity testing, histopathological studies and induction studies based on microarray techniques. Contaminated sediments, for example, have not only been

recognized as a major sink for persistent toxic substances released into the aquatic environment, but also as a potential source. There is an ongoing debate about appropriate ways to assess sediment toxicity, i.e. whether to test aqueous eluates, organic extracts or native sediment samples. Whereas aqueous eluates and organic extracts can easily be tested with the fish embryo assay (Figs. 5.16a, b), a sediment contact test, which uses zebrafish eggs to monitor the toxic effects of native sediments on a microtiter scale, has been published only recently (Figs. 5.16c, d; Hollert et al. 2003). As a definite test of genotoxicity, the micronucleus test (Al Sabti & Metcalfe 1995; Belpaeme et al. 1997; Chen & White 2004) can also be applied to 48 h old zebrafish embryos. Since erythrocytes are not accessible in adequate amounts for the micronucleus test, the liver as a rapidly developing organ may be selected as the monitoring organ. Best results were obtained by fixing with a mixture of 1.5 % formaldehyde and 1.5 % glutardialdehyde and embedding in moderately hard Spurr's resin (Spurr 1969). After removal of the resin by incubation in sodium hydroxide, sections were stained with ferric haemalaun by Weigert (Böck & Romeis 1989) for 5 - 10 min (Fig. 5.17). Results of the micronucleus assay can be confirmed by data obtained with the alkaline comet assay (Schnurstein & Braunbeck 2001; Singh et al. 1988). *In ovo* exposure of zebrafish embryos for only 1 h to fixed concentrations of the known genotoxin hydrogen peroxide results in a clear-cut dose-dependent increase in the tail moment as the product of tail length and relative DNA density within the tails (Fig. 5.18). The heterogeneous single cell suspension from the embryos can be prepared by simple mechanical isolation from anaesthetized 48 h old embryos (Kosmehl et al. 2006).

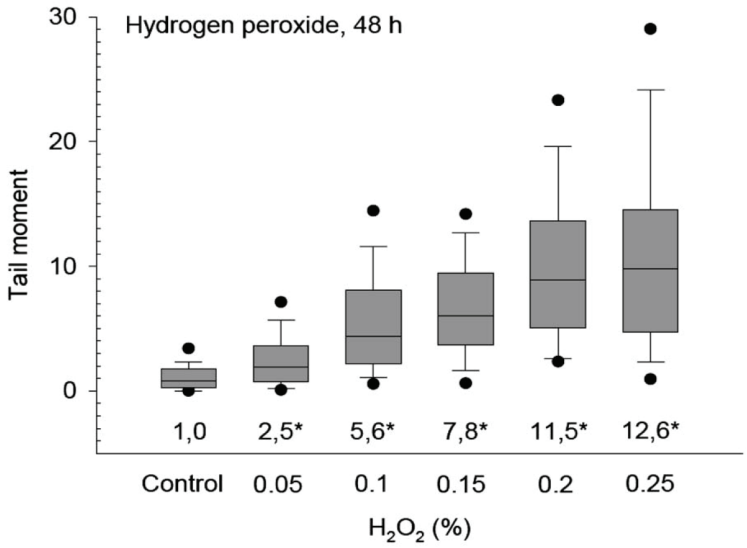

Fig. 5.18: Genotoxic effects of hydrogen peroxide in a heterogeneous cell suspension obtained from zebrafish embryos after 1 h of incubation. Cells were obtained by mechanical isolation from anaesthetized embryos at the age of 48 h. Data are given as box plots with median (central line), 25 and 75 percentiles (grey boxes), standard deviation as well as 5 and 95 % values.

5.6 Future perspectives and conclusions

For sewage treatment testing, the (zebra-) fish embryo (egg) test according to DIN 38415-T6 (2001) has been obligatory in Germany since 2002. Yet, in case of overstepping of threshold values, the fish embryo results had to be verified in the conventional acute fish test. Since January 2005, however, the fish embryo test stands on its own, i.e. the acute fish test has been banned from the routine sewage testing procedure in Germany. Until the end of 2005, all administrative procedures will have to be adapted to the new protocols. With respect to ISO standardization, the fish embryo test for sewage testing passed all discussion stages and has reached ISO discussion, i.e. the second last step to international standardization. As already pointed out by Nagel (2002), however, the embryo test has its place not only in sewage, but also in chemical testing and potentially in QSAR (quantitative structure-activity relationship) procedures. The present update documents that the fish embryo test protocol can readily be applied to other OECD species, i.e. fathead minnow and medaka. Further points of criticism by experts addressing the impact of disturbing factors such as illumination, temperature, oxygen supply or osmotic stress are currently under investigation. Additional experimental data for highly lipophilic compounds, substances with specific modes of action as well as chemicals that are specifically toxic or nontoxic to fish will be provided by summer 2005 and a modified draft guideline will be submitted to the OECD together with a draft detailed review paper on fish embryo toxicity testing by the end of 2005. With respect to additional fields of application for the fish embryo test, more-in-depth research is required. First results for its applicability to genotoxicity and mutagenicity testing are encouraging and will be pursued further.

Chapter 6

Final discussion and conclusion

In 2007, REACH (Registration, Evaluation, Authorization and Restrictions of Chemicals) has come into force. It is a “volume-triggered notification system” approach, and all chemicals on the European market or produced or imported in volumes above 1 ton need to be registered. Manufacturers and importers are required to gather information on the properties of their substances and to submit the information in the form of a registration dossier to a central database managed by the European Chemicals Agency (Brown 2003; Combes et al. 2003; Petry et al. 2006). Since the earlier chemical testing regime was less rigorous in its testing requirements, there is concern that a substantial number of the existing chemicals on the EU market may not have been sufficiently tested and could therefore be harmful due to so-called data gaps in the information body on their hazard potential (Combes et al. 2003). These data gaps now need to be filled.

Within the framework of REACH, animal testing should - whenever possible - be reduced or even replaced by alternative methods (according to the concept of 3Rs by Russell & Burch 1959; EC 2001), and already the Animal Welfare Guideline 86/609/EC in 1986 (EC 1986) highly promoted the development and validation of alternatives to animal testing by EU institutions (Lilienblum et al. 2008). However, the 96 h acute fish test is still a mandatory component in the “base set” of data requirements of chemical and risk assessment. Concerning animal welfare and the fact that an increasing number of researchers suggest that some form of pain perception, similar to what is present in mammals, may be present in bony fish (Chandroo et al. 2004; Huntingford et al. 2006; Sneddon et al. 2003), this test should be replaced by an alternative. Since in Germany the 96 h acute fish test has already been replaced by the fish embryo toxicity test (FET) for whole effluent testing, this approach seems to be a good candidate to replace the acute fish test also in chemical testing. Nagel (2002) and Braunbeck et al. (2005) already demonstrated that the FET is a very promising tool to replace the acute fish test in chemical testing.

However, the correlation between the alternative procedure and the test to be replaced is one of the most important criteria for a test to be accepted as an alternative to a conservative test. Therefore, a preliminary analysis of existing data for the FET and the 96 h acute fish test has been performed by Ratte & Hammers-Wirtz (2003) and resulted in a R^2 of 0.854. Nevertheless, the study included data from both personal communications and acute fish test data from non-verified sources and, thus, some outliers were left. A thorough re-evaluation of FET and acute fish test data has been conducted and resulted in an overall R^2 of 0.90 for the comparison of FET data and acute fish test data. Restricting the data to zebrafish (*Danio rerio*) FET data and all fish data, the correlation resulted in an R^2 of 0.87. In general, all correlations with different restrictions of the complete dataset (e.g. only zebrafish FET and zebrafish acute fish test data, only 48 h FET and 96 h fish toxicity data,

etc.) showed no R^2 lower than 0.81. For comparison between different biotests, these are definitely excellent correlations, and compared to correlations of 96 h acute fish data among different fish species, they are in the same range or even better. For example, the lowest correlation between medaka (*Oryzias latipes*) and zebrafish resulted in an R^2 of 0.719, and the highest correlation between rainbow trout (*Oncorhynchus mykiss*) and medaka gave an R^2 of 0.96. Thus, the result of the present thesis proved that the acute fish test can be replaced by the FET. The results fit in the range of general fish toxicity. Since the tremendous number of acute LC_{50} data varies by orders of magnitude not only between species, but also for the same species between laboratories, the FET would have similar predictive power for fish toxicity as the acute fish test.

However, most of the FET data have been retrieved from experiments with zebrafish. Further investigations are needed to clarify if a higher number of embryo tests with fish species other than zebrafish would modify the correlation and the quality of the correlation. Braunbeck at al. (2005) already showed that an adapted and optimized test protocol for the FET can equally be applied to the early embryonic stages of other OECD species such as the fathead minnow (*Pimephales promelas*) and the medaka. However, given its high number of eggs per spawning, the rapid development, the perfect transparency of its eggs, and the immense body of existing information on zebrafish development (Braunbeck et al. 2005; Nagel 2002), the zebrafish seems to be first choice for routine embryo toxicity testing.

However, in chemical and risk assessment, acute data from one single species are used as surrogates for entire species groups. Since the correlation between the FET and general fish toxicity is almost perfect, the FET can be used in risk assessment without any concern. Even more so, since the test would be used in a test battery in combination with the acute toxicity for daphnids (OECD 202, OECD 2004b) and the growth inhibition test with freshwater algae (OECD 201, OECD 2006). Hoekzema et al. (2006) reported that in 88 % out of 507 compounds fish were not the most sensitive compared to acute toxicity to daphnids and algae. The EC_{50}s for algae and *Daphnia* were lower than or equal to the LC_{20} for fish. For all compounds investigated in this study, the lowest LC_{50} or EC_{50} was a non-vertebrate value, and application of the acute threshold test with fish would not reduce this value (Hoekzema et al. 2006). Likewise, Weyers et al. (2000) documented that the algal growth inhibition test was the most sensitive, giving the lowest value in 43.5 % of cases. This test alone triggered most stringent classifications in 22.9 % of all cases. In contrast, fish and *Daphnia* results together led to stricter classification in only 17 % of all cases. Hence, if the FET was used in chemical hazard and risk assessment instead of the acute fish test, due to the nearly ideal correlation, the results would not change chemical classification and the outcome of risk

assessment most likely. Therefore, concerning animal welfare and the number of substances which need to be tested under REACH, the acute fish test should be replaced by the FET.

However, some outliers could still be identified. There are several hypotheses to explain the discrepancies between the acute fish test and the FET. Apart from differential metabolism and the barrier function of the chorion, restricted availability of the test substances due to the static nature of the protocol for the standard fish embryo test in polystyrene 24-well microtiter plates might be a reason. Several authors reported that polystyrene surfaces are highly hydrophobic (Koutsopoulos et al. 2007; Palmgren et al. 2006) and that lipophilic and positively charged drugs and chemicals show strong interactions with negatively charged polystyrene (Dahlström et al. 2004; Knorr & Gätzschmann 1966; Koutsopoulos et al. 2007; Palmgren et al. 2006). However, the limited binding sites can be saturated (Palmgren et al. 2006).

Therefore, in order to optimize the FET, a flow-through system on the basis of commercially available polystyrene 24-well microtiter plates was developed. In a flow-through system, the limited electrostatic binding sites of the polystyrene plates can easily be saturated, and the concentrations can be maintained constant more easily during the whole testing period. Additionally, by using a flow-through system, adequate amounts of test substance for chemical analysis could be obtained, which might be rather limiting in the static FET. Moreover, in a flow-through system oxygen deficiency is very unlikely to occur. Becker & Crass (1982) showed unacceptable dissolved oxygen declines in both static and static-renewal acute toxicity tests with the fathead minnow. In contrast, the flow-through system provides relative stable levels of dissolved oxygen. For the FET, adequate oxygen concentrations in the surrounding medium are required, since the oxygen consumption of embryos, especially if they are still in the chorion, takes place *via* passive processes depending on the oxygen gradient (Kranenbarg et al. 2003). A flow-through system usually guarantees constant oxygen concentrations, whereas in static systems oxygen gradients may occur. However, the preparation of the plates is a very time-consuming and laborious procedure, and the plates are not yet commercially available.

In order to examine if the static testing procedure is responsible for the differences in toxicity of the acute fish test and the FET, four chemicals with increasing log P_{ow} were tested in the static and the flow-through FET. Independent of chemical nature and lipophilicity, the flow-through and the static FET gave almost the same results in every test. No significant differences could be detected in the results of the flow-through FET.

Due to chemical analysis, a saturation of the flow-through system could be demonstrated. However, since the 24-well microtiter plates could also be saturated under static conditions

24 h prior to the beginning of the test, this procedure seems to be equally suitable (results of the flow-through and the static FET did not differ significantly). Hence, the flow-through system seems to be no further improvement compared to the static version plus pre-saturation. Thus, for selected compounds, the differences in results between FET and acute fish test persist.

Hence, since the differences between the acute fish test and the FET could not be explained by the static nature of the FET procedure, two hypotheses remain to be discussed. First, the chorion may act as a barrier. Already Van Leeuwen et al. (1985) reported that the chorion appears to 'protect' the embryo from the uptake of toxicants, not by completely preventing but by slowing down the intrusion. They assumed that egg membrane permeability decreases during early embryo genesis. Nevertheless, it was also reported that most lipophilic compounds can easily penetrate membranes. Léonard et al. (2005) conducted a comparative study on the acute toxic effects of two sets of selected quaternary ammonium salts and 4 groups of surfactants (anionic, cationic, non ionic and amphoteric) on zebrafish and medaka. They found that embryonic stages of both species showed similar sensitivities to the tested chemicals. Most importantly, however, for the quaternary ammonium salts, they found lower LC_{50} values for the eleutheroembryos (stage after hatch until absorption of the yolk) of either species than for the non-hatched embryonic stages. In fact, in all cases the LC_{50} values for the eleutheroembryos showed a better correlation to corresponding *in vivo* data from the OECD 203 test. As a conclusion, Léonard et al. (2005) recommended, with regard to the selective permeability of the fish eggs envelops (chorion), e.g. to quaternary ammoniums, testing on fish eleutheroembryos, which still rely on autotrophic vitellogenic supply, before they become capable of independent feeding, rather than testing with embryos inside the chorion. Additionally, from a physiological and toxicological point of view, hatch is a critical stage during embryogenesis (Van Leeuwen et al. 1985) and should be included in the testing procedure if the exposure already starts with non-hatched embryos. Scholz et al. (2008) also reported that the eggshell could provide a barrier for uptake and extrusion of chemicals, and it cannot be excluded as a reason for outliers in the correlation of the acute fish toxicity and embryo toxicity.

The differential uptake and metabolization of the chemicals in the embryos and the adults may be another reason for the differences in toxicity. It is well established that teratogenic activities are not always due to parent compounds, but may also be caused by metabolites formed by maternal metabolism (Fantel 1982; Webster et al. 1997). Parent compounds, termed proteratogens, can be bioactivated to highly teratogenic metabolites, for example, electrophiles or free radical intermediates (Wells et al. 2005). Singh et al. (2007) found teratogenic effects in embryos of pregnant Wistar rats which were given endosulfan by oral

exposure during the gestational days 6 - 20. Nevertheless, they suggest a potential maternal P-450-dependent contribution to bioactivation and induction of teratogenicity.

Busquet et al. (2008) combined the fish embryo toxicity test with a Metabolic Activation System (MAS; microsomes from cytochrome P450-activated mammalian systems) and exposed zebrafish embryos to two proteratogens (cyclophosphamide and ethanol) and MAS for 2 h. They could show that there is insufficient CYP activity in the embryos at the earlier stages (2 - 3 hpf) to bioactivate proteratogens, e.g. cyclophosphamide. The exposure of fish embryos to each proteratogen alone did not result in any significant teratogenic effect. In contrast, only the presence of the mammalian MAS triggered the formation of significant lethal or teratogenic effects in exposed fish embryos. Only the combined exposure of fish embryos to MAS and proteratogens was able to reveal the teratogenic potential of the proteratogenic compounds CPA and ethanol. This is a clear indication that the different metabolization of chemicals in the embryo and in the adult fish may be responsible for the differences in the acute fish test and the FET.

However, further investigations are necessary to clearly identify whether the chorion or the metabolism may be responsible for the differences in toxicity to chemicals of adult and embryonic fish.

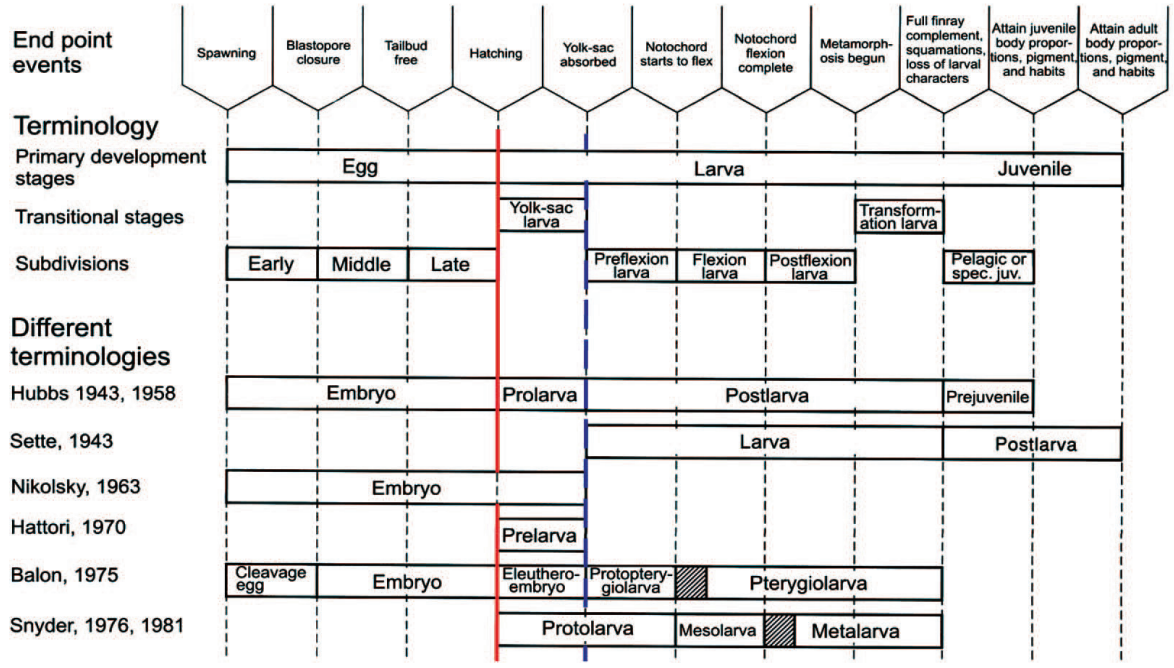

Fig. 6.1: Overview of different terminologies of a fish “embryo” and a fish “larva” (redrawn from Kendall et al. 1984).

It could be shown that the static FET definitely represents an alternative to replace the acute fish test in chemical and risk assessment. However, the question remains if the FET is a real replacement according to the principles of the 3 Rs (Russell & Burch 1959). Braunbeck et al.

(2005) reported that there is a consensus that experiments with fetuses or embryos of mammals and birds should not be regarded as animal experiments, as long as they are allocated to the first half of gestation or incubation. However, for fish there is no general and clear definition when the transition from embryo to a larva takes place. In most cases, hatching is thought to terminate embryonic life (red line in Fig. 6.1). In contrast, there are views that the transition to external feeding, i.e. using up the yolk-sac reserves, is the critical step to independence (blue line in Fig. 6.1).

In legal terms, at the moment only the UK Animal Procedures Act (UK 1986) covers fish. The protection of immature forms of fish first starts when they become capable of independent feeding. In the current standard version of the test protocol, the test with zebrafish is limited to two or three days and ends before hatch. Therefore, the test can clearly be termed as an embryo test. However, as mentioned earlier, for some chemical classes the chorion may act as a barrier (Léonard et al. 2005; Scholz et al. 2008; Van Leeuwen et al. 1985) and from a physiological and toxicological point of view hatch is a critical stage during embryogenesis (Van Leeuwen et al. 1985). Thus, the test should definitely be continued until hatch in order to exclude, that some chemicals would not be detected in chemical and risk assessment. Since independent feeding of zebrafish starts at approximately 96 h post fertilization, the test can be prolonged to that point of time. As zebrafish hatch approximately 72 h after fertilization, hatch would be included, and, according to the UK Animal Procedures Act, the test would be still classified as a non-animal test. Therefore, from a juristic point, the prolonged FET up to 96 h can be regarded as real replacement for the acute fish test.

However, apart from legal positions, there may still be ethical considerations if a fish embryo does or does not represent an animal. Braunbeck et al. (2005) stated that even an egg protected fish embryo bears both morphological characteristics and behavioral traits of an intact animal. As a consequence, the fish embryo test should be classified as a “refinement" rather than as a “replacement” of the conventional fish test.

Nevertheless, as already mentioned, the FET compared to the acute fish test covers a lot of advantages (1) A large numbers of chemicals can be tested in parallel, since a single mature female zebrafish lays 50 - 200 eggs per day (Braunbeck et al. 2005). Under laboratory conditions several thousand embryos can be produced daily and used for parallel experiments (Scholz et al. 2008). (2) By using 24-well microtiter plates, only very low volumes of test substances are required. It is particularly important when limited amounts of test solutions are available. (3) At present, the test duration of the embryo test is limited to two or three days, and is, therefore, less time-consuming compared to the conventional *in vivo* acute fish test. (4) Sublethal endpoints can easily be applied. (5) The embryos may have

not yet developed pain perception like adults, which is an important factor for animal welfare considerations. (6) The FET, compared to fish cytotoxicity tests, is more sensitive (Lange et al. 1995). (7) An optimized test protocol can easily be adapted to early embryonic stages of other OECD species such as the fathead minnow and the medaka (Braunbeck et al. 2005).

In conclusion, the FET should definitely be incorporated in routine chemical hazard and risk assessment, since the same overall results in risk assessment will be obtained. In order to test difficult substance also a flow-through system for the FET is now available, and the test is also applicable for fish species other than zebrafish. However, research is still needed why some chemicals do not elicit the same sensitive toxicity in embryos as in adult fish. It is still unknown whether the differences can be related to the presence of the chorion or if differential metabolism in embryos and adults is responsible for the different sensitivity, since the availability of test substance due to the static exposure definitely can be eliminated. Finally, it should be clarified if chemical classes or specific mode of actions of chemicals have to be identified as general outliers in the FET. Further tests with fathead minnow and medaka should be conducted to confirm existing conclusions and to check whether outliers also exist for other fish species.

Chapter 7

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