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Oral Examination:....……

Limits of the fish embryo toxicity test with *Danio rerio* as an alternative to the acute fish toxicity test

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Kirsten Henn

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Contents

Zusammenfassung

Als Folge der gegenwärtigen Entwicklungen in der europäischen Umweltpolitikpolitik in Bezug auf Chemikalien gewinnen alternative Methoden der Risikobewertung von Chemikalien in der Ökotoxikologie immer mehr an Bedeutung. So wird etwa erwogen, als Alternativmethode zur Überprüfung von Chemikalien an adulten Fischen künftig den Fischembryotoxizitätstest (FET) mit *Danio rerio* anzuwenden, da hier eine sehr gute Korrelation zwischen beiden Testsystemen gegeben ist. Allerdings ist der FET für einige „Ausreißer" unter den Chemikalien weniger empfindlich als der konventionelle Fischtoxizitätstest. Um zu der Klärung der Frage beizutragen, in welchem Ausmaß ein Schutz des Embryos durch das Chorion für solche Abweichungen verantwortlich ist, wurden im Rahmen der vorliegenden Arbeit die verfügbaren Informationen zur Entstehung und Morphologie, chemischen Zusammensetzung, biologischen Funktion und Durchlässigkeit des Fischchorions unter toxikologischen Gesichtspunkten aufgearbeitet. Für ein besseres Verständnis der chemischen Interaktionen zwischen Bestandteilen des Chorions und Xenobiotika, vor allem in Hinblick auf die Funktion und Eigenschaften der Poren, die sich über die gesamte Oberfläche des Chorions verteilen, bedarf es hier freilich noch ergänzender Forschung. Dennoch scheint bereits jetzt erkennbar, dass das Chorion für gewöhnliche Chemikalien keine Barriere darstellt. Ausnahmen sind lediglich Substanzen, die durch Komplexierung oder durch chemische Interaktion, wie zum Beispiel das Eingehen ionischer Bindungen mit anderen Molekülen, große Strukturen ausbilden können (z.B. kationische Polymere), Substanzen die mit SH-Gruppen interagieren (z.B. Schwermetalle) oder sehr große Moleküle (z.B. Polymere). Diese werden durch das Chorion blockiert. Für weiterführende Untersuchungen wurden zudem bestehende Methoden zur Dechorionierung von Embryonen auf ihre Anwendbarkeit im Rahmen des Toxizitätstests hin überprüft und in der Folge ein modifiziertes Protokoll entwickelt, das eine reproduzierbar hohe Überlebensrate in Folge der Entfernung des Chorions aufweist. Der Effekt des Dechorionierens wurde mit dem kationischen Polymer Luviquat HM 522 demonstriert, für das auf diese Weise gezeigt werden konnte, dass es – vermutlich auf Grund seines hohen Molekulargewichts von ~ 400000 Da – bei intaktem Ei durch das Chorion blockiert wird, aber nach Dechorionierung stark toxisch wirkt. Eine „kritische molekulare Größe" liegt möglicherweise zwischen 2000 und 3000 Da, wie durch Experimente mit Polyethylenglykolen gezeigt werden konnte. Ein exakter Grenzwert konnte allerdings nicht bestimmt werden, da neben dem Molekulargewicht in Da oder g/Mol auch eine Reihe anderer Faktoren zur tatsächlichen Grüße eines Moleküls beitragen.

Um einzuschätzen können, ob unterschiedliche Aufnahme, Akkumulation oder Metabolisierung einer Testsubstanz Unterschiede in den Befunden akuter Fischtoxizitätstest und Embryotoxizität erzeugen, wurde ein bekannter „Ausreißer", das Salz Kaliumchlorid im Vergleich mit anderen Kalium- und Natriumsalzen in Standard- und verlängerten Fisch-embryonentoxizitätstests sowie in Eleutheroembryotests und Dechorionierungsversuchen untersucht. Die Ergebnisse wurden mit Daten aus der US EPA Ökotoxizität-Datenbank zur akuten Fischtoxizität und Daten zur Larvaltoxizität aus offen zugänglicher Literatur verglichen. Die Ergebnisse bestätigen, dass die Periode um den Schlupf ein kritisches Stadium während der Embryogenese darstellt, das im Prüfverfahren der Chemikalientestung miteinbezogen werden sollte.

Summary

As a consequence of current development in European chemical legislation, alternative methods in ecotoxicology gain more and more importance in chemical risk assessment. As an alternative to chemical testing with adult fish, the fish embryo toxicity test (FET) with *Danio rerio* is considered since a general correlation between both protocols is very high. However, for a couple of outliers the FET were less sensitive than the conventional fish test. To contribute to the clarification, to which extent protection of the embryo by the chorion is responsible for such deviations, the available information on the genesis and morphology, chemical composition, biological function and permeability of the fish chorion was reviewed under toxicological aspects. With regard to possible chemical interactions between chorion components and xenobiotics, there is still a need for supplementary research, particularly on understanding the function and properties of the pores which disperse across fish chorions. Nevertheless, it seems that the chorion is not a major barrier for ordinary chemicals; however, there are exceptions, like substances which might form complex or large structures with other similar molecules through chemical interactions such as ionic bonding (e.g. cationic polymers), substances which likely interact with SH-groups (e.g. heavy metals) or large molecules (e.g. polymers) are blocked by the chorion. For further investigations, existing dechorionation methods were evaluated on their applicability in toxicity testing. Thereupon a modified fish embryo test protocol with reproducibly high survival rates following removal of the chorion was established. The effect of dechorionation was demonstrated with the cationic polymer Luviquat HM552, which was thus shown to be blocked by the chorion of intact eggs probably due to its high molecular weight of ~400000 Dalton, but becomes strongly toxic after dechorionation. A "critical" molecular size might lie between 2000 and 3000 Da as demonstrated with experiments using polyethylene glycols. However, a reasonable molecular size cut off value could not be determined, since beside the molecular weight in Dalton or g/mol, a number of other factors contribute to the actual size of a molecule.

In order to evaluate if differential uptake, accumulation or metabolization of the test compounds may produce differences between the results of acute fish and fish embryo toxicity, a known outlier, KCl, was investigated in comparison with other potassium and sodium salts in the standard as well as in the prolonged FET, in tests with eleutheroembryos and dechorionated embryos. Results were compared to acute fish toxicity data retrieved from the US EPA Ecotoxicity database and other data on larval toxicity available from the open literature. The results confirmed that the period around hatching is a critical stage during embryogenesis, which should be included in the testing procedure for chemical testing.

Chapter 1: Introduction

1 Structure of this thesis

This doctoral thesis consists of six chapters, beginning with a general introduction (Chapter 1), including some background information on the use of fish embryo toxicity testing for environmental risk assessment and its role as an alternative method to replace animal testing. The following four chapters (Chapters 2 to 5) are formed by scientific articles or manuscripts, designed as stand-alone manuscripts and related to this research. These chapters were written by the author of this thesis and co-workers. Chapter 6 presents an overall discussion and a final conclusion, which can be drawn from the entire study.

2 Background information on the use of fish embryo toxicity testing

The use of chemicals and their introduction into the environment can pose considerable risk to the environment and human health. Therefore, current legislation in Europe (e.g. REACH, 2006) requires the registration of chemicals, pesticides, biocides and pharmaceuticals including the acquisition of appropriate data to evaluate and assess the risk of the utilization of these substances. These data include information on the toxicity to representatives of different environmental compartments and various trophic levels and require testing with vertebrates, for the aquatic compartment in particular with fish.

However, in recent years, there has been increasing ethical concern using fish and vertebrates in general for toxicity testing, since the experimental animals may be suspected to suffer severe distress and pain (Braunbeck *et al.* 2005; Nagel 2002) although this is not conclusively answered (Ahrlinghaus *et al.*, 2007; Rose, 2007). Hence, and – not least – driven by scientific and cost interest, the idea to reduce, refine and replace animal testing by alternative test methods (Russell and Burch, 1959) was taken up again in research as well as in regulatory (Walker *et al.*, 1997). For example, in 2005, the 48 h toxicity test with fertilized eggs of the zebrafish (*Danio rerio)* has become mandatory and, thus, replaced the conventional 96 h acute fish test for the routine testing of whole effluent discharges in Germany (DIN 38415-T6; DIN 2001). With the new European chemical policy REACH (Registration, Evaluation and Authorization of Chemicals; Regulation (EC) Nr. 1907/2006) the principle has now become an inherent part of Chemical Legislation on European Level (REACH, 2006)).

Based on the protocol for waste water testing in Germany (DIN 38415-T6: DIN, 2001), which is also standardized since 2007 (ISO15088: ISO, 2007) on international levels, a proposal for a new guideline on fish embryo toxicity for the testing of chemicals has been developed and

submitted to the OECD by the German Federal Environment Agency (Braunbeck and Lammer 2006). Braunbeck *et al.* (2005) provided data substantiating that an optimized test protocol can equally be applied to early embryonic stages of other OECD species such as the fathead minnow (*Pimephales promelas*) and the Japanese medaka (*Oryzias latipes*). In the current version of the OECD test protocol, the FET is limited to two or four days and, thus, according to the rule that protection of immature forms of fish starts not until they become capable of independent feeding, classified – based on the current UK Animal Procedures Act (UK 1986) and the new EU Directive 2010/63/EU on protection of animals used for scientific purposes (EU, 2010) – as a test with non-protected life-stages.

3 The fish embryo toxicity test (FET)

3.1 Zebrafish (Danio rerio)

The zebrafish (*Danio rerio*, Hamilton-Buchanan 1822) is a small benthopelagic freshwater representative of the family of cyprinids, originating from the Ganges River system, Burma, the Malakka peninsula and Sumatra (Eaton *et al*., 1974). The fish is named for the five uniform, pigmented, horizontal blue lateral stripes extending to the end of the caudal fin, alternating with silvery bands. Particularly during spawn maturity, females can be distinguished easily from the male by their swollen bellies. Male fish are more slender and show an orange to reddish tint in the silvery bands along the body. The mean adult length varies between 3 and 5 cm. The approximate generation time is 3 - 4 month at 26°C in both soft and hard waters. The species is easily obtainable, inexpensive and readily maintainable. Females are able to spawn as often as 2 – 3 days with 50 – 200 eggs per day. Thus, under appropriate conditions, a large number of non-adherent, fully transparent eggs can be obtained all-seasonally (Laale, 1977). The embryonic development is short and has been described in detail in numerous studies (Hisaoka, 1958; Laale, 1977; Kimmel *et al*., 1995; Westerfield, 2007); Kimmel *et al*., 1995); it rapidly progresses with precursors to all major organs appearing within 36 hours of fertilization. Already after five days post fertilization, at the time of complete yolk consumption and start of external feeding, organogenesis of major organs is completed (Scholz *et al*. 2008). Due to these characteristics, as well as the eggs transparency enabling the easy observation of development (Laale, 1977; Kimmel *et al*., 1995), the zebrafish has become a major model in neurobiology and toxicology as well as in general molecular and developmental biology (Laale, 1977; Nüsslein-Volhard, 1994; Kimmel *et al*., 1995; Westerfield, 20075; Lele and Krone, 1996; Langheinrich, 2003; Hill *et al*., 2005).

3.2 Embryonic development of zebrafish

A detailed description of the embryonic development of zebrafish can be found in several studies (Hisaoka, 1958; Laale, 1977; Kimmel *et al*., 1995). 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). Selected major stages of zebrafish development are given in Fig. 1 and Fig. 2

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

Time	Stage	Characterization after Kimmel *et al.* (1995)
0	Fertilization	Zygote
0	Zygote period	Cytoplasma accumulated at the animal pole; one-cell-stage
0.75	Cleavage period	Discoidal partial cleavage
1		1. median vertical division: two-cell-stage 2. vertical division: four-cell stage
1.25		3. vertical and parallel to the plane of the first: eight-cell-stage
1.5		4. vertical and parallel to the second plane of division: 16-cell-stage
2	Blastula period	Start of blastula stage
3		Late cleavage; blastodisc contains approximately 256 blastomers
4		Flat interface between blastoderm and yolk
5.25	Gastrula period	50 % of epibolic movements; 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 segment or metamere
20		Muscular twitches; sacculus; tail well extended
22		site to side flexures; otoliths
24	Pharyngula period	Phylotypic stage, spontaneous movement, tail is detached from the yolk; early pigmentation
30		Reduced spontaneous movement; retina pigmented, cellular degeneration of the tail end; circulation in the aortic arch 1
36		Tail pigmentation; strong circulation; single aortic arch pair, early motility; heart beating starts
72-96	Hatching period	Heart-beat regularly; yolk Extension beginning to taper; dorsal and ventral stripes meets at tail; segmental blood vessels: thickened sacculus with two chambers; foregut developmental; neuromasts

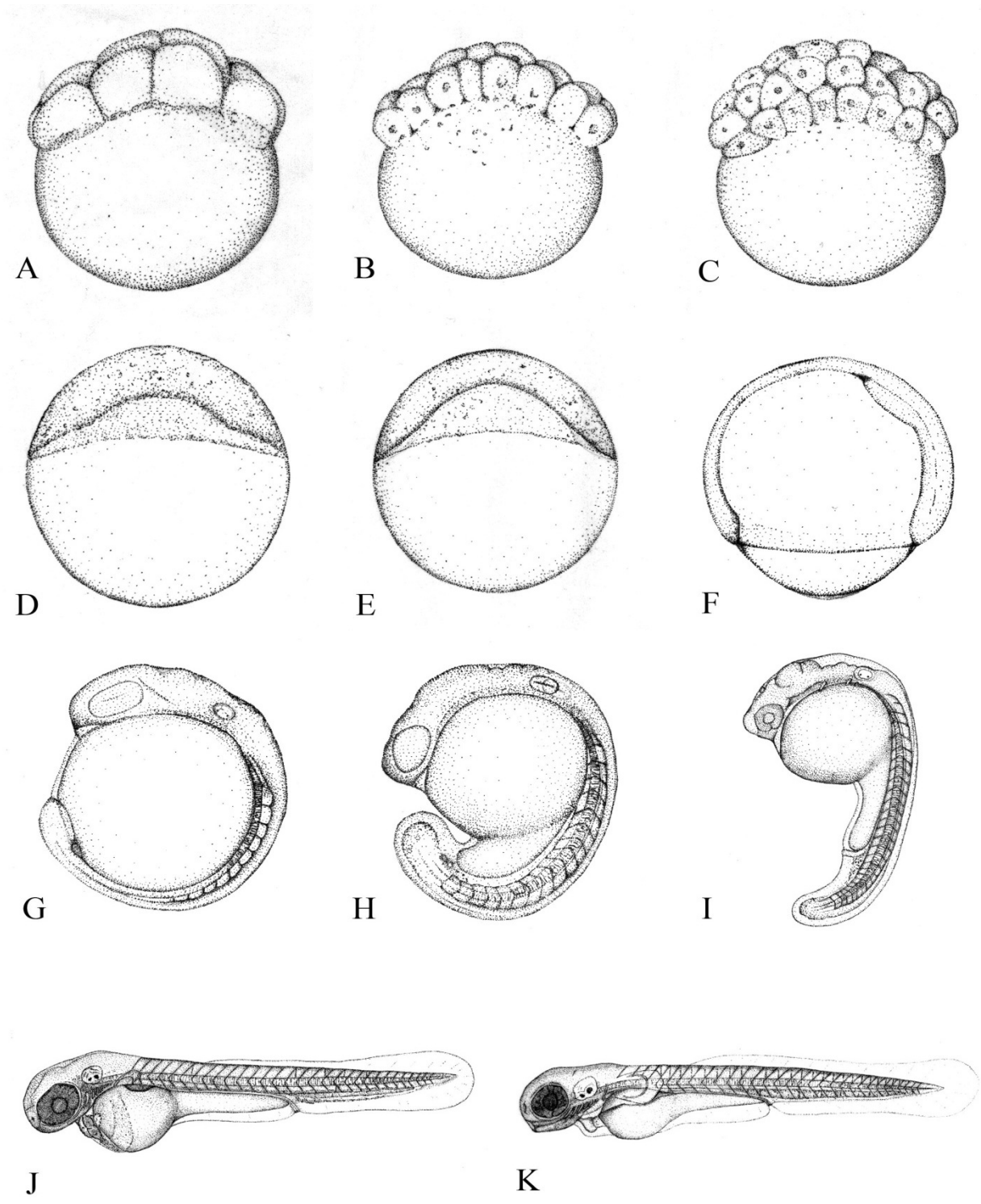

Fig. 1: Selected stages of the embryonic development of *Danio rerio* – illustrations without Chorion (Kimmel *et al*., 1995):

A 8-cell stage (1.25 hpf) **B** 15-cell stage (1.5 hpf) **C** 32-cell stage (1.75 hpf)
D beginning epiboly (4.3 hpf) **E** 30 % epiboly stage (6 hpf) **F** 75% epiboly stage (8 hpf)
G embryo at 14 hpf **H** embryo at 18 hpf **I** embryo at 22 hpf
J embryo at 48 hpf **K** embryo at 72 hpf
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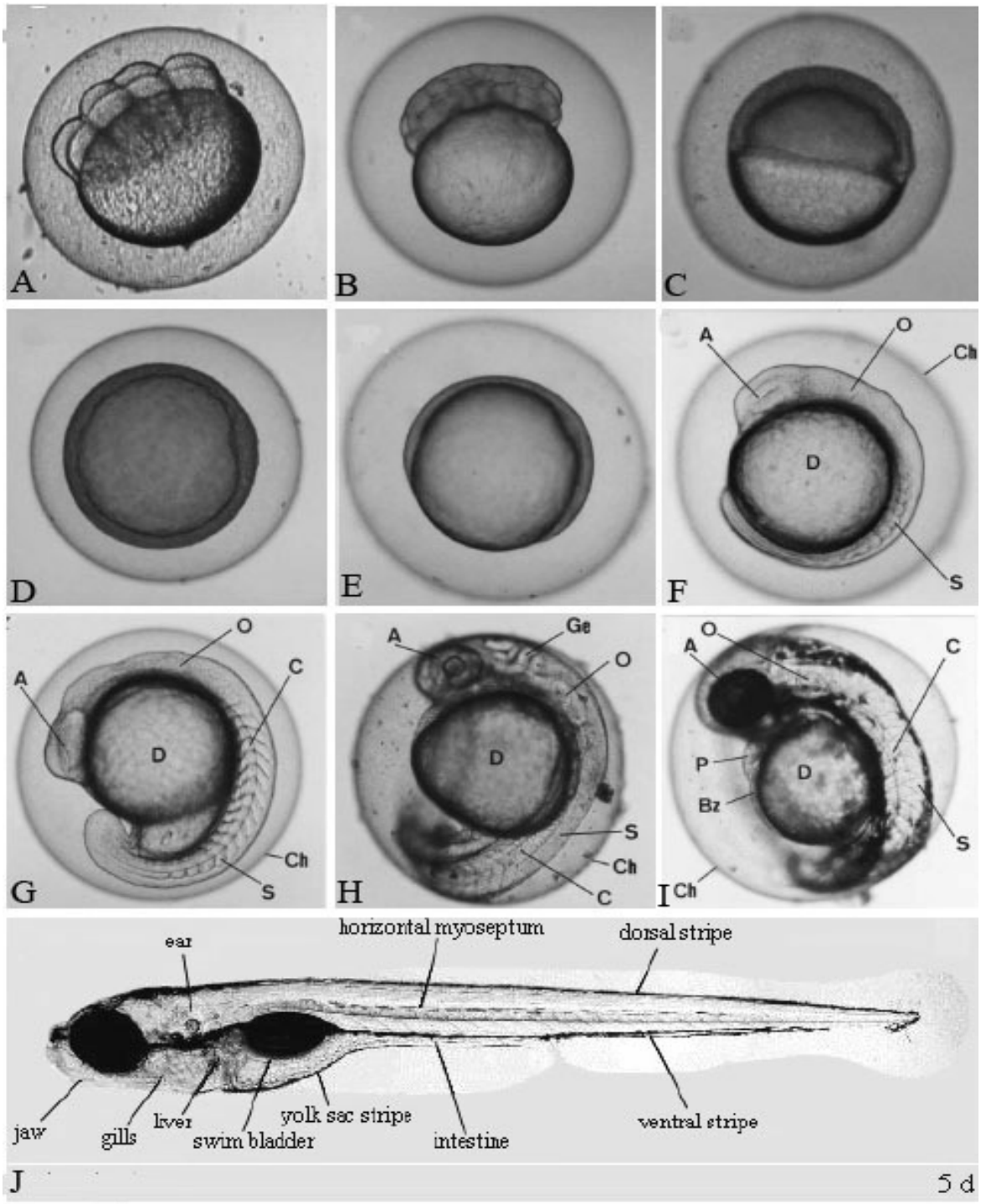

Fig. 2: Selected stages oft he embryonic development of Danio rerio. Photos (A – I): Rudolf (2000) ; Photo (J) Haffter et al. (1996) :

A 8-cell stage (1.25 hpf) **B** 32-cell stage (1.75 hpf) **C, D** 50 % epiboly stage (6 hpf)
E 75 % epiboly stage (8 hpf) **F** embryo at 16 hpf **G** embryo at 18 hpf
H embryo at 24 hpf **I** embryo at 48 hpf **J** embryo at 120 hpf

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

3.3 Principles of the fish embryo toxicity test (FET)

The FET with zebrafish (*Danio rerio*) embryos has become a mandatory component in routine whole effluent testing in Germany since 2005 (DIN 38415-T6: DIN, 2001) and was standardized at an international level in 2007 (ISO 15088: ISO, 2007). 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 and Lammer, 2006). The principles of the fish embryo test has been described in Schulte (1997) and Nagel (2002): fertilized zebrafish eggs are exposed as soon as possible after fertilization to at least 5 different concentrations of the test substance, a positive control substance (3.7 mg/L 3,4-dichloroaniline), a negative control and – if applicable – a solvent control. Regularly divided embryos in the 32-cell stage are then chosen using a dissecting microscope and 10 eggs for each concentration are transferred to 24-well microtiter plates, each well incubated with 2 ml of the respective test solutions. Sublethal and lethal endpoints (Tab. 2) can easily be detected and recorded after 24, 48, 72 and 96 h; four lethal endpoints which are coagulation of eggs, non-development of somites, non-detachment of the tail and absence of heartbeat are defined. Furthermore, a couple of the sublethal and teratogenic endpoints (Tab. 2) can be detected, including effects which might provide additional information on sublethal or teratogenic modes of action of the sample tested (Küster and Altenburger, 2007).

Tab. 2: Lethal and sublethal endpoints for evaluating the toxicity and teratogenicity of chemicals on zebrafish embryos as listed by Nagel (2002)

	Exposure time			
	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.

As mentioned above, in the current version of the test protocol the FET is limited to two or three days. 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), which should be included in the testing procedure. Based upon data found by Belanger *et al.* (2010) it is recommended that testing of developing zebrafish embryos should be

terminated between 24 and 48 hr after hatching in order to be compliant with above mentioned animal welfare legislation within Europe.

For that purpose the standard fish embryo test can be adapted: the test duration can be prolonged up to 144 hours of exposure and additional examinations can be done at 96, 120 and 144 h in the so-called "prolonged FET". For evaluation of toxicity the same endpoints can be used as for 48 hours and additionally hatching or lack of hatching should be recorded.
To enable a comparison between the sensitivity of juvenile/eleutheroembryos with pre-hatched embryos, eggs can be raised in artificial water until hatch (72 hpf) and then be exposed to chemicals as eleutheroembryos. In this case, eleutheroembryos are checked after 24, 48 and 72 h of exposure, equivalent to an age of 96, 120 and 144 hpf, respectively.

4 The scope of this thesis

Probably the most important feature 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: a first evaluation of the existing data base from the fish embryo test (Ratte and Hammers-Wirtz, 2003), followed by a comprehensive statistical analysis (Lammer *et al.* 2009) documented that the correlation between the fish embryo test and the acute fish toxicity test is comparable to those between conventional acute fish toxicity tests with different species. The prospects of fish embryo testing as an alternative method in hazard and risk assessment and scientific research has been reviewed by Embry *et al.* (2010). Nevertheless, there are some questions surrounding the sensitivity of fish embryos when compared with whole fish: a small number of substances was identified for which the embryonic and adult toxicity differ significantly (Braunbeck *et al.*, 2005). Several reasons might be assumed to account for such outliers: (1) restricted availability of the test substances due to the static nature of the standard FET (Lammer *et al.*, 2009), (2) differences in uptake, accumulation and metabolization of the test compounds between embryonic and adult stages (Barry *et al*., 1995; Ensenbach, 1987; Van Leeuwen, 1985), and (3) protection of the embryo by the chorion (Barry *et al*., 1995; Braunbeck *et al.*, 2005; Cheng *et al*., 2007, Creton *et al.*, 2004, Gellert and Heinrichsdorff, 2001).

In order to address the first point Lammer *et al.* (2009) developed adaptations to the standard FET to allow testing under flow-through conditions. However, for no substance tested so far a significant difference in the results of the FET could be documented under flow-through conditions (Lammer, 2009).

The investigations described herein focus on a potential barrier function of the chorion for which evidences can be found in a couple of studies (Braunbeck *et al*., 2005; Léonard *et al*., 2005; Rudolf, 2000; Scholz *et al*., 2008; Wendler, 2006) as well as on understanding of developmental stage-specific toxicity as found e.g. by Ensenbach (1987), González-Doncel *et al*., (2004) and Van Leeuwen (1985).

In order to determine the influence of the chorion on chemical toxicity for zebrafish embryos, this thesis first reviews the available information on the fish chorion structure and permeability under toxicological aspects. Following this, dechorionation methods are evaluated for application in the fish embryo test with the zebrafish. As next steps, the assumption that molecules of high molecular are not able to pass the chorion is pursued and the reason for the discrepancy between adult fish toxicity and fish embryo toxicity data for an identified outlier (from the correlation) are investigated.

Chapter 2: The fish chorion under toxicological aspects

1 Introduction

With the new European chemical policy REACH (registration, evaluation and authorization of chemicals), for the first time an important aspect was incorporated into EU horizontal chemicals legislation: the protection of laboratory animals (REACH, 2006)). In case of risk assessment concerning aquatic environments, particularly acute toxicity tests with fish (as vertebrates) are of great interest to be replaced by alternative methods. A very promising potential alternative is a toxicity test using fish embryos instead of adult fish (Nagel, 2002; Braunbeck *et al*., 2005). A modified version of the whole effluent test (DIN, 2001) with zebrafish (*Danio rerio*), the fish embryo toxicity test (FET), has been submitted to the OECD by the German Federal Environment Agency as a draft guideline for an alternative to chemical testing with intact fish (Braunbeck *et al*. 2006). In the current version of the test protocol the FET limited to two or three days and classified as a non-animal test in legal terms; protection of immature forms of fish starts first when they become capable of independent feeding (EU, 2010). Although it could be shown that a good correlation between acute fish toxicity tests and embryo toxicity tests is given (Ratte and Hammers-Wirtz, 2003; Braunbeck *et al*., 2005; Lammer *et al*., 2009), dispersed through literature on fish embryo morphology and development as well as ecotoxicological research there is growing evidence that the chorion may represents an effective barrier for a couple of substances. It could, however, not be clarified unequivocally whether the chorion represents an effective barrier and, thus, protects the embryo from exposure to distinct chemicals (Braunbeck *et al.*, 2005; Léonard *et al.*, 2005; Rudolf 2000; Scholz *et al.*, 2008; Wendler, 2006). An understanding of the structure and function of the chorion would enable a better understanding of the protective properties of this potential barrier to entry of certain chemicals. There are a couple of articles summarizing general chorion development (Mommsen *et al.*, 1988), structure and properties of various species, mainly focusing on fertilization, hardening and hatching (Kunz, 2004a; Kunz, 2004b; Laale, 1980; Schoots *et al.*, 1982; Yamagami, 1988; Yamagami *et al.*, 1992). Most of the research that has been performed on investigating the permeability of fish embryos has been associated with the cryopreservation of fish embryos (Cabrita *et al.*, 2003; Harvey *et al.*, 1983; Robles *et al.*, 2003; Zhang and Rawson, 1996a; Zhang and Rawson, 1996b). The focus of this review is therefore to give an overview on the available information on the genesis and morphology, chemical composition, biological function and permeability of the fish chorion with regard to toxicological aspects. Special attention will be paid to the zebrafish chorion.

2 The chorion

2.1 Genesis and morphology

2.1.1 Genesis

The chorion may be defined as the acellular, highly structured envelope enclosing an ovulated egg or developing embryo, separating it from the external environment (Hart *et al.* 1984). In literature this envelope is also referred to as *zona radiata*, *zona pellucida*, vitelline envelope, primary envelope, etc (Laale, 1980). The development of the zebrafish chorion is described in detail by Selman *et al.* (1993). Its earliest manifestation, the vitelline envelope can be detected at the end of the primary growth stage of oocyte development as an amorphous electron-dense material appearing between short microvilli extending from the oocyte surface toward and the overlying follicle cells (*zona radiata interna* 1: Ulrich, 1969). During the cortical alveolus stage, the vitelline envelope becomes tripartite by developing a second more electron-lucent layer (*zona radiata interna* 2: Ulrich, 1969) between the initial layer and the oolemma, and, finally, a third layer (*zona radiata externa*: Ulrich, 1969), consisting of numerous horizontal laminae close to the oolemma. The envelope is perforated by pore canals containing long microvilli originating from both the oocyte and the follicle cells. In some species, at the end of maturation, the microvilli from both oocyte and follicular cells are withdrawn from the pore canals and the outer openings may become blocked with “plugs” (Dumont and Brummet, 1980; Flügel 1964; Flügel 1967; Hart and Donovan, 1983; Stehr and Hawkes, 1979), in some cases microvilli from the oocyte remain penetrating the vitelline envelope, partly beyond ovulation. In the zebrafish most of each pore canal remains open except for the plug (Hart and Donovan, 1983). The mature zebrafish egg, ovulated into the ovarian lumen and capable of fertilization, is about 0.75 mm in diameter (Selman *et al.*, 1993). The chorion remains tripartite with aggregates of dense material at its external surface. It is perforated by a passage, the micropyle, through which a spermatozoa can penetrate the chorion for fertilization. Already after ovulation hardening of the chorion begins and is continued following fertilization by the “cortical reaction” (Coward *et al.*, 2002; Gilkey, 1981): Penetration by the spermatozoan causes an increase of the calcium content of the peripheral ooplasm, causing cortical alveoli to fuse with the oolemma and discharge an acidic glycosaminoglycan into the space between the plasma membrane and the chorion (perivitelline space), which is too large to penetrate the chorion. Thus, osmolarity of the perivitelline fluid is increased and an influx of water begins, increasing the hydrostatic pressure in the perivitelline space, forcing the chorion to lift away from the plasma

membrane. The chorion loses its elasticity and becomes tough and rigid (Gray, 1932; Hayes, 1942; Kim *et al.*, 2004; Yamagami *et al.*, 1992; Zotin, 1958). The thickness and complexity of fish chorions shows wide variation (Kunz, 2004b; Lønning, 1972; Stehr and Hawkes, 1979).

2.1.2 Morphology

The morphology has been described early for various species (Cherr and Clark, 1982; Cotelli *et al.*, 1988; Dumont and Brummet, 1980; Hart and Donovan, 1983; Hart *et al.* 1984; Hisaoka, 1958; Kobayashi and Yamamoto, 1981; Kuchnow and Scott, 1977; Rawson *et al.* 2000; Shanklin, 1959; Stehr and Hawkes, 1979; Schoots *et al.* 1982; Wolenski and Hart, 1987; Yamamoto and Yamagami, 1975). The ultrastructure of the chorions appears rather to be determined by reproduction strategies and habitat of the species (Riehl, 1991; Stehr and Hawkes, 1979) than by their taxonomic position. Most teleostean chorions consist of more than one layer (Kunz, 2004b). The outer surface usually shows distinct patterns, often covered with accessory structures in form of threads, filaments, fibrils, spikes, or jelly coats, deriving from different origins (Ludwig, 1874) like the oocytes themselves (primary envelope/membrane), the follicles cells (secondary envelope/membrane), or the oviduct or other structures (tertiary envelope/membrane). The layers are pierced by pore canals (Fig. 3), which are more or less or completely closed

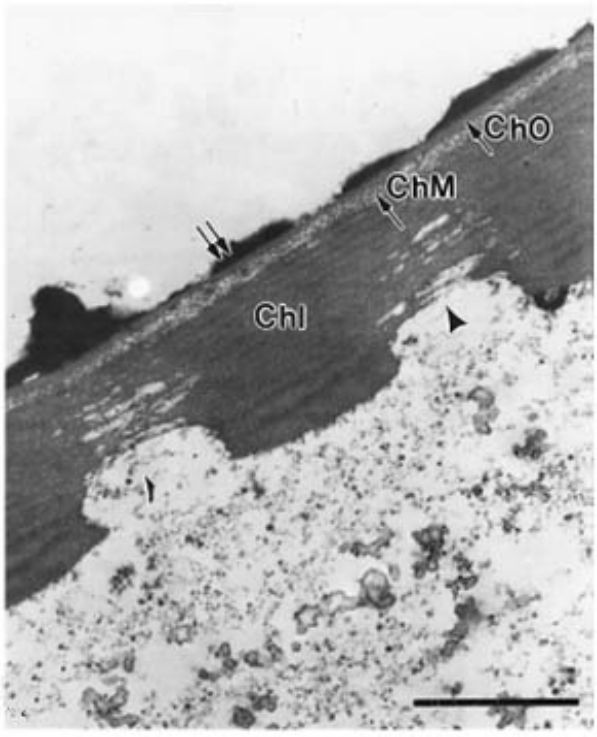

Fig. 3: TEM view of gastrula-stage embryo: the outer (ChO; 0.2 – 0.3 µm) and innermost layers (ChI; 1.0 – 1.6 µm) are electron-dense as a result of the high osmium uptake, and separated by a low contrast middle layer (ChM; 0.3 – 0.6 µm). The middle and inner layers are pierced with pores (▲). The outermost layer of the chorion is visible as distinctive projections (↑↑). Bar = 1µm
Photo: Rawson *et al.*, 2000

The zebrafish chorion is said to be derived soley from the primary envelope (Hart and Donovan, 1983). Its morphology was investigated in several studies (Hart and Collins, 1991; Hart and Donovan, 1983; Hisaoka, 1958; Rawson *et al.*, 2000; Schoots *et al.* 1982; Wolenski and Hart, 1987). The outer chorion membrane complex with a thickness of 1.5 - 2.5 µm consists of three layers: electron-dense outer and innermost layers with a thickness of 0.2 – 0.3 µm and 1.0 - 1.6 µm, respectively, separated by an electron-lucent middle layer (0.3 - 0.6 µm in

thickness); the middle and inner layers are pierced by pore canals while the outermost layer is covered with projections of 2.0 - 3.0 µm in diameter (Fig. 3).

These plugs obfuscate the pore canals at intervals of 2.0 - 10.0 µm (Fig. 4). The diameter of the outer openings of the pore canals is 0.5 - 0.7 µm, the centre-to-centre distance 1.5 - 2.0 µm. The pores are cone-shape with a larger diameter at the inner surface, which is lined spirally with lamella in a corkscrew-like manner (Fig. 4). The middle layer is organized in bundles of fibers with about 0.05 µm to 0.06 µm in diameter, crisscrossing around the pore canals. The inner layer, comprising most of the chorion, consists of 16 horizontal electron-dense lamellae of about equal thickness alternating with 15 interlamellaes of lower density.

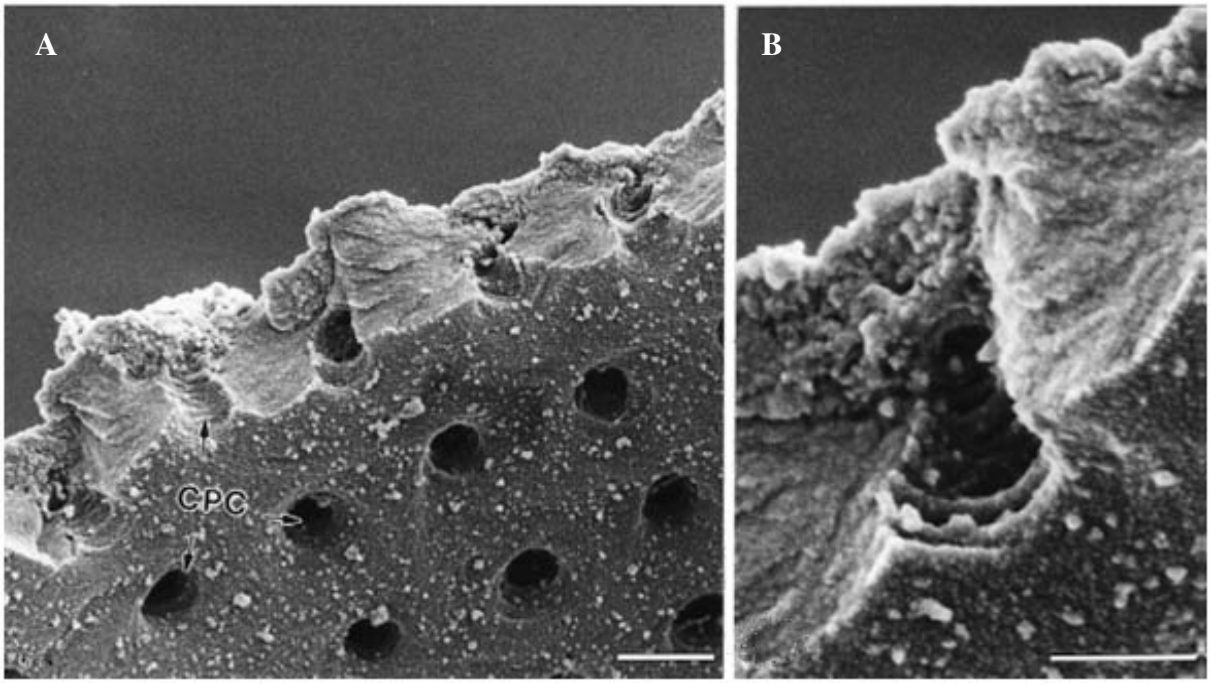

Fig. 4: FE-SEM view. (**A**) Inner and fracture surface of the chorion showing the pore canals (CPC). Bar = 1 µm. (**B**) Detail of a pore canal with a typical cone shape. Bar = 0.5 µm
Photo: Rawson et al. 2000

The most recent investigations on the structure of the chorion can be found in Lilicrap (2010). The SEM images affirm that the internal surface of the chorion is permeated with numerous pores, which could be the route of aqueous transfer into and out of the embryo across the chorion. The glycoproteins, which constitute the outer layer of the chorion, are clearly visible and it is shown that the external surface is a flexible membrane to which particles and micro organisms adhere. However, these glycoproteins possibly prevent the transfer of aqueous material into and out of the embryo.

2.2 Chemical composition

Trough histochemical techniques components of the chorion were revealed to be mucopolysaccharides, glycoproteins, carbohydrate proteins, proteins and polysaccharides (Anderson, 1967; Arndt 1960; Guraya, 1965; Hagenmaier, 1974a; Iuchi *et al.*, 1976; Tesoriero, 1977), differing in their individual combination between the different layers and species. A lot of studies on various fish species (Begovac and Wallace, 1989; Brivio *et al.*, 1991; Cotelli *et al.*, 1988; Hyllner *et al.*, 1991; Hyllner *et al.*, 1995; Masuda *et al.*, 1991; Oppen-Bernsten *et al.*, 1990; Scapigliati *et al.*, 1994) could show, that the major molecular constituents of the egg envelope in teleost fish are 3 - 4 major proteins with between about 40 and 250 kDa, many of them glycosylated. The protein composition, glycosylation pattern and analysis of amino acid content of the zebrafish (*Danio rerio*) egg chorion were investigated by Bonsignori *et al.* (1996). Four major polypeptides (116, 97, 50 and 43 kDa) were found. In contrast to the 97 kDa and the 43 kDa polypeptides, the 116 kDa and 50 kDa proteins were stained by lectins (concanavalin agglutinin (Con A), *Galanthus nivalis* agglutinin (GNA), *Sambucus nigra* bark agglutinin (SNA) and *Ricinus communis* agglutinin (RCA 120)), suggesting them to be N-linked glycoproteins. Results of amino acid content investigation showed, that the zebrafish chorion contains in comparison to other species more serine, but less proline. Beyond that, Pulella et al (2006) demonstrated that DSSA-probes bind with high affinity to the chorion, suggesting the presence of thiol-rich proteins.

2.3 Biological function

Various functions can be assigned to the chorion: specific sperm binding and guidance of the sperm to the micropyle (Dumont and Brummet, 1980), functional buoyancy (Podolsky, 2002), protection against microorganisms and protozoans (Hisaoka, 1958; Schoots *et al.*, 1982) - the chorion even possess bactericidal properties (Kudo and Yazawa, 1997) - and diffusive exchange of gases and physical protection (Grierson and Neville, 1981; Stehr and Hawkes, 1979). An essential function is the mechanical protection of the embryo (Riehl, 1991) as well as - in case of demersal eggs - binding to the substratum by external chorion filamentous and ornate structures. A couple of authors (Blaxter, 1988; Braunbeck *et al.*, 2005; Léonard *et al.*, 2005; Lillicrap, 2007; Mizell *et al.*, 1996; Mizell and Romig, 1997; Von Westernhagen, 1988; Weis and Weis, 1989;) furthermore assume the chorion to offer an undefined protection against pollutants.

2.4 Permeability

Already in 1958 Hisaoka stated, that the relative size of the chorion pores indicate, that, from a purely physical standpoint, the chorion must be a leakage membrane (Hisaoka, 1958). Although the pore canals are closed or obliterated, the chorion seems to be freely permeable to water, electrolytes and small molecules (Coward *et al.*, 2002, Potts and Eddy. 1972; Rawson *et al.*, 2000; Scapigliati *et al.* 1994; Yamagami *et al.* 1992; Zhang and Rawson. 1996), however, it is not equal at all embryonic stages and for all groups of substances.

2.4.1 Fertilization to hatching and alterations of the chorion structure

According to Hagedorn *et al.* (1997), membrane permeability of the zebrafish embryo changes during development. At ovulation, when the fish eggs get in contact with the external medium, it hardens. This process is accompanied by structural changes of the chorion regarding protein composition (Iuchi *et al.*, 1991; Iwamatsu *et al.*, 1995; Masuda *et al.*, 1991; Oppen-Bernsten *et al.*, 1990), involving differences in chorion solubility before and after hardening (Yamagami *et al.*, 1992). Reduction/oxidation reactions (Ohtsuka, 1960; Zotin, 1958), and the formation of disulfide or isopeptide cross-links between chorion proteins (Ha and Iuchi, 1998; Hagenmeier *et al.*, 1976; Lønning *et al.*, 1984; Ohtsuka, 1960) are involved in the mechanism, supposingly supported by transglutaminases (Ha and Iuchi, 1998; Oppen-Bernsten *et al.*, 1990; Yamagami, 1988). According to several authors, the permeability of the plasma membrane decreases noticeably following activation and fertilization (Gerking and Lee, 1982; Humphrey and Klumpp, 2003; Mangor-Jensen, 1987; Potts and Eddy, 1972; Terner, 1968; Van Leeuwen *et al.*, 1985; Villalobos *et al.*, 2000; Vuorinen and Vuorinen, 1987). Gellert and Heinrichsdorff (2001) for example found very early stages of the zebrafish embryo (< 1h) to be more sensitive against chemical influence than later stages (4 h). It has been postulated by Longwell (1977) that pollutants, along with imbibed water, may enter the egg during hardening. A possible entry route for fluid and other materials in to the embryo is via the micropyle.

There is furthermore evidence, that after hardening, as the embryo develops, the permeability gradually increases again although remaining significantly lower than before activation and fertilization (Mangor-Jensen, 1987; Rudy and Potts, 1969). Cabrita *et al.* (2003) showed that cryoprotectant permeability of turbot embryos (*Scophthalmus maximus*) was stage development dependent since embryos at the tail bud free stage (G stage) were more permeable, followed by embryos at the F and E stages. An explanation may be found in a study of Rubstov (1981), who observed that the diameter of the pores of the carp egg chorion

(*Cyprinus carpio*) increase during development. The openings take a higher percentage of the eggs surface than before fertilization, accompanied by the disappearance of partitions from the canals of the inner membrane. For zebrafish embryos Harvey *et al.* (1983) reported that the permeability increases between the early cleavage stage of development and closure of the blastopore, and that permeability is maximal during epiboly. After the epibolic stage permeability seems to decrease again (Herrmann, 1993).

At an advanced stage of development the hatching process begins with a partial (thin chorion) or complete (thick chorion) enzymatic dissolution of the inner chorion layer by the hatching enzyme (Schoots *et al*., 1982; Yamamoto and Yamagami, 1975), followed by mechanical breaking and osmotic rupture. The hatching enzyme, in many cases a metalloprotease (Hagenmaier, 1974a; Schoots and Denuce, 1981; Yamagami, 1981) is synthesized in specific hatching gland cells (De Gaspar *et al*., 1999; Hagenmaier, 1974b; Schoots *et al*., 1982; Schoots *et al*., 1983). The thinning process, called "chorion softening", can be observed by measurement of penetration forces of puncturing the chorion at different developmental stages. Penetration forces for puncturing the zebrafish chorion at the blastula stage are 1.3 times higher than those at the pre-hatching stages (Kim *et al*., 2004). With the alterations in structure and mechanic properties, also chemical properties change. Guadagnolo *et al*. (2000) discovered that the concentration of silver in the chorion of silver-exposed rainbow trout embryos increased through whole development, but a few days before hatch the ratio between chorion and whole embryo silver concentration increased. It is assumed, that various cross-links of cysteine residues within the chorion which do not possess available sulfhydryl groups to bind silver are broken down during the proteolytic hatching process (Yamagami, 1988). Consistently with a study of Harvey and Chamberlain (1982), Adams *et al.* (2005) found permeability of the zebrafish chorion to be noticeably higher at later stages. Cypermethrin exposure of medaka (*Oryzias latipes*) embryos at different stages showed that the later stages (stage 34) which involve advanced organogenetic stages in which the chorion is partially degraded before hatching were the most sensitive.

2.4.2 Permeability – Environmental Conditions

In addition to stage-specific alterations of the chorion, the environmental conditions in which embryos grow may affect chorion permeability (Alderdice, 1988). Fertilized eggs of *Salmo salar*, are more permeable for water when placed in a modified salmon Ringer solution (isotonic to coelomic fluid) than in river water, distilled water or isotonic glucose (Rudy and Potts, 1969). Similarly, embryos of *Esox lucius* at various developmental stages were more

permeable for water when placed in 100% salmon Ringer solution than in 7.5% Ringer solution (Loeffler, 1971). Cameron and Hunter (1984) found medaka embryo sensivity for amiloride being influenced by the cation-concentration of external medium. Water permeability of embryos of the euryhaline *Oreochromis mossambiscus* decreased when transferred from freshwater to seawater (Miyazaki *et al*., 1998) and embryos of *Scophthalmus maximus* showed no change in permeability over a range of salinities (Tytler and Ireland, 1993). Adams *et al*. (2005) showed that in contrast to zebrafish embryos at young developmental stages and dechorionated embryos permeability in intact embryos was influenced by changes of external medium composition. An explanation of the membrane permeability changes may be the concentration of ions in the external medium. A low concentration of calcium or sodium in the medium delayed the phase of decreasing permeability in fertilized eggs of the Atlantic salmon (Rudy and Potts, 1969), as well as reducing the concentration of calcium in the external medium increased membrane water permeability in the embryos of the *Gadus morhua* (Mangor-Jensen, 1987) and the European turbot at some developmental stages (Tytler and Ireland, 1993).

2.4.3 Permeability – Cryoprotectants

Harvey *et al.* (1983) reported that DMSO, glycerol and methanol permeate to relatively low levels in zebrafish embryos. Methanol permeates the most effectively; glycerol entered the embryo relatively easily but only reached about 8% of the equilibrium level after two hours (equilibrium relates to the external concentration of the glycerol solution). DMSO reached about 2.5% equilibrium in the same period of time, however, dechorionation of the zebrafish embryos seems to increase the amount of DMSO permeating in to the embryo. Harvey *et al.* (1983) suggested that these findings indicated that the chorion retards the free exchange of solute. Robles *et al.* (2003) indicated that this retardation by the chorion might be caused preventing water and solute movement in and out of the embryo. According to a suggestion of Lilicrap (2010) glycoproteins, which constitute the outer layer of the chorion, are possibly responsible for the prevention of the transfer of aqueous material into and out of the embryo. Therewith these authors' opinions are contrary to Zhang and Rawson (1996) who reported that the chorion is effectively freely permeable to water and cryoprotectants. Accordingly, the actual barrier to cryoprotectants is the inner vitelline membrane and the pores are actually the major pathway for cryoprotectants to enter into the egg. They conclude that permeability is not just influenced by the chorion but rather by a combined effect of the chorion and the perivitelline fluid, controlling the osmotic influx of chemicals in fish embryos.

2.4.4 Permeability – Heavy Metals

A few studies have shown that metals that are incorporated into the egg during oogenesis can produce malformations in the embryo (Zn: Speranza *et al*., 1977; Hg: Birge *et al*., 1979; McKim *et al*. 1976), as well as exposure before hardening (González-Doncel *et al*., 2003), but once the chorion is elevated and hardened, it can act as a barrier (Ozoh, 1980). On the other hand, some studies showed that dechorionated embryos were less susceptible to the toxic effect than embryos with intact chorions (Cd: Rombough and Garside, 1983; Zn sulphate: Skidmore, 1966). An explanation by Rombough (1985) applies to the Donnan equilibrium: cations with negative standard electrode potentials (e.g. Zn^{2+}. Cd^{2+}, Pb^{2+}) would easily pass the chorion (which acts as an ion exchanger) and would be accumulated in the perivitelline fluid. Thus, embryos with a chorion were more susceptible to these ions than those without. In contrast, cations with positive standard electrode potentials (e.g. Hg^{2+}, Cu^{2+}, Ag^{2+}) with high affinities to sulfhydryl groups would bind to the chorion, which thus would acts as a barrier. Results of studies with different heavy metals and various fish species only partly corroborate to these assumptions (Blaylock and Frank, 1979: *Cyprinus carpio*, Ni; Dave, 1985: *Danio rerio*, Cd, Fe, Al; Dave and Xin, 1991: *Danio rerio*, Hg, Cu, Ni, Pb, Co; Fraysse *et al*., 2006: *Danio rerio*, Cd; Hallare *et al*., 2005: *Danio rerio*, Cd; Middaugh and Dean, 1979: *Fundulus heteroclitus* and *Menidia menidia*, Cd; Ngguyen *et al*., 1999: *C. gariepinus*), Cr; Ozoh, 1980: *Danio rerio*, Co, Pb; Shazili and Pascoe, 1986: *Salmo gairdneri*, Cu, Cd, Zn; Van Leeuwen *et al*., 1985: *Salmo gairdneri*, Cd; Von Westernhagen, 1988: *Pleuronectes flesus*, Cd;). Guadagnolo (2001) confirmed that silver accumulates in the chorion. Similar observations, however, were made for cadmium (Beattie and Pascoe, 1978; Michibata, 1981; Von Westerhagen *et al*., 1974), cobalt (Kunze *et al*., 1978), lead (Holocombe, 1976; Stouthart *et al*., 1994), and zinc (Wedemeyer, 1968). Anyway, it is suggested by most of the authors that an anionic charged group, possibly sulfhydryl (pK about 8) in the chorion is responsible for binding, which is affirmed by several observations: Michibata (1981) could show that cadmium bound to the chorion can be removed by washing the egg in an acid solution. Metal accumulation in the chorion seems to be strongly pH dependant – the lower the pH is, the more metal is bound to the chorion (Dave, 1985; Stouthart, *et al*., 1994; Stouthart *et al*., 1996; Wedemeyer *et al*., 1968). Wedemeyer *et al*. (1968) furthermore revealed that exposure to iodoacetate greatly reduced zinc binding by the chorion, presumably by -SH blockade, allowing a substantial diffusion of zinc into the perivitelline fluid. Recently Pulella *et al* (2006) demonstrated the presence of thiol-rich proteins in the chorion of the zebrafish. Nevertheless, also here other factors can influence the chorion binding and the uptake of

metals into the egg. Sensitivity to Cd for example is dependent on salinity (Von Westerhagen *et al*. 1974), as well as on water hardness (Michibata, 1981), temperature (Hallare *et al*., 2005) and the presence of dissolved organic matter (Burnison *et al*., 2006; Meinelt *et al*., 2001).

2.4.5 Permeability – Physico-chemical properties of test substances

2.4.5.1 Lipophilicity

Some authors suggest that the toxicity of substances to fish embryos exposed at different embryonic stages is likely to be linked to the physico-chemical properties of these chemicals (e.g. Braunbeck *et al.,* 2005, Nguyen *et al.,* 1999). For example Helmstetter and Alden (1995a) state that the amount of organic compounds that penetrate fish eggs is controlled by their lipid solubility. Braunbeck *et al.* (2005) compared the results of zebrafish acute embryo toxicity tests and prolonged exposure of dechorionated embryos conducted with potassiumchromate, 4-chloroaniline, 3,4-dichloroaniline and lindane, concluding that a barrier function of the chorion may increase with lipophilicity. Prolonged exposure of dechorionated embryos to the relatively hydrophilic potassium chromate produced severe disturbances to swimming equilibrium in hatched larvae. In contrast, for rather lipophilic 4-chloroaniline and lindane, a significant increase in toxicity could already be recorded for the core endpoints of the fish embryo test. In a couple of studies with different fish species highly lipophilic pesticides like e.g. esfenvalerate, chlorpyrifos, alpha-cypermetrhin, alpha-endosulfan etc. were demonstrated to be significantly more toxic to fish adults and larvae than to embryos (Barry *et al*., 1995; Hansen *et al.,* 1983, Lammer *et al.*, 2009, Wendler, 2000).

In a couple of studies radioactive marked lipophilic substances were purposeful investigated on their ability to pass the chorion of eggs of various fish species and on their fate and distribution in the embryo (Broyles and Noveck, 1979; Calamari *et al*., 19981 Galassi *et al*. 1982; Klaverkamp *et al*. 1977; Korn and Rice 1981; Marchetti 1965; Skidmore, 1966; Van Leeuwen *et al*., 1985; Wiegand *et al*., 2000): in all cases, the substances were able to pass the chorion and accumulated in the yolk. Embryos became more sensitive with increasing age, suggesting that the substances are delayed in their effectiveness due to their accumulation in the yolk, which is consumed by the sac fry during growth. Ensenbach (1987) investigated acute toxicity, uptake and eliminations kinetics as well as distribution of .pentachlorophenol (5-CP), 4-chlorophenole (4-CP), phenol (P), 4-nitrophenole (4-NP) and methanol in the zebrafish embryo. Although phenols were less toxic for embryos compared to adults an assumed barrier function of the chorion was not confirmed. As expected due to the fact that the yolk consists mainly of phospholipids and triglycerides (Guiney and Peterson, 1980), with

increasing lipophilicity more substance was accumulated in the embryo, especially in the yolk. However, measurement of the elimination ratios indicate that also differences in permeability of chorion and embryonic membranes exist: for 5-CP and 4-CP the chorion and embryonic membrane were equally permeable, whereas for 4-NP and methanol the embryonic membrane was better permeable and for P the chorion was better permeable.

2.4.5.2 (Molecular) Size

It is suspected, that the chorion pores potentially restrict the uptake of compounds depending on their size. This was found for fluorescent dextrans exceeding 3 kDa (Creton, 2004) as well as for polymers, higher molecular weight surfactants and nanoparticles. A comparison between embryo and eleutheroembryo toxicities of polymers resulted in considerably higher sensitivity of the latter (Léonard *et al*., 2005). Nanoparticles of 39.4 – 42 000 nm in diameter were adsorbed on the chorion of medaka eggs, accumulated in oil droplets and distributed size-dependently in the organism (Kashiwada, 2006). Cheng *et al.* (2007) examined the interaction between the zebrafish chorion and raw single-walled carbon nanotubes (SWCNT), ranging in size from several to several hundred micrometers, using scanning-electron microscopy. SWCNT agglomerates could adhere only to the outer layer of the chorion and were too large to enter the chorion. Exposure of *Fundulus heteroclitus* embryos, larvae, and adults to aqua-nC60 resulted in very little mortality and aggregates did adhere to the chorion, not affecting development of the embryos or their hatching success. Movements of aqua-nC60 through the chorion and into the embryo tended to increase with higher exposure levels; however, the concentrations were extremely low and did not differ significantly (Blickley and McClellan-Green, 2008). Some hints on the chorion acting as a barrier for polyethylene glycols of higher molecular weight were found by Lillicrap (2010).

2.5 Manipulation of permeability

Unlike other commonly used carriers, such as acetone or methanol, DMSO can permeate biological membranes without damage to the integrity of these “barriers” (Rammler and Zaffaroni, 1967) and does not structurally modify the test substance, providing the physical or chemical properties. The effect of facilitated uptake in the egg is already used as a noninvasive alternative method to direct injection of chemicals through the chorion, since during development of an injection method by Black *et al.* (1985), it was observed that a drop of the solvent carrier, DMSO placed on the egg surface, elicited a vigorous motor response from the embryo. Due to these observations and the well-known membrane penetration properties of DMSO, it was hypothesized that chemicals could be carried across the chorion

by DMSO resulting in exposure of the developing embryo to the corresponding chemical (Metcalfe and Sonstegard, 1984). Finally, Helmstetter and Alden (1995a; 1995b) used DMSO topical treatment to carry test substances across the egg chorion of the medaka (*Oryzias latipes*), establishing a new technique in aquatic toxicology (Helmstetter *et al.* 1996). In a study of Kais *et al*. (pers com) using fluorescence microscopy, DMSO was demonstrated to influence the uptake of fluorescein across the chorion of zebrafish eggs. DMSO is known to cross the dermal barriers rapidly in high concentrations, which is the result of reversible configurationally changes of proteins by substitution of water by DMSO (Rammler and Zaffaroni, 1967) and, thus, DMSO might also change the proteins of the chorion by water retention, causing a steric change e.g. in the pore canals.

Lillicrap (2010) investigated if chorion permeability of zebrafish embryos could be manipulated by using either pronase or saponin. Findings were that both chemicals had a substantial effect on the outer structure of the chorion, but techniques were too invasive.

3 Conclusion

The morphological structure of fish chorions in general and of the zebrafish embryo in particular is clarified to a large extent, but with regard to toxicological aspects there is still demand of investigations on the chemical composition. Without knowledge of the nature of chorion proteins and other components it is not possible to draw a conclusion on possible chemical interactions between chorion components and xenobiotics and their potential ability to cross the chorion. Most of all the composition of the chorion pores as the only “passage” through the chorion would be of great interest. Although it is known, that permeability of the chorion changes during development and is influenceable by environmental conditions, it is not declared for which kind of molecules the chorion is freely permeable and for which it may act as a barrier. For differences in the sensitivity of early embryos, the ontogenetic stage at begin of exposure is rather responsible than differences in chorion permeability. At least for lipophilic substances, most of which can penetrate membranes easily, it seems rather applicable that with increasing lipophilicity the substance is accumulated in the yolk and becomes available slowly at the beginning of yolk consumption. Nevertheless, differences in permeability of chorion and embryonic membranes for different substances exist, somehow dependant on hydrophilicity and lipophilicity, as well as on other properties of the chemical. Identified exceptions, however, are heavy metals, for which is known that they can be blocked by the chorion via binding. Most likely a complexation with anionic charged groups, possibly

thiol-groups, which are abundantly present in the chorion takes place. It is not declared yet if this accounts only for heavy metals with positive standard electrode potentials, since studies are contradictory and binding and permeability are dependent on salinity, as well as on water hardness, temperature and the presence of dissolved organic matter. Furthermore it is suspected, that the chorion pores potentially restrict the uptake of compounds depending on their size and that permeability is influenceable by use of DMSO, supposingly interacting with the chorion pores.

Chapter 3: Dechorionation as a tool to improve the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*)

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1 Introduction

Historically, fish have always been used for evaluating acute and chronic toxicity and have been accepted as vertebrate representatives for the aquatic environment by the scientific community, regulatory bodies and chemical industries. However, in recent years, there has been an increasing ethical concern, since fish exposed to acutely toxic concentrations of chemicals may be suspected to suffer severe distress and pain. The idea to reduce, refine and replace animal testing by alternative test methods, (Russell and Burch, 1959) has now become an inherent part of the new European Chemical Legislation, REACH. In 2005, the 48 h toxicity test with eggs of the zebrafish (*Danio rerio*) has become mandatory and, thus, replaced the 96 h acute fish test for the routine testing of whole effluent discharges in Germany (DIN, 2001). Based on this protocol, a proposal for a new guideline on fish embryo toxicity for the testing of chemicals has been submitted to the OECD by the German Federal Environment Agency (Braunbeck and Lammer, 2006). Braunbeck *et al*. (2005) provided data substantiating that an optimized test protocol can equally be applied to early embryonic stages of other OECD species such as the fathead minnow (*Pimephales promelas*) and the Japanese medaka (*Oryzias latipes*). In a statistical analysis of existing data, Lammer *et al*. (2009) documented that the correlation between the fish embryo test and the acute fish toxicity test is just as good as the correlation between conventional acute fish toxicity tests with different species, and the scope of fish embryo tests as an animal alternative method in hazard and risk assessment and scientific research has been reviewed by Embry *et al*. (2010). However, a small number of substances were identified to differ significantly with respect to embryonic versus adult toxicity. This observation reignited a discussion, which had been initiated decades ago (Battle and Hisaoka, 1952; Braunbeck *et al*., 2005; Harvey *et al*., 1983; Mizell and Romig, 1997): As all other fish embryos, the zebrafish embryo is surrounded by an acellular envelope, the chorion the intermediate and outermost layers of which are pierced by pore canals. The structure of the chorion is described in detail by Donovan and Hart (1983), Hart and Collins (1991), Rawson et al. (2000) and Cheng et al. (2007). Despite repeated

speculations, it could not be clarified unequivocally whether this chorion represents an effective barrier and, thus, effectively protects the embryo from exposure to distinct chemicals. The pores have been reported to potentially restrict the uptake of compounds depending on their size, as could be shown for fluorescent dextrans exceeding 3 kDa (Creton, 2004).

Moreover, polymers and higher molecular weight surfactants are also suspected to be blocked by the chorion, since a comparison between embryo and eleutheroembryo toxicities resulted in a considerably higher sensitivity of the latter (Léonard *et al*., 2005). For definition of embryos and eleutheroembryos, i.e., developmental stages depending on external food uptake see Belanger *et al*. (2010). There is limited evidence that the permeability of the chorion changes during the embryonic development and that, after hardening, the chorion is less permeable (Gellert *et al*., 2001). It then functions as a barrier for even smaller molecules in the zebrafish embryo (Braunbeck *et al*., 2005; Harvey et al., 1983) as well as in other teleost species (Gonzalez-Doncel *et al*., 2004; Villalobos *et al*., 2000). Especially heavy metals seem to be blocked by the chorion via accumulation at or within the chorion (Stouthart *et al*., 1994; Ozoh, 1980; Van Leeuwen *et al*., 1985; Wedemeyer, 1968). The most obvious approach to investigate the role of the chorion for chemical toxicity is to remove the chorion and expose the "naked" embryo. Several protocols exist for fish embryo dechorionation, using either trypsin/EDTA (Collodi *et al*., 1992) or pronase (Stuart *et al*., 1990; Westerfield, 2007) solutions to digest or even dissolve the chorion. Most studies aimed at recovering embryos for genetic engineering or generating embryonic cells for cell cultures, but only few investigations were carried out to reveal the influence of chemicals on dechorionated embryos (Braunbeck *et al*., 2005; Mizell and Romig, 1997; Ozoh, 1980). According to Westerfield (2007), embryos can also be dechorionated mechanically by use of forceps without any enzymatic digestion. For the specific purposes of these studies, it seemed sufficient to select undamaged embryos or embryonic cells for further operations, and the actual survival rates of dechorionated embryos in all studies mentioned above, however, have never been given special attention never been subjected to statistical analysis. For chemical toxicity assessment, however, a reproducibly high survival rate is of fundamental importance. Therefore, the present study was designed to develop and optimize a dechorionation procedure with reproducibly high survival rates. The success of the dechorionation procedure was tested with a cationic polymer (Luviquat HM 552), which has been suspected to be blocked by the chorion (Léonard *et al*., 2005).

2 Material and methods

2.1 Chemicals and materials

The polystyrene 24-well microtiter plates were provided by Renner (TTP; Dannstadt, Germany), self-adhesive foil (clear polyester sealing tapes) were purchased from Nunc (Wiesbaden, Germany). Agarose with a gelling temperature of 40.5 °C–43.5 °C was provided by SeaKem (HGT Agarose, Cambex BioScience Rockland, ME, USA). Acetone (p.a., Sigma-Aldrich (Deisenhofen, Germany) was tested on embryos dechorionated at 24 hpf exposure at concentrations of 0.5, 1, 1.5, 2 and 2.5%, corresponding to 3.95, 7.9, 11.85, 15.8 and 19.75 g/L. The positive control 3,4-dichloroaniline (p.a.) as well as Luviquat HM522 (20% in H2O) were purchased from Sigma-Aldrich. Luviquat HM 522 (CAS 95144-24-4;MW~400,000 g/mol)was tested at concentrations of 10.5, 21, 42, 84, 168 and 336 mg/L in the standard fish embryo toxicity test (FET) and at concentrations of 1.875, 2.625, 3.75, 5.25, 7.35, 10.29, 14.4 and 20.16 mg/L in a prolonged fish embryo test as well as in tests with eleutheroembryos (embryos post-hatch, but prior to external feeding) and dechorionated embryos. All other chemicals used were purchased from Sigma-Aldrich at the highest purity available. The artificial water used for dilution of the test compounds corresponded to reconstituted water according to ISO 7346/3 (ISO 1996; 294.0 mg/L $CaCl_2 \times 2\ H_2O$; 123.3 mg/L $MgSO_4 \times 7\ H_2O$; 63.0 mg/L $NaHCO_3$; 5.5 mg/L KCl). Before use, the pH was adjusted to 7.8±0.2.

2.2 Fish maintenance and egg production

For details on fish maintenance and egg production, see Lammer *et al*. (2009).

2.3 Dechorionation methods

2.3.1 Enzyme-supported dechorionation

Enzyme-supported dechorionation was carried out according to Westerfield (2007): Under a stereo microscope, 30 min after spawning, fertilized, regularly dividing eggs at the 8- to 32-cell stages were selected and transferred by use of pipettes with a terminal opening of ≥1.5 mm to Petri dishes filled with aerated artificial water and incubated at 26.0±1.0°C. Dechorionation with pronase treatment was performed in 6 h post-fertilization (hpf) embryos (epiboly stage) in 5 replicates with 50 embryos each. Embryos were transferred to agarose-coated Petri dishes filled with temperature-adjusted artificial water and incubated with pronase (protease from *Streptomyces griseus*) with an activity of 4 units/mg diluted to a final concentration of 1 mg/L pronase. When, by occasional agitation, the first embryos dropped out of their chorion, most of the medium was carefully decanted, and the embryos were rinsed

at least 3 times with artificial water to remove excess pronase. After dechorionation, 10 - 12 undamaged embryos per replicate treatment and controls were transferred by plastic pipettes to separate agarose-coated 24-well microtiter plates filled with 2 mL of artificial water. Both the Petri dishes and the multiwell plates were coated with 2% agarose to avoid injury of the dechorionated embryos, which otherwise might occur through direct contact with glass or plastic materials (Igel, 2002). The 24-well plates were covered with self-adhesive foil and incubated at 26.0±1.0°C. The early development was inspected at 24, 48 and 72 h after fertilization. Endpoints for evaluating the influence of enzyme supported dechorionation are described below.

2.3.2 Mechanical dechorionation

In order to elucidate the impact of embryonic age on survival at the time of dechorionation, dechorionation was performed with embryos at 2 hpf (cleavage: ~64-cell stage according to Kimmel *et al.*, 1995), 4 hpf (blastula period: transition between the high and oblong stages), 6 hpf (early gastrula period: 40-50% epiboly) and 8 hpf (late gastrula period: 60-70% epiboly) as well as 24 hpf (pharyngula period: Prim-5 stage). Dechorionation at 2, 4 and 6 hpf was performed with 5 replicates (20 eggs each), whereas dechorionation at 8 and 24 hpf was analyzed in 7 and 12 replicates, respectively (10 eggs each) in order to confirm the reproducibility of the high survival rates. For dechorionation experiments after 2, 4 and 6 hpf, the number of eggs was duplicated in order to increase the chance of surviving embryos. Under a stereo microscope, 30 min after fertilization, fertilized, regularly dividing eggs at the 8- to 32-cell stage were transferred by use of pipettes to Petri dishes filled with aerated artificial water. At different points of development (2, 4, 6 and 8 hpf, respectively), about 50 eggs were transferred to agarose-coated 20 cm Petri dishes, and chorions were removed with sharply pointed forceps (type Dumont™ No. 5) by piercing the chorion gently and - after internal pressure adjustment - enlarging the cleft carefully by means of the forceps. Lifting the chorion and turning it upside down allows the embryo to drop out of its chorion (Fig. 5). During dechorionation, it is important to create a hole big enough for the embryo to leave the chorion undamaged by the edges of the chorion; likewise, excess pressure during manipulations should be avoided. After dechorionation, embryos were carefully transferred by means of plastic pipettes (diameter of the terminal opening: 1.5 mm) to agarose-coated wells of 24-well microtiter plates filled with 2 mL of artificial water per well. During transfer, care should be taken to avoid contact with atmospheric air, since zebrafish embryos tended to inflate upon air contact. The multiwell plates were then covered with self-adhesive foil.

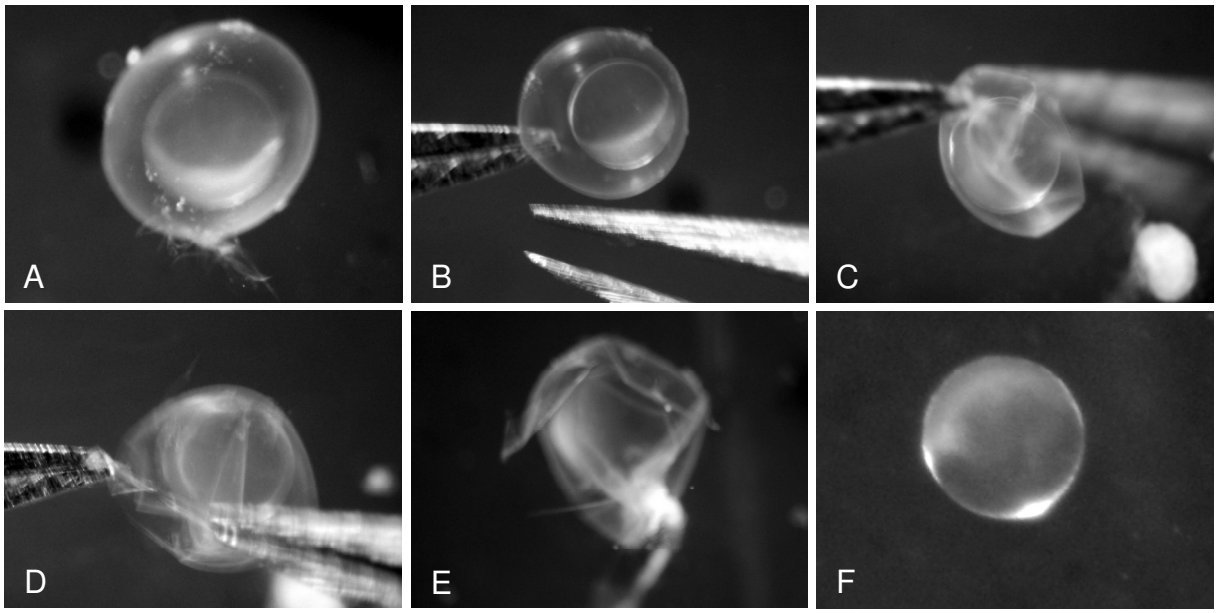

Fig. 5: Mechanical dechorionation using Dumont^{TM} no. 5 forceps: **A:** intact embryo 6 h after fertilization; **B:** egg fixed with one pair of forceps; **C**: piercing the chorion with a second pair of forceps; **D:** enlarging the cleft by pulling apart the chorion by means of two forceps; **E:** chorion with cleft before lifting; F: dechorionated embryo. Average diameter of the embryo with chorion: 0.7 mm.

The embryos were incubated at 26.0 ± 1.0°C. For the embryos dechorionated at 2, 4, 6 and 8 hpf, the early development was inspected at 24, 48 and 72 hours after fertilization, for the embryos dechorionated at 24 hpf, development was checked at 48, 72 and 96 h after fertilization, i.e., after 24, 48 and 72 h of incubation.

Endpoints for the evaluation of development were selected according to DIN 38415-T6 (DIN, 2001) and ISO 15088 (ISO, 2007). For all tests, non-dechorionated embryos in artificial water were used as negative control. Positive controls were tested simultaneously only for 8 and 24 hpf. Preliminary tests (data not shown) discovered a lack of sensitivity of embryos exposed to 3.7 mg/L 3,4-dichloroaniline (the standard positive control in the standard FET (DIN, 2001; ISO, 2007) at an age of 24 hours. With regard to a potential application of dechorionated embryos in toxicity testing, it was therefore decided to verify the effects of 3,4-dichloroaniline at 3.7 mg/L on embryos dechorionated and exposed at 8 and 24 hpf. Additionally, for embryos exposed at 24 hpf tests with 0.5 - 2.5 % acetone (corresponding to 3.95 - 19.75 g/L) were conducted in order to find a suitable concentration to serve as positive control.

2.4 Fish embryo tests with Luviquat HM 522

2.4.1 Standard and prolonged fish embryo toxicity tests with Luviquat HM 522

The standard fish embryo toxicity test (FET) with LuviquatHM522 was conducted in three replicates according to DIN 38415-T6 and ISO 15088 (DIN, 2001; ISO, 2007) as described in Lammer et al. (2009): At least 20 freshly spawned zebrafish eggs were selected per concentration and transferred to 60 mm crystallization dishes containing 100 mL of the different Luviquat HM 522 concentrations and the negative control, respectively. Tests with acetone served as the positive control. Subsequently, 10 fertilized eggs were selected for each test concentration and the control (pure dilution water) and transferred to 24-well plates filled with 2 mL freshly prepared test solutions or dilution water each. For pre-saturation of plastic materials, the 24-well plates had been pre-incubated with the respective concentrations 24 h prior to the exposure of the embryos. After addition of the embryos, the 24-well plates were covered with self-adhesive foil and incubated at 26.0±1.0°C. In the prolonged fish embryo test, the same embryos were examined after 24 and 48 h; in addition to the standard protocol, embryos were inspected after 72, 96, 120 and 144 h. Evaluation of the development was done according to DIN 38415-T6 and ISO 15088 (DIN, 2001; ISO, 2007) for the standard FET; endpoints for older endpoints were selected according to Nagel (2002). The LC_{50} was calculated using probit analysis (Finney, 1952). Embryo tests were classified as valid, if mortalities in negative controls were <10% and positive controls gave mortalities between 20 and 80% (Lammer et al., 2009).

2.4.2 Eleutheroembryo toxicity tests with Luviquat HM 522

For the eleutheroembryo tests, egg were collected the same day as eggs for the prolonged fish embryo test and incubated at 26.0±1.0°C in artificial water until 72 hpf, when most embryos had hatched. In three replicates, for each test concentration, 10 hatched embryos each were selected, transferred to pre-saturated 24-well plates with the test concentrations listed above, covered with self-adhesive foil and incubated at 26.0±1.0 °C. The embryos were inspected after 24, 48, and 72 h of exposure equivalent to an age of 96, 120 and 144 h, respectively. Evaluation of the development was again performed according to DIN 38415-T6 (DIN, 2001), ISO 15088 (ISO, 2007) and Nagel (2002). The LC_{50} was calculated using probit analysis (Finney, 1952). Eleutheroembryo test were classified as valid, if mortalities in negative controls were <10%.

2.4.3 Fish embryo toxicity tests with dechorionated embryos and Luviquat HM 522

For dechorionation tests with Luviquat HM 522, embryos were dechorionated mechanically at an age of 24 hpf as described above. Eggs were collected about 30 min after fertilization the day before testing and kept in artificial water at 26.0±1.0 °C for 24 h. For each of the three replicates per test concentration as well as the negative control (artificial water), 10 embryos were dechorionated as described above and transferred to pre-saturated, 24-well plates covered with self-adhesive foil and incubated at 26.0±1.0 °C. In order to prevent mechanical damage to the very sensitive embryos, the 24-well plates had been pre-coated with 2% agarose. Endpoint recording and determination of LC_{50} calculations were carried out as described above.

2.5 Statistics

Differences in survival and hatching rate between embryos dechorionated at different time points were evaluated by Kruskal-Wallis one-way analysis of variance on ranks ($p = 0.001$) followed by all pairwise multiple comparison (Dunn's method, $p \leq 0.05$).

3 Results

3.1 Enzyme-supported dechorionation

The final number of successfully dechorionated embryos (by means of pronase incubation) ranged between only 6 and 12 out of 50 individuals for each replicate. A statistical evaluation of the results was therefore not possible. One day after dechorionation, 5 of 6, 3 of 8, 8 of 12, 4 of 10, and 5 of 9 of the embryos had developed normally, whereas two days after enzyme treatment not more than 1 - 2 of the embryos in each replicate survived. Sublethal effects did not become evident. Attempts to

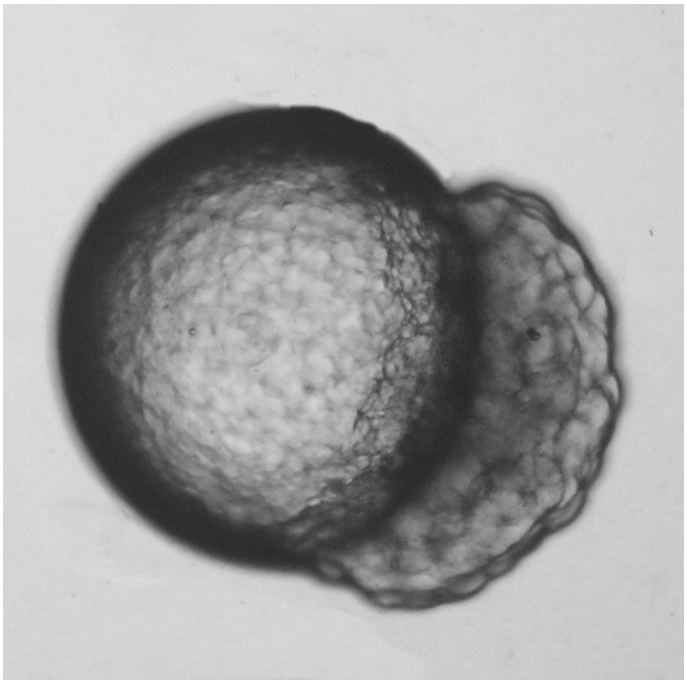

Fig. 6: Intact embryo (256-cell stage) 0.5 h after successful dechorionation by means of pronase treatment.

dechorionate even younger stages by means of pronase treatment mostly resulted in dechorionated, but damaged embryos. In only one exceptional case, pronase treatment of 100 embryos resulted in a total of 24 embryos, which were not damaged by the procedure itself (Fig. 6). On the day after dechorionation, 54% of the embryos were coagulated; the rest, however, developed normally, even at the second and third day after dechorionation. Preliminary tests with pronase pretreatment at the 16- and 32-cell stages to soften the chorion, but not to completely dechorionate the embryos resulted in similar mortality rates as seen after complete dechorionation with pronase (details not shown). Interestingly, after pronase softening, some embryos at first developed normally and left - as intended - the chorion very early; however, after 24 h they were clearly underdeveloped, and a secondary "envelope" with the shape of the embryo could be identified (Fig. 7).

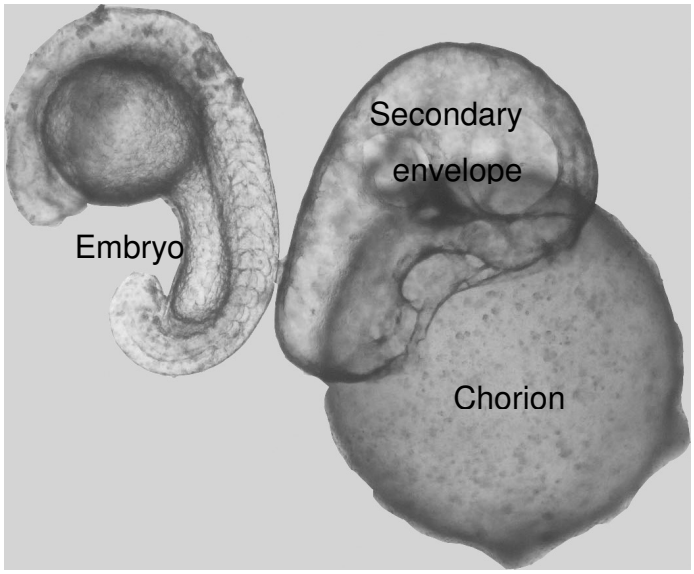

Fig. 7: 24 h after dechorionation with pronase pretreatment, the embryo has left the chorion and developed a secondary "envelope" produced by the embryo after dechorionation.

3.2 Mechanical dechorionation

Dechorionation at 2, 4, 6 and 8 h as well as 24 h post-fertilization (hpf) resulted in low rates of sublethal effects of < 5 %. Mortality was restricted to rapid (24 h) coagulation, which always showed typical appearance of complete disintegration (Fig. 8).

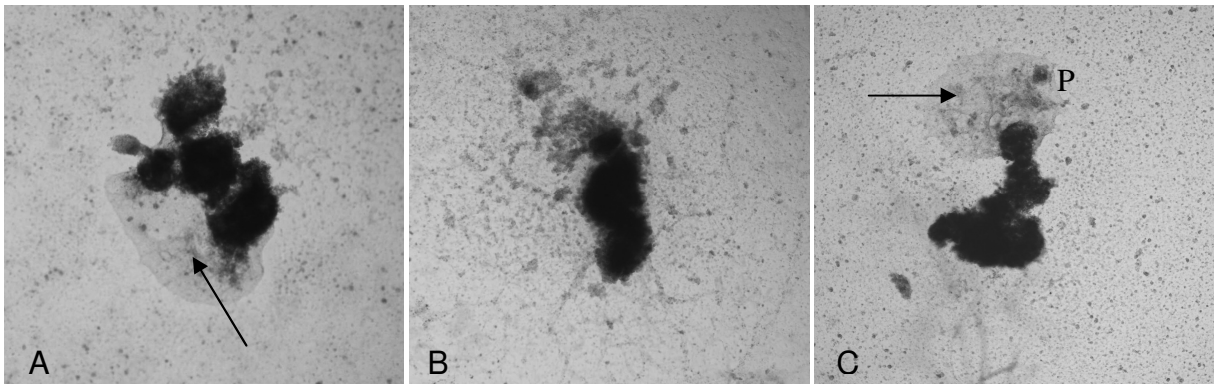

Fig. 8: Typical appearance of coagulated embryos at 24 h as a consequence of inadequate mechanical dechorionation after 2 (**A**), 4 (**B**) and 6 h (**C**) post-fertilization: embryos did not only appear coagulated, but are characterized by total leakage of the embryo; at arrows probable remains of the integument

There was no significant increase of mortality after periods of up to two days. For dechorionation at 2, 4 and 6 hpf, mean survival rates on the second day after dechorionation were 61, 54 and 62%, respectively, whereas for dechorionation at 8 hpf, the mean survival rate reached 80%. An evaluation of the individual replicates (Tab. 3) showed that the survival rate fluctuated considerably between test replicates for dechorionation experiments at 2, 4 and 6 hpf, but became more constant for dechorionation experiments at 8 hpf.

Tab. 3: Percentage of normally developed zebrafish embryos 2 days after mechanical dechorionation (n = 20 for each replicate)

Replicate no.	Time of dechorionation (h post fertilization)			
	2h	4h	6h	8h
1	30	20	30	80
2	80	75	75	70
3	100	80	75	80
4	35	40	70	70
5	60	57	62	90
6	-	-	-	90
7	-	-	-	80
Mean	61	54.4	62.4	80

Dechorionation at 24 hpf resulted in an average survival rate of 90% at 48 h after dechorionation, i.e. compatible with survival rates required for negative control embryos in the standard fish embryo test (Lammer *et al.* 2009). A comparison of normal development, mortality and sublethal effect rates for the second day after dechorionation for all dechorionation times is shown in Fig. 9. For comparison, data for non-dechorionated embryos have been added.

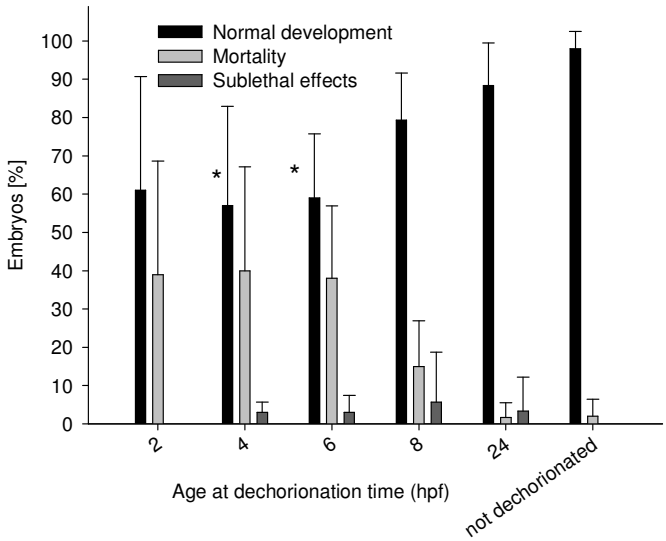

Fig. 9: Correlation between the age of zebrafish embryos at time of dechorionation and the ratio of normal development versus mortality as well as sublethal effect rates on the second day after dechorionation. For comparison, data for non-dechorionated embryos have been added.*Results statistically different compared to the group dechorionated 24 hpf (Dunn's method, $p<0.05$).

3.3 Positive controls (3,4-dichloroaniline, acetone)

After 48 h, exposure to 3,4-dichloroaniline at 3.7 mg/L, i.e. resulted in mortalities of 36% for embryos dechorionated at 8 hpf. This is within the range expected for the positive control in the standard fish embryo test. Typically, about 20% of the embryos exposed to 3,4-dichloroaniline showed additional sublethal effects: After 24 h of exposure, 20% of the embryos displayed lack of spontaneous movement and about 4% showed retardation in tail development; after 48 h of exposure, predominant sublethal effects were edema formation at the pericardium and/or the yolk sac, impairment of cardiovascular functions and development of general malformations (Fig. 10).

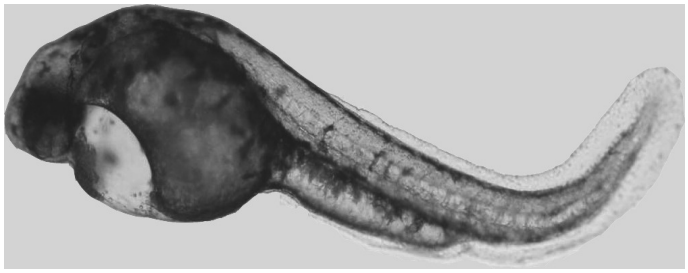

Fig. 10 After 48 h exposure to 3.7 mg/l 3,4-dichloroaniline, zebrafish embryos dechorionated at the age of 8 h after fertilization show edemata and tail malformation as well as a lack of heart beat and blood circulation.

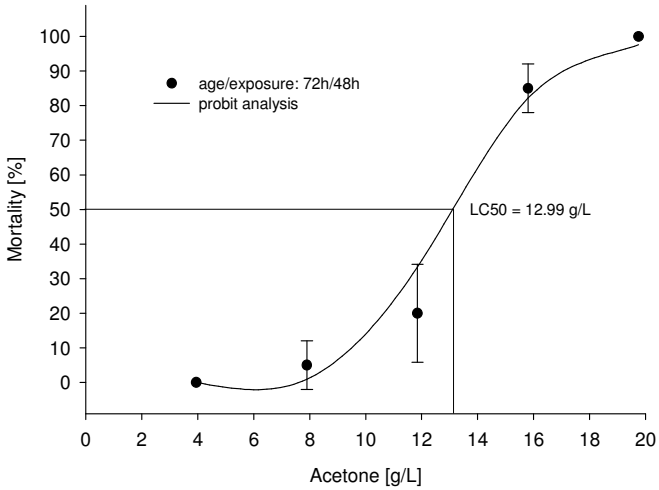

Fig. 11. Mortality of 72 h old dechorionated zebrafish embryos exposed to acetone for 48 h from the age of 24 hpf. LC_{50} was calculated using probit analysis (Finney, 1952).

However, 3,4-dichloroaniline exposure of embryos dechorionated at 24 hpf resulted in mortality rates of about 8%, i.e. comparable to the mortality rates of negative controls. Moreover, the overall rates of sublethal effects (12 and 32% after 24 and 48 h of exposure, respectively) were even lower than expected from standard fish embryo tests and were restricted to edema formation.

Fig. 12: After 48 h exposure to 15.8 mg/l acetone, zebrafish embryos dechorionated at the age of 24 h after fertilization show complete lack of pigmentation as well as lack of heartbeat and blood circulation.

In contrast, acetone exposure following dechorionation at 24 hpf resulted in an LC_{50} value of 12.99 g/L for 48 hours of exposure (Fig. 11), thus corresponding to literature values of the standard fish embryo test, which range between 10.5 g/L (Maiwald, 1997) and 13.1 g/L (Bachmann, 2002). Sublethal effects by acetone included edema formation, reduced pigmentation, and a negative impact on heartbeat and blood circulation (Fig. 12) as well as

tail deformations. With prolonged exposure, these effects became more prominent and, as an additional effect, coagulation could be observed.

3.4 Results of fish embryo tests with Luviquat HM 522

3.4.1 Standard and prolonged fish embryo tests with Luviquat HM 522

In the standard 48 h fish embryo test, an LC_{50} value for Luviquat could not be determined within the range of the concentrations tested. Therefore, LC_{50} was determined to be ≥ 336 mg/L). Prolongation of the exposure to an age of 72 h (i.e. including hatching) resulted in 100% mortality at all concentrations except for the lowest (10.5 mg/L) with a mortality of 80% (Fig. 13). No sublethal effects were observed.

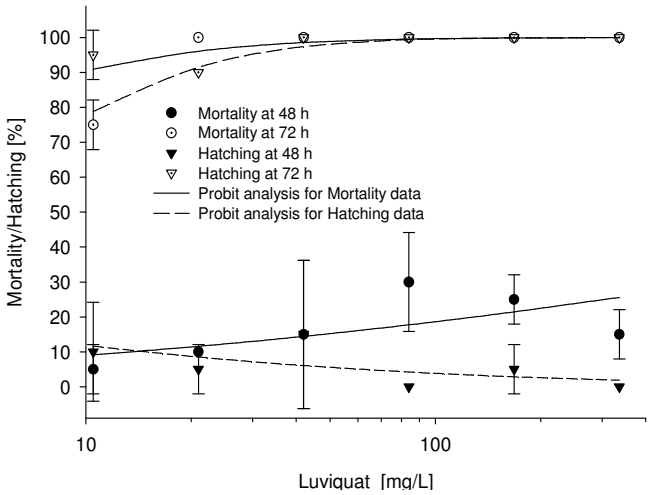

Fig. 13: Mortalities of zebrafish embryos (—) after 48 (●) and 72 h (○) and corresponding hatching rates (- - -) after 48 h (▼) and 72 h (▽) of exposure to Luviquat. Data fitted by probit analysis (Finney, 1952).

Additional prolonged tests in refined and lower concentration ranges confirmed that at lower concentrations mortality never occurs before hatch (Fig. 14): After 24 h of exposure, no mortality was observed. After 48h of exposure, concentrations between 7.35 and 20.16 mg/L stimulated not only the beginning of hatch, but also an average of 30% mortality at the highest concentration. After 72 h of exposure, almost all embryos had hatched, and an LC_{50} of 9.5 mg/L could be determined. LC_{50} values for 96 and 120 h of exposure were calculated at 6.5 mg/L, respectively. The major lethal effect was coagulation, and in some cases an influence on heartbeat and blood circulation was evident. Sublethal effects such as edema formation and malformations of the tail occurred sporadically without an apparent dose-response relationship.

3.4.2 Eleutheroembryo toxicity tests with Luviquat HM 522

In order to exclude the possibility that the increased toxicity of Luviquat after hatch was a consequence of prolonged exposure, separate tests with eleutheroembryos exposed from an age of 72 h (i.e., exposure exclusively after hatch) were conducted. After 24 and 48 h of exposure (i.e., at a total age of 96 and 120 h), the LC_{50} could be determined at 2.9 and 2.8 mg/L, respectively. Again, the major lethal effect was coagulation, and in some cases impairment of heartbeat and blood circulation was seen. The dose–response relationship was

comparable to that of the prolonged fish embryo test (Fig. 14). Sublethal effects (edemata, tail malformations) occurred at low rates, but did not prove dose-dependent.

3.4.3 Fish embryo toxicity test with Luviquat HM 522 in dechorionated embryos

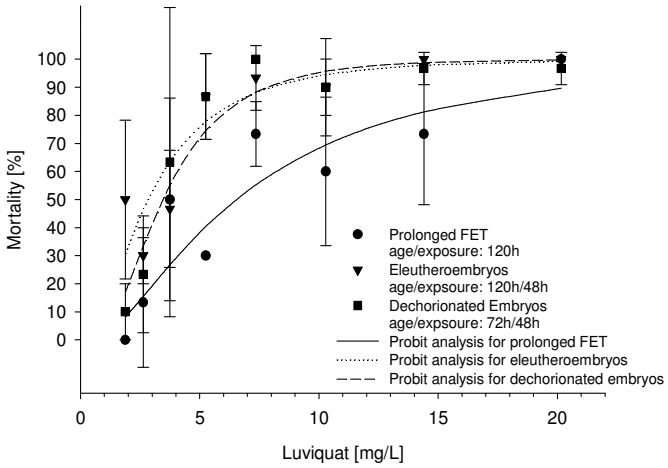

Fig. 14: Comparison of mortalities after 120 h of exposure to Luviquat in nondechorionated embryos (●), after 48 h of exposure to Luviquat in eleutheroembryos exposed from the age of 72 h (total age of embryos: 120 h; ▼), and after 48 h of exposure to Luviquat in embryos dechorionated at 24 h post fertilization (total age of embryos: 72 h; ■). Data fitted by probit analysis (Finney, 1952).

Exposure of embryos dechorionated at an age of 24 hpf resulted in a dose–response relationship almost identical to that of the eleutheroembryo tests (Fig. 14). The LC_{50} values for Luviquat after 24 h and 48 h of exposure (embryonic age: 48 and 72 h, respectively) were 3.5 mg/L. Again, lethal effects were restricted to coagulation, and sublethal effects only occurred sporadically.

4 Discussion

Dechorionation of zebrafish embryos by incubation in pronase with reproducibly high survival rates turned out to be difficult. Especially early developmental stages could not be dechorionated successfully with a satisfactory amount of embryos. Embryos incubated with pronase at 6 hpf displayed survival rates as low as 20% and could, thus, not be used reliably for chemical testing. Likewise, tests with pronase pretreatment at 16- to 32-cell-stages to soften the chorion prior to subsequent mechanical dechorionation resulted in similarly low survival rates. Interestingly, some embryos released by pronase treatment first developed normally and left – as intended – the chorion prematurely, but were clearly underdeveloped after 24 h and formed a “second envelope” with the shape of the embryo. Overall, results thus indicate that pronase treatment is detrimental for zebrafish embryos, even though they might first look undamaged. Similar observations were made by Morrison *et al.* (2003) for dechorionation of Nile tilapia (*Oreochromis niloticus*) embryos. Only in exceptional cases, attempts to digest the chorion by pronase treatment at the cleavage or blastula stage resulted in survival for one or two days, but embryos did not reach normal hatching stage. As with zebrafish, partial chorion digestion at the gastrula or early segmentation stages still affected survival until hatching stage (Morrison *et al.*, 2003). Since the influence of pronase on normal

embryonic development is that erratic, pronase treatment did not prove a suitable method for dechorionation with the purpose of providing dechorionated embryos for ecotoxicological testing. Pretests with trypsin solutions (details not shown) indicated similarly poor results. Enzyme activity is generally difficult to control: The precise point of time when the chorion is degraded sufficiently without affecting the plasmalemma differs strongly with the enzyme batch and with the specific egg batch. Whether other digestive enzymes would give better results, needs to be elucidated.

In contrast, the delicate process of mechanical dechorionation with fine forceps at 2, 4, and 6 hpf turned out to be generally feasible. The mean survival rates, however, showed a high variability around 60% and were, thus, not acceptable for toxicological purposes. A pilot study into mechanical dechorionation of zebrafish at 4 hpf by Marguerie *et al.* (2007) gave similar results. For this reason, dechorionation at younger stages should only be applied in exceptional cases, especially when an influence on very early development is suspected.

For routine testing, however, only mechanical dechorionation at 8 hpf and 24 hpf resulted in reproducibly high survival rates of 80% and ≥90%, respectively. Normally, mechanical damage of embryos due to the dechorionation procedure itself can easily be identified by their typical appearance, i.e. coagulation in combination with complete disintegration of the embryo. As a rule, with increasing practice of the experimenter, survival rates tended to be higher and more stable. Thus, survival rates of ≥90% might be suspected also for embryos dechorionated at 8 hpf.

In the standard fish embryo test protocol as submitted to the OECD (Braunbeck and Lammer, 2006), one validity criterion for the test is a negative control (artificial water; ISO 7346) with ≥90% survival and normal development of the embryos up to at least 48 h of exposure. Thus, the threshold for validity of a dechorionation protocol should also be ≥90% survival. According to the existing DIN (2001) protocol for whole effluent testing, exposure to the positive control 3.7 mg/L 3,4-dichloroaniline should result in 20 to 80% mortality of zebrafish embryos. In embryos dechorionated at 24 hpf and exposed from 24 hpf, however, exposure to 3.7 mg/L of 3,4-dichloroaniline did not show any mortality. On the other hand, embryos dechorionated mechanically at an earlier stage, e.g. already at an age of 8 hpf showed mortality rates comparable with those of the standard fish embryo assay. These results indicate that damage by 3,4-dichloroaniline is exerted at very early stages of zebrafish development, which is in line with results by Lahnsteiner (2008), who reported that exposure of non-dechorionated zebrafish embryos to 3.6 mg/L 3,4-dichloroaniline at an age≤12 hpf

resulted in mortality rates as expected from the positive control of the standard fish embryo test (20 – 80%; ISO, 2007), whereas embryos exposed at ≥12 hpf showed only minor mortality, if any. As a consequence, for zebrafish dechorionated at 24 hpf, 3,4-dichloroaniline does not appear to be a suitable positive control.

As an alternative candidate as a positive control substance for the dechorionation tests, acetone was selected, since it is easily available and unproblematic with respect to handling and disposal. Acetone has repeatedly been tested in the fish embryo test with LC_{50} values between 8.2 and 13.09 g/L (Maiwald, 1997; Bachmann, 2002); the correlation to the conventional fish toxicity test (LC_{50} values ranging between 6.21 and 10.7 g/L; US EPA, 2002) is excellent. Likewise, the toxicity of acetone to zebrafish embryos dechorionated at the age of 24 h old resulted in an LC_{50} value of 12.99 g/L.

Overall, the potential barrier function of the chorion as a confounding factor in embryo tests with zebrafish can be overcome by dechorionation of 24 h old embryos. However, given the fact that successful dechorionation not only requires considerable experimental experience, but is also quite time-consuming, a modified FET protocol including dechorionation will probably be restricted to specific purposes, but hardly become a routine method. Moreover, a modified FET protocol for dechorionated embryos, which would only allow toxicant exposure after 24 hpf, would only be considered very cautiously since it would, firstly, exclude every other chemical showing higher toxicity before than after 24 hpf, and, secondly, likely raise the number of false negatives for teratogenic chemicals. Part of these restrictions, however, could be overcome by exposure of the eggs prior to dechorionation at 24 hpf. Alternatively, in cases when such problems were suspected, dechorionation might also be carried out at 8 hpf.

For pilot tests with dechorionated zebrafish embryos Luviquat HM522 was selected, a cationic polymer which has been suspected to be blocked by the chorion due to its molecular weight (approx. 400,000 g/mol). A comparison of adult fish toxicity values (LC_{50} for *Oncorhynchus mykiss* is 0.56 – 1 mg/L (MSDS)) with the LC_{50} of the standard 48 h fish embryo test (≥336 mg/L) showed that nondechorionated embryos were significantly less sensitive than adult fish. Prolongation of embryo exposure beyond hatch as well as eleutheroembryo tests with embryos exposed from hatch at 72 hpf to 120 hpf resulted in LC_{50} values of 6.5 and 2.8 mg/L, respectively. Thus, an immediate effect of exposure periods longer than 72 h could be excluded; rather, the chorion is, indeed, an effective barrier for Luviquat HM 522, which could definitely be confirmed by exposure of zebrafish, which had

been dechorionated at the age of 24 hpf and proved as sensitive as eleutheroembryos (LC_{50} 3.4 mg/L).

5 Conclusions

The present investigation demonstrates that a modified fish embryo test can be conducted with zebrafish embryos dechorionated at 24 hpf with reliably high survival rates. However, the standard positive control test substance, 3,4-dichloroaniline, must be replaced, since this substance exerts its toxicity during the first 24 h of development. As an alternative, acetone can be recommended. Dechorionation of younger stages is basically possible, but with decreasing survival rates; in zebrafish, 8 hpf seems to be the lower limit for dechorionation. Cationic polymers with high molecular weights such as Luviquat HM 522 can be demonstrated to be blocked by the chorion, but to exert full toxicity (1) in embryos that are allowed to hatch normally (extended fish embryo test), (2) in embryos dechorionated at the of 24 hpf, as well as (3) in eleutheroembryos exposed from beyond hatch for up to 48 h.

Chapter 4: Investigations if the uptake of chemicals into the embryo through the chorion is limited by the molecular size of the molecule

1 Introduction

As all other fish embryos, the zebrafish embryo is surrounded by the chorion, in the case of zebrafish a 1.5 - 2.5 µm thick acellular envelope, which consists of electron-dense outer and innermost layers (thickness: 0.2 - 0.3 and 1.0 -1.6 µm, respectively) and an electron-lucent intermediate layer (thickness: 0.3 - 0.6 µm; (Hart and Donovan, 1983; Hart and Collins, 1991; Rawson *et al*., 2000). The intermediate and inner layers are penetrated by pore canals with a dimension of 0.17 µm² (Cheng *et al*., 2007). This semi-permeable membrane was shown to be freely permeable for water, ions and small molecules and it is believed that the pores in the chorion may be one possible passage of entry for chemicals into the embryo (Hisaoka, 1958; Rawson *et al*., 2000; Zhang and Rawson, 1996). However, there appears to be some properties associated with certain chemicals which limit uptake across the chorion: e.g. the chorion pores may potentially restrict the uptake of compounds depending on their size. This was found for nanoparticles (Blickley and McClellan-Green, 2008; Cheng *et al*., 2007; Kashiwada, 2006), polymers and higher molecular weight surfactants (Henn and Braunbeck, 2011; Léonard *et al*., 2005) as well as for fluorescent dextrans (Creton, 2004). Creton (2004) noticed using fluorescent dextrans as molecular probes for Ca^{2+} imaging that dextrans of a size of 3 kDa can diffuse through the chorion of zebrafish embryos, while for 10 kDa dextrans the chorion was not permeable.

This chapter investigates if there exists a “critical molecular size”, which would not allow a substance to pass the chorion. To locate this “critical molecular size” polymers present themselves to be an ideal test substance: a polymer is a macromolecule composed of repeating identical structural units typically connected by covalent chemical bonds, which are available in various molecular weights. Since the basic unit is always the same, no additional functional groups which might interact with the chorion are introduced. Polyethylene glycols were chosen as the test substance due to its availability in different varieties of molecular weight and since they are perfectly soluble in water. Due to the size evidences of between 3 and 10 kDa (Creton, 2004), in this study polyethylene glycols of between 2 and 12 kDa are tested on their ability to pass the chorion.

2 Material and methods

2.1 Chemicals and materials

The polystyrene 24-well microtiter plates were provided by Renner (TTP; Dannstadt, Germany), the self-adhesive foil (clear polyester sealing tapes) were purchased from Nunc (Wiesbaden, Germany). Agarose was provided by SeaKem (HGT Agarose, Cambex BioScience Rockland, ME, USA; gelling temperature: 40.5°C - 43.5°C). The positive control 3,4-dichloroaniline (p.a.) were purchased from Sigma-Aldrich at the highest purity available. Polyethylene glycols (CAS 25322-68-3) PEG 2000 (average Mn 1900 - 2200), PEG 3000 (average Mn 3,015 - 3,685), PEG 4000 (average Mn 3500 - 4500), PEG 6000 (average Mn 5000 - 7000), PEG 8000 (average Mn 8000), and PEG 12000 (average Mn 11000 - 15000) were tested at concentrations of 1, 5, 25, 50, 75, and 100 g/L in the standard fish embryo toxicity test (FET), in a prolonged fish embryo test, in recovery investigations as well as in tests with dechorionated embryos. The artificial water used for dilution of the test compounds corresponded to reconstituted water according to ISO 7346/3 (ISO,1996; 294.0 mg/L $CaCl_2$•2 H_2O; 123.3 mg/L MgSO4•7 H_2O; 63.0 mg/L $NaHCO_3$; 5.5 mg/L KCl), which was diluted 1:5 using double-distilled water. Before use, the pH was adjusted to 7.8 ± 0.2.

2.2 Fish maintenance and egg production

For details on fish maintenance and egg production, see Lammer et al. (2009).

2.3 Fish embryo testing

2.3.1 Standard and prolonged fish embryo toxicity test

The standard FET was conducted according to DIN 38415-T6 and ISO 15088 (DIN, 2001; ISO, 2007): Embryo tests were initiated at latest 2 h after fertilization (~ 64 cell stage). Due to preliminary investigations, pre-exposure was abandoned. 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 pre-saturation, 24-well plates had been 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 26.0 °C ± 1.0 °C. The embryos were examined after 24, 48, and 72 h in the standard FET, and additionally at 96, 120 and 144h in the prolonged fish embryo test. Evaluation of the development was done according to DIN 38415-T6 and ISO 15088 (DIN, 2001; ISO, 2007). The LC_{50} was determined graphically by linear interpolation. As a negative control, artificial water was used, as a positive control 3,4-dichloroaniline at 3.7 mg/L was tested. Embryo tests were

classified as valid, if the mortality in the negative control was less than 10 %, and the positive control gave mortalities between 20 and 80 %.

2.3.2 Investigations on recovery

Recovery tests were prepared like the standard fish embryo toxicity test, conducted with the same concentrations including a positive control (3,4-dichloroaniline) and a negative control (artificial water). However, in contrast to the standard test, the embryos were examined after 2 h of exposure on effects, and afterwards transferred to 24-well plates filled with 2 ml dilution water. After another 2 h, embryos were examined on recovery. Additionally, embryos were examined at 24 h and 48 h. Evaluation of the development was done following the standard procedure.

2.3.3 Fish embryo toxicity test with dechorionated embryos

For dechorionation tests, embryos were dechorionated mechanically as described in Henn and Braunbeck (2011) vat an age of 24 hours. Eggs were collected at about 30 minutes after fertilization the day before testing and incubated at 26.0 ± 1.0 °C in artificial water. For each test concentration and the negative control (artificial water), 10 embryos were dechorionated and transferred to pre-saturated, 24-well plates covered with self-adhesive foil and incubated at 26.0 ± 1.0°C. The embryos were examined after 24, 48, 72, 96, and 120 hours of exposure. Endpoint recording and determination of LC50s were carried out as described above.

3 Results

3.1 Standard and prolonged fish embryo toxicity test

Exposure of 48 hours with PEG 2000, PEG 3000, and PEG 4000 in concentration of between 1 - 100 g/L resulted in no or very low mortality. No LC_{50} could be determined. Exposure to PEG 6000, PEG 8000, and PEG 12000 in the same concentration range resulted in higher mortalities, allowing determination of LC_{50} values (Fig. 15): LC_{50}s (48h) for PEG 6000, PEG 8000, and PEG 12000 were calculated as 90.8 g/L (15. 1 mmol/L), 36.3 g/L (4.5 mmol/L), and 38.8 g/L (3.2 mmol/L), respectively.

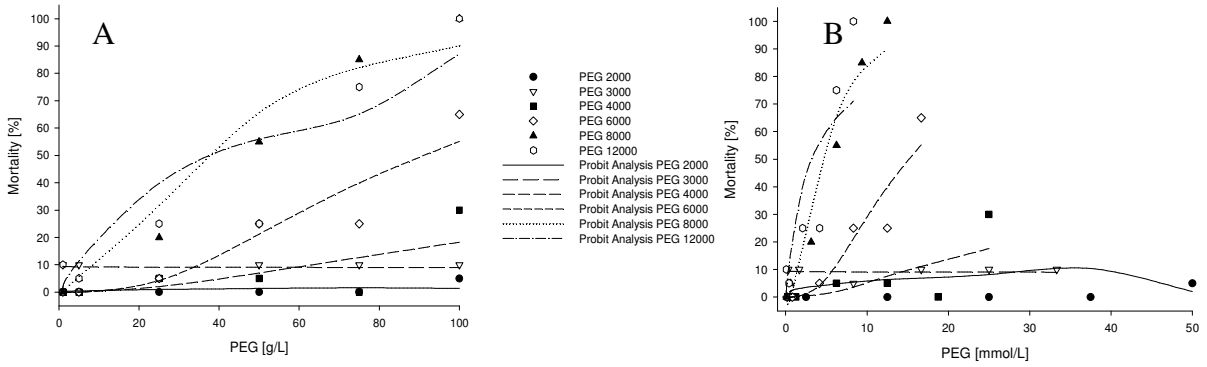

Fig. 15: Comparison of mortalities after 48 h of exposure to polyethylene glycols of different molecular weights (**A:** PEG concentration in g/L; **B:** PEG concentration in mmol/L): exposure to PEG 2000 (average Mn 1900 - 2200; ●), to PEG 3000 (average Mn 3,015 - 3,685; ▼), to PEG 4000 (average Mn 3500 – 4500; ■), to PEG 6000 (average Mn 5000 – 7000, ♦), to PEG 8000 (average Mn 8000; ▲), and to PEG 12000 (average Mn 11000 – 15000; □); Data fitted by probit analysis (Finney, 1952).

The only lethal effect observed, was coagulation. In addition to occasionally appearing edema, sometimes accompanied by a slight influence on blood circulation, the only sublethal aberrations of embryonic development observed, were general deformations of the embryo without any specific occurrence (Fig. 16).

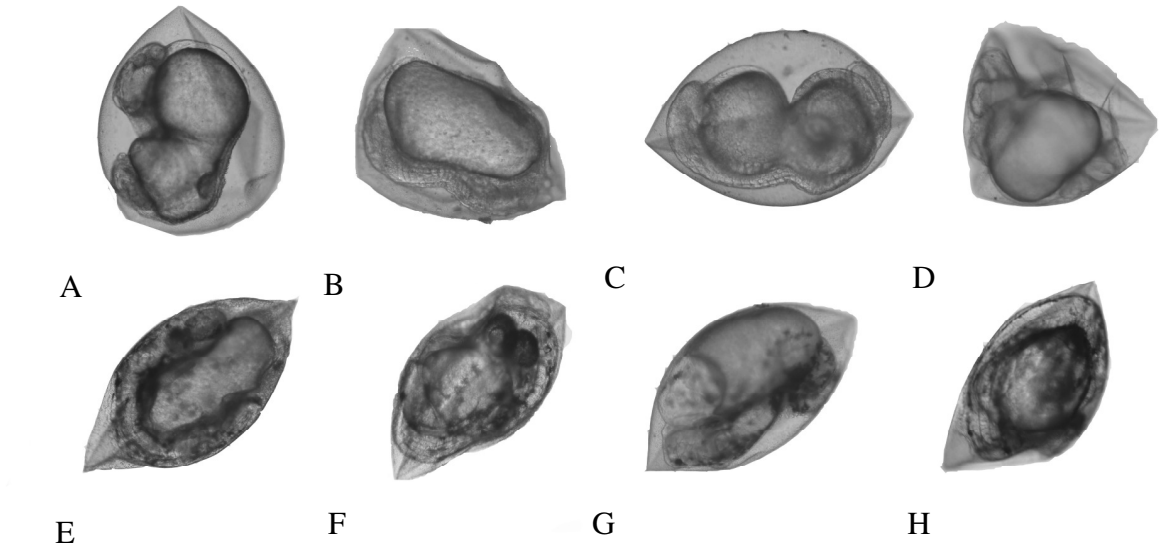

Fig. 16: Various malformations of zebrafish embryos after 24 h (**A-D**) and 48 h (**E-H**) of exposure to polyethylene glycols of different molecular weights; **A:** exposure to 25 g/L PEG 4000; **B:** exposure to 50 g/L PEG 4000; **C:** exposure to 5 g/L PEG 6000; **D:** exposure to 25 g/L PEG 8000; **E:** exposure to 50 g/L PEG 12000; **F:** exposure to 25 g/L PEG 8000; **G:** exposure to 25 g/L PEG 8000; **H:** exposure to 50 g/L PEG 12000.

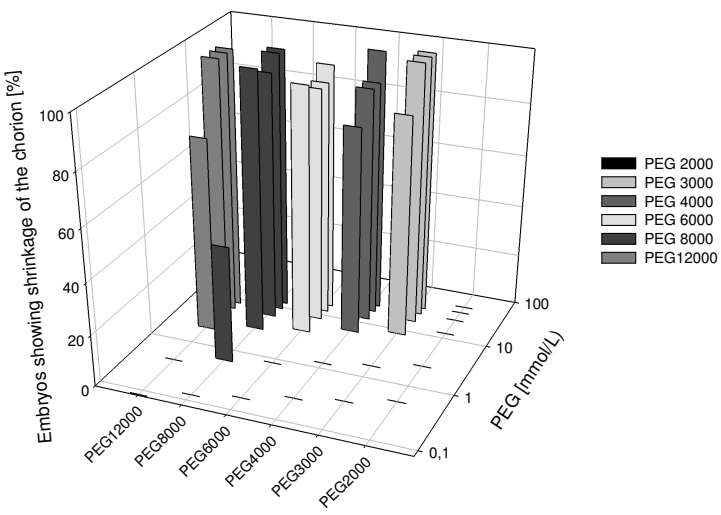

Fig. 17: Embryos showing a shrinkage of the chorion after 24 h of exposure to polyethylene glycols of different molecular weights: exposure to 1 – 100 g/L PEG 2000 (average Mn 1900 - 2200), PEG 3000 (average Mn 3,015 - 3,685), PEG 4000 (average Mn 3500 – 4500), PEG 6000 (average Mn 5000 – 7000), PEG 8000 (average Mn 8000) and PEG 12000 (average Mn 11000 – 15000).

Fig. 17 shows the percentage of embryos exhibiting shrinkage of the chorion for all tested PEGs in all test concentrations. Interestingly, for exposure to PEG 2000 (Fig. 18) no shrinkage of the chorion was observed, whereas exposure to all other tested polyethylene glycols in concentrations > 5 g/L resulted in light (Fig. 19) to heavy deformations (Fig. 20) of the chorion.

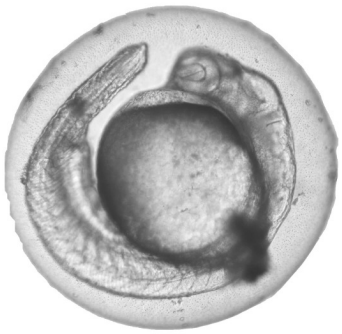

Fig. 18: Embryo after 24 h of exposure to 25 g/L PEG2000 – no deformations of the chorion

Fig. 19: Embryo after 24 h of exposure to 25 g/L PEG3000 – light deformations of the chorion

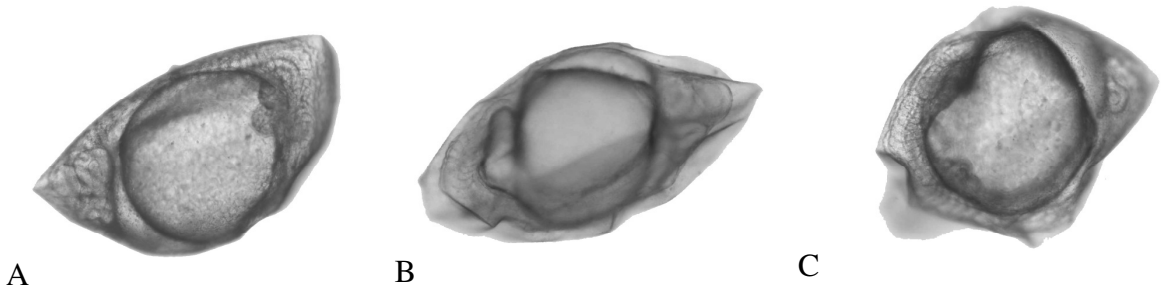

Fig. 20: Embryos after 24 h of exposure to 25 g/L polyethylene glycol of (**A**) PEG 6000 (average M_n 5000 – 7000), (**B**) PEG 8000 (average M_n 8000) and (**C**) PEG 12000 (average M_n 11000 – 15000) – heavy deformations of the chorion

Preliminary tests discovered a beginning of this phenomenon shortly after start of exposure (Fig. 21).

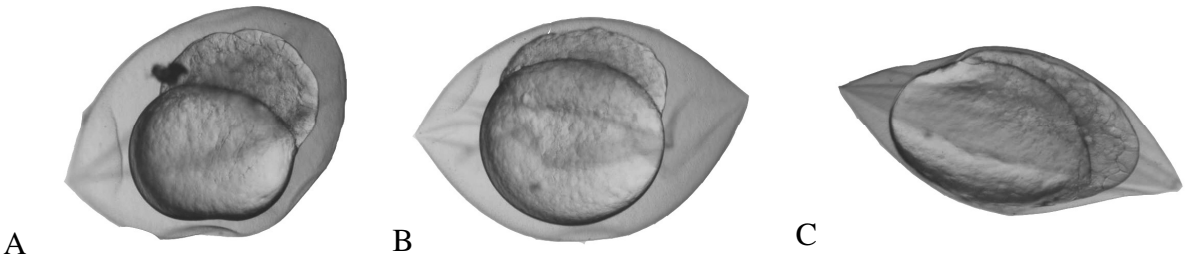

Fig. 21: Embryos after 10 minutes of exposure to PEG 12000 (average Mn 11000 – 15000); **A:** exposure to 25 g/L; **B:** exposure to 50 g/L; **C:** exposure to 75 g/L

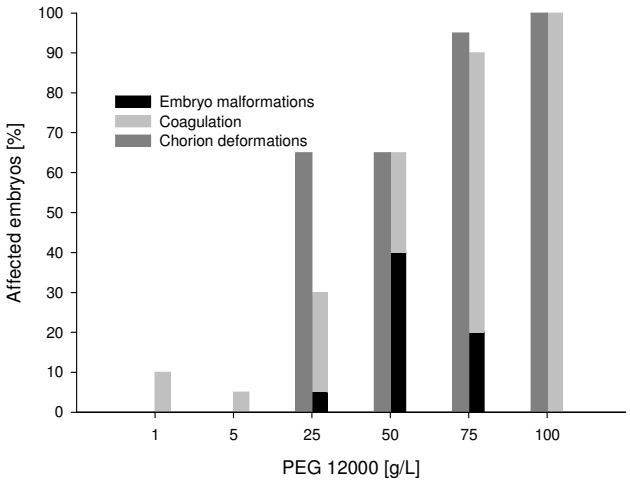

Fig. 22: Comparison of chorion deformations after 24 h of exposure, embryo malformations and coagulation rate after 48 h of exposure to PEG 12000 in non-dechorionated embryos.

Since eggs were highly sensitive during pipetting then, for all following tests, pre-exposure was dispensed with. Interestingly, with increasing number of embryos with deformed chorion, also the number of coagulated and malformed embryos increased. Beyond that, the sum of embryos malformed and coagulated in each concentration equals the number of embryos with deformed chorion as is shown in Fig. 22 for exposure to PEG 12000. Prolongation of exposure did not result in increased mortality for any of the tested polyethylene glycols: For PEG 2000, PEG 3000, and PEG 4000 no LC_{50} could be calculated since lethal effects were ≤ 50% for all concentrations tested. For PEG 6000, 8000, and 12000 LC_{50} values were calculated as 52.1 g/L (8.7 mmol/L), 23.2 g/L (2.9 mmol/L) and 26.3 g/L (2.2 mmol/L), respectively, and thus, in the same range as after 48 hours of exposure. Except for general malformations, other sublethal effects occurred only sporadically. The general malformations were of various appearances, without any regularity, varying from simple malformations of the spine or the tail to severe deformations of the whole larvae, to larvae without eyes or even a head (Fig. 23).

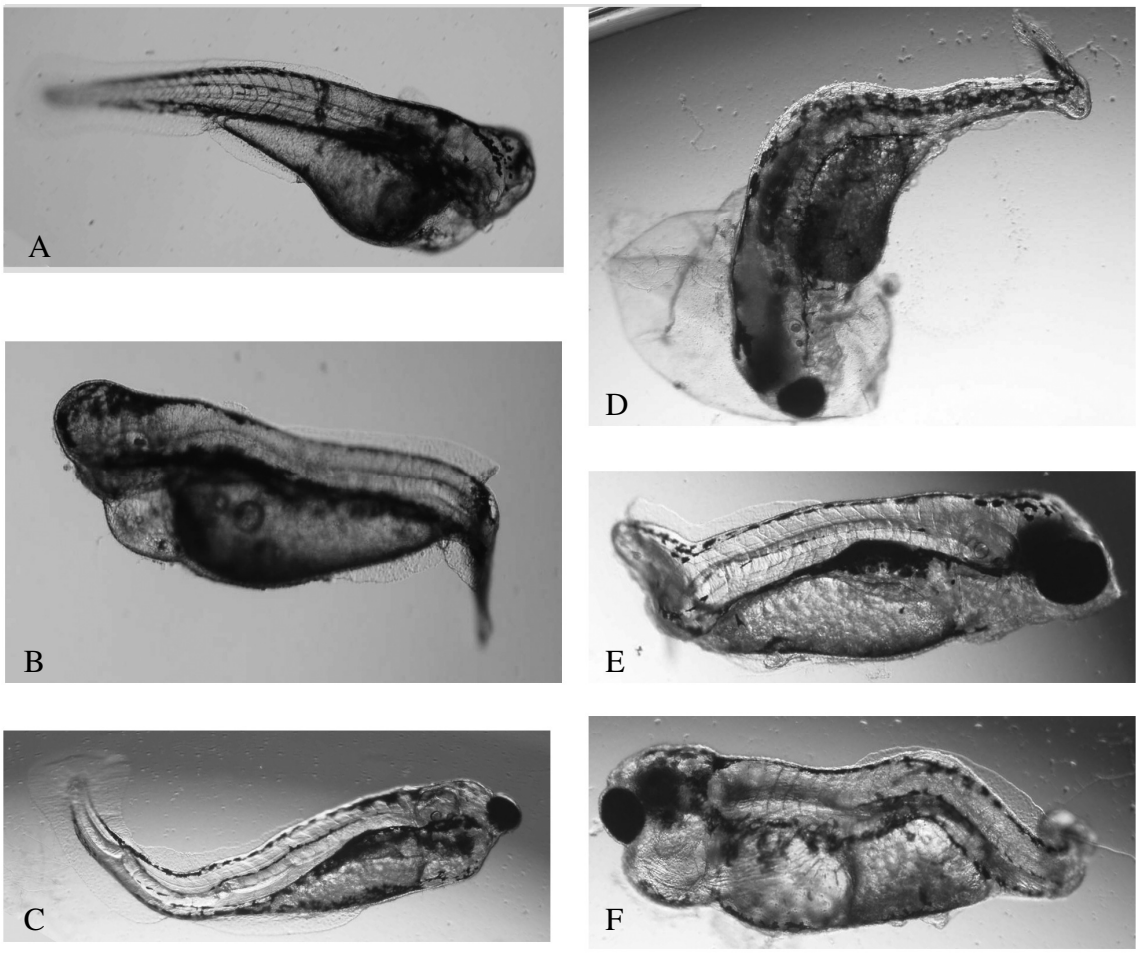

Fig. 23: Embryos after 120 h of exposure to different concentrations of polyethylene glycols of different molecular weights; **A:** exposure to 25 g/L PEG 6000 (average Mn 5000 – 7000); **B:** exposure to 75 g/L PEG 6000 (average Mn 5000 – 7000); **C:** exposure to 5 g/L PEG 12000 (average Mn 11000 – 15000); **D, E:** exposure to 25 g/L PEG 12000 ((average Mn 11000 – 15000); **F:** exposure to 5 g/L PEG 8000 (average Mn 8000).

3.2 Investigations on recovery

Recovery studies were conducted with PEG 3000, PEG 4000, PEG 6000, PEG 8000, and PEG 12000. After 2 h of exposure to the different polyethylene glycols, all embryos exposed to ≥ 5 g/L showed chorion deformations (Fig. 24A). Embryos were then washed and transferred to dilution water. After another 2 h almost all embryos' chorions recovered completely (Fig. 24B).

Some of the embryos, on the other hand, seemed to be damaged irreversibly and did not recover, as could be seen at additional controls after 24 and 48 hpf. Mortality rates fluctuated between the replicates and neither a dose-dependency nor a dependency on molecular size of the different polyethylene glycols could be observed (Fig. 25).

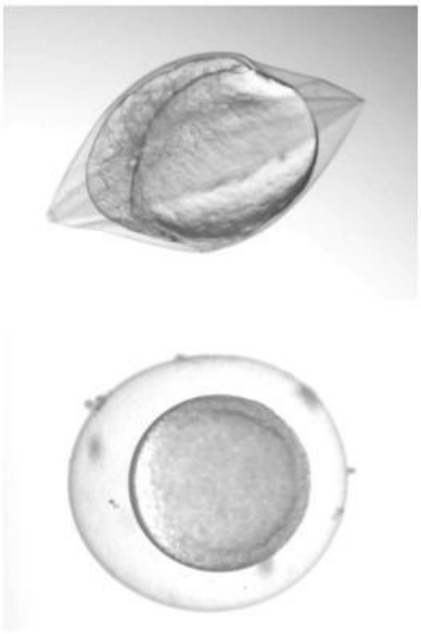

Fig. 24: Embryo after 2 hours of exposure to PEG 4000 (**A**) and after transfer to dilution water and 2 h recovery (**B**)

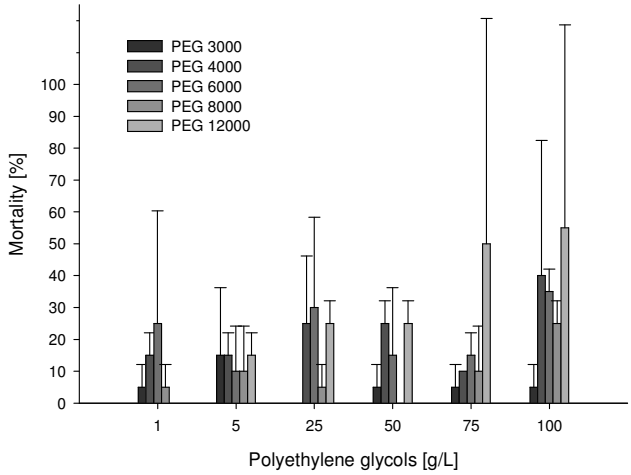

Fig. 25: Comparison of mortalities of non-dechorionated embryos after 48 h, exposed to polyethylene glycols of different molecular weights for 2 h directly after fertilization and transferred to dilution water afterwards.

3.3 Fish embryo toxicity test with dechorionated embryos

Dechorionation studies were conducted with PEG 3000, PEG 4000, PEG 6000, PEG 8000, and PEG 12000. Exposure to PEG 4000 and PEG 12000 did not cause mortality exceeding negative control mortality rates during the whole test period. For PEG 3000 and PEG 6000 no mortality exceeding negative control mortality rates could be observed for 48 hours of exposure, however, after 120 h of exposure, a slight increase of mortality could be seen. For PEG 3000 only in the highest concentration tested (100 g/L) a mortality of ≥50% (55%) was reached after 120 h of exposure; for PEG 6000, a slight increase of mortality could be seen in both highest concentrations (75 and 100 g/L), however, at 120 h of exposure (embryonic age: 144 h), mortality did not exceed 20% for exposure to ≤ 75 g/L, whereas it was 75%, respectively in the highest concentration (100 g/L). Basing on these data no LC_{50} could be calculated for both PEGs using probit Analysis (Finney, 1952). Exposure of dechorionated embryos to PEG 8000 for 48 h resulted in no mortality except for the highest concentration were a mortality of 55% was reached. After 120 h of exposure (embryonic age: 144 h) mortality had increased in all concentrations tested and an LC_{50} of 77.8 g/L could be determined.

4 Discussion

A semi-permeable membrane, as the chorion is generally considered to be (Blaxter, 1988; Von Westernhagen, 1988), is a membrane that will allow certain molecules or ions to pass through it by diffusion or specialized facilitated diffusion. The rate of passage depends on the pressure, concentration, and temperature of the molecules or solutes on either side, as well as the permeability of the membrane to each solute. Depending on the membrane and the solute, permeability may depend on solubility, chemical properties, or simply of solute size. The latter aspect is the one, this chapter is focusing on. As measurement parameter for the substance size, the molecular weight was consulted. By choosing polyethylene glycols of different molecular weights as test substances, differences in physiological and chemical properties not depending on the molecular size should be fairly excluded.

Toxicity of PEGs is believed to be low at high concentrations (Sheftel, 2000). This seemed to be confirmed by standard fish embryo testing, resulting in LC_{50} values of between 36.3 to > 100 g/L and prolongation of exposure did not increase mortality for any of the tested polyethylene glycols.

However, eggs were placed in the relatively high concentrated solutions of polyethylene glycols of different molecular weight, for exposure to polyethylene glycols with ≥ 3000 g/mol, a depression of the chorion was observed, increasing until the chorion adjoined the body of the embryo. The light to heavy deformations of the chorion became more prominent with increasing concentrations. Preliminary tests discovered a beginning of this phenomenon shortly after start of exposure (Fig. 21).

The only prominent sublethal aberrations of embryonic development observed, were general malformations of various appearances of the embryo, without any regularity, varying from simple malformations of the spine or the tail to severe deformations of the whole larvae or to larvae without eyes or even a head (Fig. 16). No specific occurrence and no observable dependency on concentration or exposure duration were detected. However, one dependency was observed: with increasing number of embryos with deformed chorion, also the number of coagulated and malformed embryos increased and, beyond that, the sum of embryos malformed and coagulated in each concentration at 48 hours of exposure equals the number of embryos with deformed chorion at 24 hours of exposure (Fig. 22). These observations lead to the conclusion that the observed effects are a consequence of mechanical damage originating from the shrinking of the chorion rather than from toxicological properties of the polyethylene glycols.

Additional evidences for mechanical damage originating from the shrinking of the chorion as responsible factor for most of the observed effects were found in recovery tests and dechorionation tests. Although in the recovery tests embryos were exposed for only two hours to PEGs, even so coagulation and malformations were observed without any dose-dependency nor a dependency on molecular size of the PEGs (Fig. 25). In comparison with the results of the standard fish embryo test, dechorionated embryos seem to be less sensitive against the polyethylene glycols.

A direct impact of polyethylene glycols on the chorion itself causing the shrinkage seems to be unlikely. The shrinkage rather results from an efflux of water, caused by osmotic pressure: The internal osmolality of the zebrafish is unknown, however, vapor pressure osmometer measurements suggest an osmolality of approximately 0.240 Osm (Hagedorn *et al*., 1998). Osmolality of the test solutions was not measured; however, since PEGs are not dissociated in water and dissolve completely, test solution osmolarity must be similar to the molarity of the test solutions which have been calculated. Thus, osmolarity of test solutions with between 5 and 100 g/L exhibit the zebrafish egg osmolarity. When the eggs with depressed chorions were placed in dilution water again after an exposure of 2 hours, the spherical shape was restored – accordingly - by endosmosis of water. Since these shrinking and swelling procedures could be observed for polyethylene glycols of all tested molecular weights, except for PEG 2000, it seems likely, that the chorion presents a barrier for all PEGs tested with more than 2000 Da. Given that the internal egg osmolality is about 0.240 Osm (Hagedorn *et al*., 1998), by transferring the egg into a highly concentrated PEG solution, a concentration gradient and thus the aspiration of adjustment of diffusion equilibrium is introduced. Assuming that the chorion is not permeable for polyethylene glycols ≥ 3000 Da, osmotic pressure is generated and, since polyethylene glycols cannot pass the chorion and get into the egg, the water molecules pass through the chorion from an area of low solute concentration (inside the egg) to one of high solute concentration (outside the egg). Thereby, egg internal "pressure" is reduced and the chorion shrinks.

With regard to the uptake of PEGs of different molecular size, Lillicrap (2010) made contradictory observations, suggesting that the uptake of PEG4000 is reduced whereas PEG40000 (average Mn: 40000 Da) is able to cross the chorion and enter the embryos. In the authors opinion a possible reason for this increased uptake is possibly due to the PEG40000 becoming smaller if the molecules folded in onto each other, resulting in a reduced actual size of the molecule.

5 Conclusion

In this study, it could be shown that the size of a molecule can be a limiting factor regarding the uptake of a molecule through the chorion. A “critical” molecular size might lie between 2000 and 3000 Da for polyethylene glycols. Nevertheless, it should be regarded that beside the molecular weight in Dalton or g/mol, a number of other factors contribute to the actual size of a molecule: two molecules of the same molecular weight but of different composition can exhibit absolutely different topologies and, thus, differ strongly in their actual size. Moreover, when a substance is dissolved in water, the water molecules in the direct environment of the solute react by restructuring of their hydrogen bond network. Thus, in the environment of the solved molecules, the arrangement of the water molecules differs, and specific hydrate coverings form around the molecules of the solved material. These effects play a crucial role within the procedure of dissolving and affect as well the actual molecule size.

Chapter 5: Differential toxicity of inorganic potassium and sodium salts in the fish embryo test (FET) with the zebrafish (*Danio rerio)*

Kirsten Henn, Thomas Braunbeck

1 Introduction

As a consequence of current development in European chemical legislation, which meets ethical concerns regarding an expected increase in the number of animal experiments (Bhogal *et al*., 2005; Breithaupt, 2006, Rovida and Hartung, 2009), alternative methods in ecotoxicology gain more importance in chemical risk assessment (e.g. Regulation (EC) Nr. 1907/2006 (REACH, 2006)). In the case of risk assessment concerning aquatic environments, acute toxicity testing with fish (as vertebrates) has become a prime target for such alternative methods which can be seen in embryo toxicity tests (Nagel, 2002; Braunbeck *et al*., 2005; Lammer *et al*., 2009; Leonard *et al*., 2005). In 2005, the fish embryo toxicity test (FET) with the zebrafish embryo (*Danio rerio*) has become a mandatory component in routine whole effluent testing in Germany (DIN, 2001) and has been standardized on international level since 2007 (ISO 15088: ISO, 2007). A modified version of the test protocol, which can easily be adapted to early embryonic stages of other OECD species (Braunbeck *et al*., 2005), has been submitted by the German Federal Environment Agency as a draft guideline for an alternative to chemical testing with intact fish (Braunbeck *et al*., 2006). In the current version of the OECD test protocol, the FET is limited to two or four days and, thus, classified – based on the current UK Animal Procedures Act (UK, 1986) and the new EU Directive 2010/63/EU on protection of animals used for scientific purposes (EU, 2010) – as a test with non-protected life-stages; protection of immature forms of fish starts when they become capable of independent feeding (Strähle *et al*., 2011).

Probably the most important requirement for a test to be accepted as an alternative to a conventional test - a good correlation between both procedures - is fulfilled by the FET (Lammer *et al*., 2009). Nevertheless, there are a number of substances, for which the embryo test is significantly more or less sensitive (Braunbeck *et al*., 2005). Several reasons have been suspected to be responsible for such outliers: (1) differential uptake, accumulation and metabolization of the test compounds into embryonic and adult stages; (2) restricted availability of the test sub-stances due to test procedure conditions and (3) protection of the embryo by the chorion (Braunbeck *et al*., 2005; Leonard *et al*., 2005). A statistical analysis identified potassium chloride (KCl) as such an outlier (Ratte and Hammers-Wirtz, 2003). The

present study was designed to investigate if this discrepancy is restricted to KCl or if it also applies to other mineral salts, namely K_2SO4, $KHCO_3$ and the corresponding sodium salts NaCl, Na_2SO4, and $NaHCO_3$. For this end, standard fish embryo toxicity tests (FET) with the zebrafish embryo were carried out according to the standard FET test protocol (Braunbeck *et al*., 2006), and results were com-pared to acute fish toxicity data retrieved from the US EPA ECOTOX database (US EPA, 2002) or the ECETOC Aquatic Toxicity (Solbe *et al*., 1998). In addition, prolonged fish embryo toxicity tests as well as tests with eleutheroembryos were conducted in order to elucidate whether there is only delayed toxicity for KCl. Finally, in case prolonged and eleutheroembryo tests did result in an approximation of embryo and adult toxicity data, additional tests with dechorionated embryos were carried out to investigate the contribution of the chorion.

2 Material and methods

2.1 Chemicals

The polystyrene 24-well microtiter plates were provided by Renner (TTP; Dannstadt, Germany), the self-adhesive foil (clear polyester sealing tapes) were purchased from Nunc GmbH & Co KG (Langenselbold, Germany). Agarose was provided by SeaKem (HGT Agarose, Cambex BioScience Rockland, ME, USA; gelling temperature: 40.5°C - 43.5°C). The positive control, 3,4-dichloroaniline (p.a.), the test substances potassium chloride (KCl; p.a.; CAS 7447-40-7; MW 74.55 g/mol), potassium sulfate (K_2SO_4; p.a.; CAS 7778-80-5; MW 174.26 g/mol), potassium bicarbonate ($KHCO_3$; p.a.; CAS 298-14-6; MW 100.12 g/mol), sodium chloride (NaCl; p.a.; CAS 7647-14-5; MW 58.442 g/mol), sodium sulfate (Na_2SO_4; p.a.; CAS 7757-82-6; MW 142.04 g/mol) and sodium bicarbonate ($NaHCO_3$; p.a.; CAS 144-55-8; MW 84.01 g/mol) as well as all other chemicals used (all highest grade available) were purchased from Sigma-Aldrich (Deisenhofen, Germany).

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 was adjusted to 7.8 ± 0.2.

2.2 Fish maintenance and egg production

Fish maintenance and as egg production was performed as described in Lammer *et al*. (2009).

2.3 Fish embryo testing

2.3.1 Standard and prolonged fish embryo toxicity test

The standard fish embryo toxicity test (FET) was conducted according to DIN 38415-T6 and ISO 15088 (DIN, 2001; ISO, 2007) as described in Lammer *et al*. (2009). Test concentrations for the tested salts in both test systems are summarized in Tab. 4. The embryos were examined after 24 and 48 hours post-fertilization (hpf) in the standard FET, and additionally at 72 hpf. The prolonged FET was performed identically, however, with extra effect recordings at 96, 120 and 144 hpf. Evaluation of embryonic development was carried out as described in Lammer et al (2009) for the standard FET, and endpoints for embryos > 72 hpf were choosen according to Nagel (2002). The LC_{50} was calculated using probit analysis (Finney, 1952). All embryo tests were classified as valid, if mortalities in negative controls were < 10% and positive controls (3,4-DCA) gave mortalities between 20 and 80% (Lammer *et al*., 2009).

Tab. 4: Test concentrations (g/L) of the tested salts in the Standard FET and the Prolonged FET

	Test concentrations [g/L]	
Test substance	**Standard FET**	**Prolonged FET**
KCl	5, 7.5, 10, 12.5, 15, 17.5, 20	0.5, 0.65, 0.8, 0.95, 1.1, 1.25, 1.4, 1.55
K_2SO_4	17, 19, 21, 23, 25, 27, 29, 31	0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.25
$KHCO_3$	1, 3, 6, 9, 12, 15, 18, 21	0.6, 0.75, 0.9, 1.05, 1.2, 1.35, 1.5
NaCl	3.5, 5.3, 8.0, 12.0, 18.0, 27.0	3.5, 5.3, 8.0, 12.0, 18.0, 27.0
Na_2SO_4	10, 12.5, 15, 17.5, 20, 22.5, 25	5, 8, 11, 14, 17, 20, 23
$NaHCO_3$	6, 8, 10, 12, 14, 16	0.5, 0.8, 1.1, 1.4, 1.7, 2.0, 2.3

2.3.2 Eleutheroembryo toxicity test

For eleutheroembryo tests, eggs were collected on the same day as for the prolonged FET and incubated at 26.0 ± 1.0°C in artificial water until 72 hpf (i.e., without salt exposure). At this time, most of the embryos had hatched. Test concentrations for the tested salts are given in Tab. 5. For each test concentration, 10 hatched embryos were selected and transferred to pre-saturated 24-well plates covered with self-adhesive foil and incubated at 26.0 ± 1.0°C. The embryos were checked after 24, 48, and 72 h of exposure equivalent to an age of 96, 120 and

144 hpf, respectively. Assessment of effects was done according to the endpoints recommended by Nagel (2002). The LC_{50} was calculated by probit analysis (Finney, 1952).

Tab. 5: Test concentrations (g/L) of the tested salts in the Eleutheroembryo test

	Test concentrations [g/L]
Test substance	**Eleutheroembryo Test**
KCl	0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.25
K_2SO_4	0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.25
$KHCO_3$	0.6, 0.75, 0.9, 1.05, 1.2, 1.35, 1.5
NaCl	3.5, 5.3, 8.0, 12.0, 18.0, 27.0
Na_2SO_4	5, 8, 11, 14, 17, 20, 23
$NaHCO_3$	0.5, 0.8, 1.1, 1.4, 1.7, 2.0, 2.3

2.3.3 Fish embryo toxicity test with dechorionated embryos

For dechorionation tests, embryos were dechorionated mechanically as described in Henn *et al*. (2011) at an age of either 8 or 24 hpf. Eggs were collected at about 30 minutes after fertilization the day before testing and incubated at 26.0 ± 1.0°C in artificial water. Test concentrations for the tested potassium salts were the same as in the eleutheroembryo tests (Tab. 5). For each test concentration and the negative control (artificial water), 10 embryos were dechorionated and transferred to pre-saturated, agarose-coated 24-well plates covered with self-adhesive foil and incubated at 26.0 ± 1.0°C. The embryos were examined after 24, 48, 72, 96, and 120 hours of exposure. Endpoint recording and determination of LC_{50}s were carried out as described above.

3. Results

3.1 Results of fish embryo testing with potassium salts

Results for all experiments with potassium salts are shown in Fig. 26 - Fig. 28 and summarized in Tab. 6. In the standard FET (48 hpf) with all three potassium salts, sublethal effects occurred only sporadically and the most prominent lethal effect was coagulation. LC_{50} values for 48 hpf were calculated as follows: KCl 14.95 g/L, K_2SO_4 23.7 g/L, and $KHCO_3$ 11.6 g/L. The prolonged FET in refined and lower concentration ranges showed that high

mortality rates at lower concentrations never occurred at stages younger than 96 or 120 hpf. LC_{50} values for 96 hpf could not be determined for $KHCO_3$ and KCL, since in the highest concentration tested (1.55 g/L and 2.55 g/L, respectively) mortality only reached 40%. For K_2SO_4, it was 1.84 g/L. LC_{50} values for 120 h of exposure were calculated as follows: KCl 0.85 g/L, K_2SO_4 0.89 g/L, and $KHCO_3$ 1.38 g/L. Lethal effects were both coagulation and lack of heartbeat (the latter more often in the lower concentrations).

Additional tests with eleutheroembryos exposed from an age of 72 hpf (i.e. after hatch) documented that the increased toxicity of potassium salts after 96 hpf and 120 hpf was not a consequence of prolonged exposure. After 24 h of exposure (i.e., at a total age of 96 hpf), no remarkable mortality occurred. At 48 h of exposure (i.e., at an age of 120 hpf), on the other hand, the LC_{50} values could be determined to be only slightly lower than the LC_{50} derived from prolonged exposure up to the same embryonic age.

LC_{50} values for dechorionated embryos exposed for 48 h were calculated as 1.11 g/L for KCl, 1.0 g/L for K_2SO_4, and 1.34 g/L for $KHCO_3$, respectively. Again, the major lethal effect was coagulation. Exposure of embryos dechorionated at an age of 24 hpf did not result in mortalities before an embryonic age of 96 hpf. For embryos at 96 hpf (i.e., 72 h exposure), the LC_{50} values could be determined as follows: KCl 2.68 g/L and K_2SO_4 1.76 g/L. For embryos at 120 hpf (i.e., 96 h exposure) mortality increased in all concentrations and the LC_{50} value – almost identical to that of the eleutheroembryo tests – were calculated as 0.94 g/L for KCl and 0.91 g/L for K_2SO_4. For $KHCO_3$, an LC_{50} was not determinable until an age of 144 h (i.e., 120 h of exposure) and was calculated as 1.30 g/L. Dechorionation tests with embryos at an age of 8 hpf to exclude early effects gave similar results as experiments with embryos dechorionated at 24 hpf (data not shown).

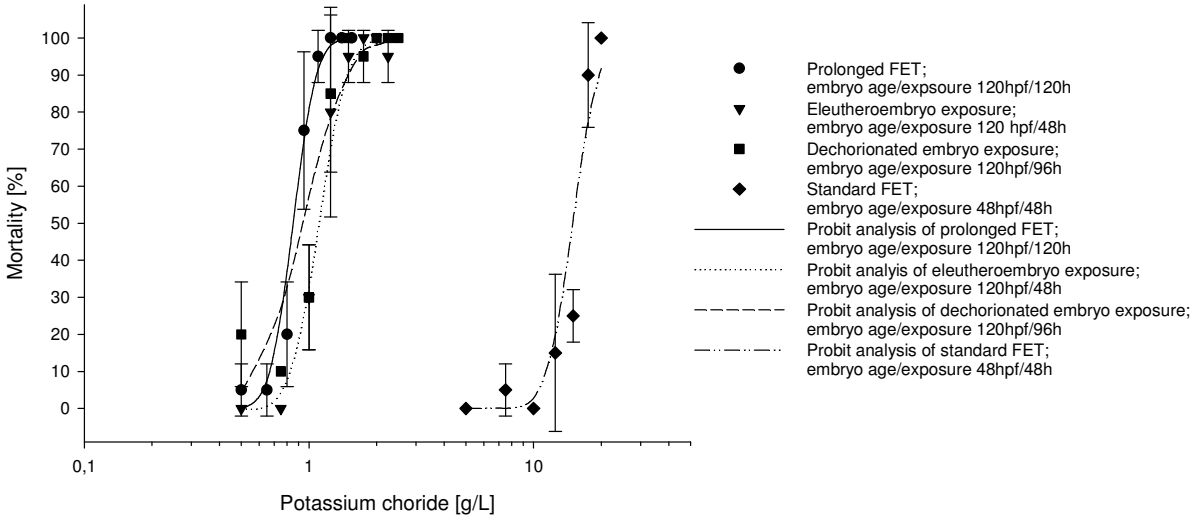

Fig. 26: Comparison of mortalities after 120 h of exposure to Potassium chloride in non-dechorionated embryos (●), after 48 h of exposure to Potassium chloride in eleutheroembryos exposed from the age of 72 h (age: 120 h; ▼), after 96 h of exposure to Potassium chloride in embryos dechorionated at 24 h post fertilization (age: 120 h; ■), and after 48 h of exposure to Potassium chloride in non-dechorionated embryos (standard FET, ♦).

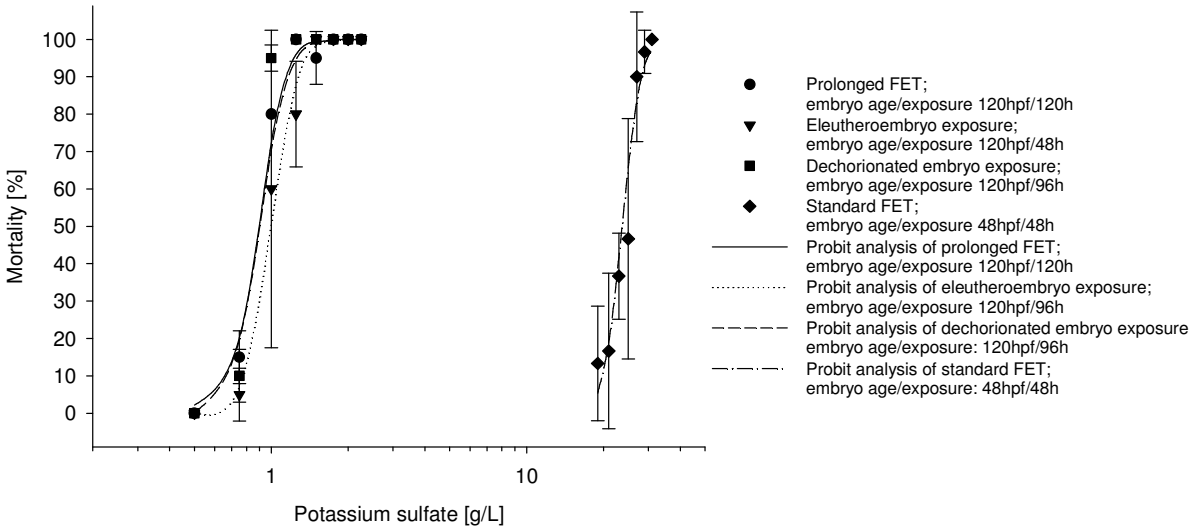

Fig. 27: Comparison of mortalities after 120 h of exposure to Potassium sulfate in non-dechorionated embryos (●), after 48 h of exposure to Potassium sulfate in eleutheroembryos exposed from the age of 72 h (age: 120 h; ▼), after 96 h of exposure to Potassium sulfate in embryos dechorionated at 24 h post fertilization (age: 120 h; ■), and after 48 h of exposure to Potassium sulfate in non-dechorionated embryos (standard FET, ♦).

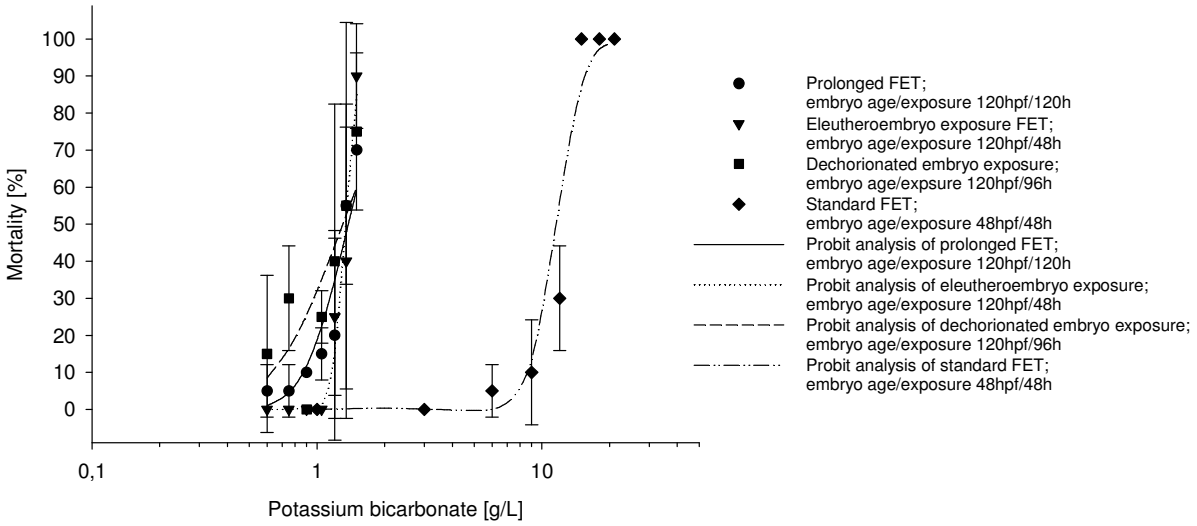

Fig. 28: Comparison of mortalities after 120 h of exposure to Potassium bicarbonate in non-dechorionated embryos (●), after 48 h of exposure to Potassium bicarbonate in eleutheroembryos exposed from the age of 72 h (age: 120 h; ▼), after 120 h of exposure to Potassium bicarbonate in embryos dechorionated at 24 h post fertilization (age: 144 h; ■), and after 48 h of exposure to Potassium bicarbonate in non-dechorionated embryos (standard FET, ♦).

3.2 Results of fish embryo testing with sodium salts

The results for all sodium experiments are shown in Fig. 29 - Fig. 31 and summarized in Tab. 6. In the standard FET with the sodium salts, sublethal effects occurred only sporadically or, in the case of $NaHCO_3$, as edema formation without dose-dependency. The major lethal effect was coagulation. The LC_{50} values for 48 h of exposure were calculated as 7.09 g/L for NaCl, 19.35 g/L for Na_2SO_4 and 8.83 g/L for $NaHCO_3$. In contrast to the tests with K_2SO_4, for prolonged exposure to Na_2SO_4 only a slight increase of mortality was observed. Sublethal effects like edema formation occurred more frequently, but were not dose-dependent. LC_{50} for Na_2SO_4 at 120 h was 12.35 g/L. Furthermore, at 120 h of exposure, from a concentration of 8 g/L, all non-coagulated embryos showed effects like edemata and impaired heart beat and blood circulation. The same was found for NaCl exposure: LC_{50} for 120 h was 6.06 g/L. For $NaHCO_3$, however, a continuously increase of mortality under prolonged exposure was observed, resulting in an LC_{50} for 120 h of exposure of 2.41 g/L. Nevertheless, additional tests with eleutheroembryos, exposed from an age of 72 h (i.e. after hatch) showed similar results as the prolonged exposure: At 120 hpf (i.e., 48 h of exposure), LC_{50} values were calculated as 12.14 g/L for NaCl, 15.60 g/L for Na_2SO_4 and 2.55 g/L for $NaHCO_3$.

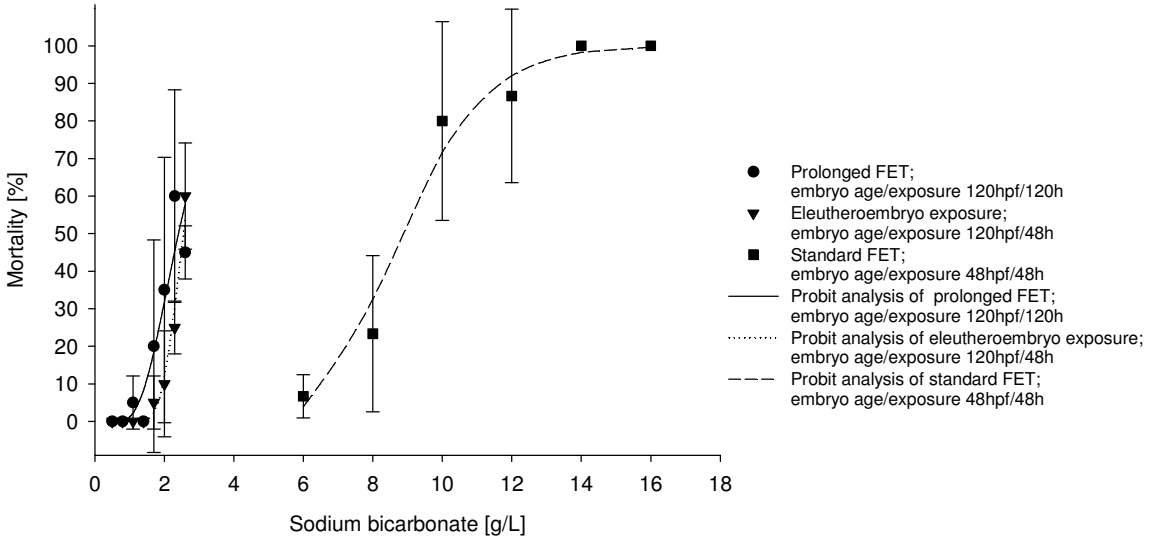

Fig. 29: Comparison of mortalities after 120 h of exposure to Sodium bicarbonate in non-dechorionated embryos (●), after 48 h of exposure to Sodium bicarbonate in eleutheroembryos exposed from the age of 72 h (age: 120 h; ▼), and after 48 h of exposure to Sodium bicarbonate in non-dechorionated embryos (standard FET, ■).

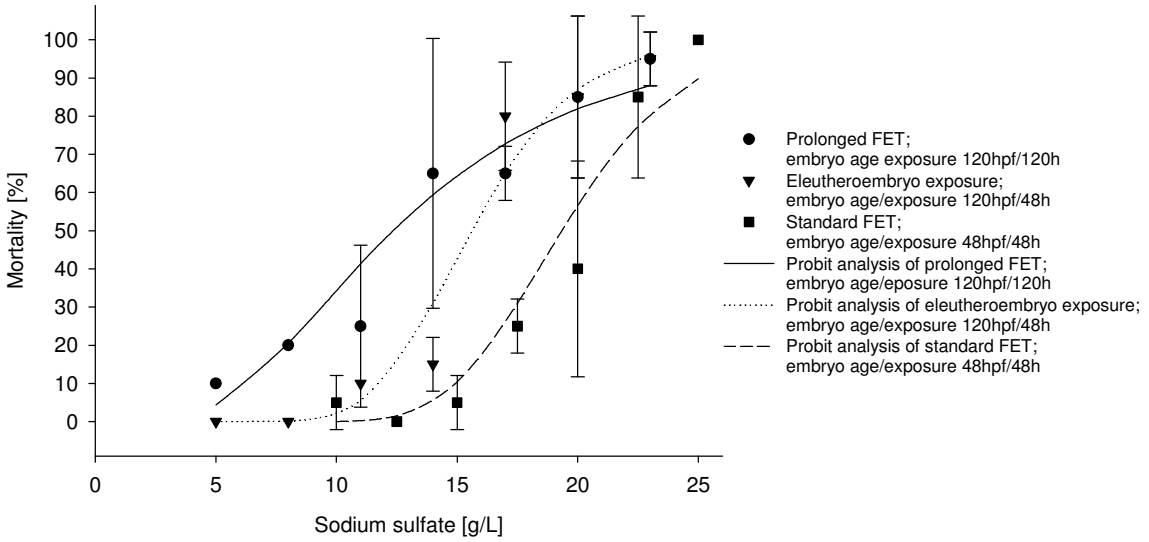

Fig. 30: Comparison of mortalities after 120 h of exposure to Sodium sulfate in non-dechorionated embryos (●), after 48 h of exposure to Sodium sulfate in eleutheroembryos exposed from the age of 72 h (age: 120 h; ▼), and after 48 h of exposure to Sodium sulfate in non-dechorionated embryos (standard FET, ■).

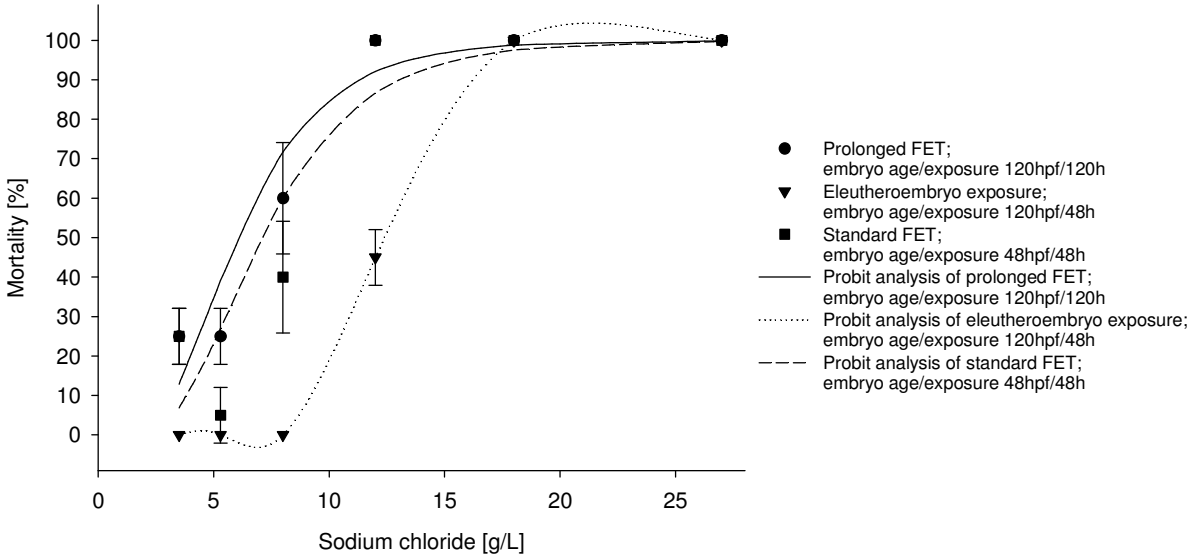

Fig. 31: Comparison of mortalities after 120 h of exposure to Sodium chloride sulfate in non-dechorionated embryos (●), after 48 h of exposure to Sodium chloride in eleutheroembryos exposed from the age of 72 h (age: 120 h; ▼), and after 48 h of exposure to Sodium chloride in non-dechorionated embryos (standard FET, ■).
Fig. 8:

4. Discussion

An evaluation of the toxicity of more than 2,900 ion solutions for daphnids as well as for juveniles of the fathead minnow (*Pimephales promelas*) showed that, generally, Na^+ is not a major contributor to aquatic toxicity (Mount *et al.* 1997): In the case of NaCl, the associated anion, Cl^-, is more toxic than Na^+, although Na^+ has a role in ion deficiency-related toxicity and sulfate (SO_4^{2-}) is less toxic than chloride (Cl^-). The relative ion toxicity was therefore found to be $K^+ > HCO_3^{2-} > Cl^- > SO_4^{2-} >> Na^+$ (Mount *et al.*, 1997). The LC_{50} values were quite similar to those found in our study for the eleutheroembryos, for the prolonged fish embryo test, for adult toxicity values derived from the US EPA ECOTOX (US EPA, 2002) database and – in case of the potassium salts – for the prolonged test with dechorionated embryos (Tab. 6). On the basis of these results, we assume that in the case of the potassium salts toxicity is driven by the cation (K^+), whereas in the case of the sodium salts toxicity is driven by the corresponding anions (HCO_3^{2-}, Cl_2^-,SO_4^{2}): For eleutheroembryos, the toxicity of potassium salts was in the same concentration range (1.00 - 1.34 g/L), whereas the range for sodium salts was broader (2.54 - 15.60 g/L).

Tab. 6: Acute toxicity (g/L) of selected potassium and sodium salts to zebrafish (Danio rerio) embryos, to adults of various fish species as well as larvae of fathead minnow

	Zebrafish (*Danio rerio*) embryo toxicity				OECD EPA [1)]	*P. promelas* (Mount *et al.* 1997)	
	Standard FET	Prolonged FET	Eleuthero-embryos	Dechorionated			
Age	48hpf	120hpf	120hpf	120hpf	Adult	Larvae	
Exposure	48h	120h	48h	96h	96h	48h	96h
Substance							
$KHCO_3$	11.6	1.38	1.34	1.30[2)]	-	0.82	< 0.51
K_2SO_4	23.7	0.89	1.0	0.90	2.12	0.86	0.68
KCl	14.95	0.85	1.119	0.94	1.38	0.91	0.88
$NaHCO_3$	8.83	2.41	2.54	-	8.61	2.5	0.85
NaCl	7.09	6.06	12.14	-	7.95	6.51	6.39
Na_2SO_4	19.35	12.35	15.6	-	10.08	> 7.96	7.96

1) Mean value of all available 96 h LC_{50} data for the 5 OECD key species *Danio rerio*, *Lepomis macrochirus*, *Pimephales promelas*, *Oncorhynchus mykiss* and *Oryzias latipes* derived from ECOTOX database (EPA, 2002)
2) Embryo total age: 144 hpf, exposure: 120 h
n.c. Test were not conducted.
n.a. No data available from database.

Apart from this, the comparison between standard FET results in our study for three potassium salts (KCl, K_2SO_4, and $KHCO_3$) and corresponding adult fish toxicity results derived from the EPA ECOTOX Database (US EPA, 2002; Tab. 6) revealed differences of a factor of up to 10 between adult fish and embryo toxicity for KCL (adult fish toxicity: 1.51 g/L; embryo toxicity 14.95 g/L) and K_2SO_4 (adult fish toxicity 3.55 g/L; embryo toxicity: 23.70 g/L). For $KHCO_3$, no adult fish toxicity values could be found in the databases. However, a comparison of results between our standard and prolonged embryo exposure tests (up to 120 hpf) and toxicity data of *Pimephales promelas* larvae (Mount *et al.*, 1997; Tab. 6) suggested that also for $KHCO_3$ adult fish toxicity would be much higher than embryo toxicity. Most importantly, for all the potassium salts tested, prolongation of the standard FET to up to 120 hours of exposure resulted in almost identical LC_{50} values compared to adult fish toxicity (KCl: 0.88 g/L; K_2SO_4: 0.75 g/L; $KHCO_3$: 1.22 g/L). Since the LC_{50} values (KCl: 1.00 g/L; K_2SO_4: 1.12 g/L; $KHCO_3$: 1.34 g/L) derived from tests with eleutheroembryos (embryonic age: 120 hpf; exposure duration: 48 h) were both comparable to results of adult fish toxicity and prolonged embryo toxicity, prolongation of exposure *per se* is not responsible for the enhanced toxicity, rather the sensitivity of zebrafish embryos to potassium salts significantly increases with age. Likewise, dechorionation of embryos at 24 hpf and subsequent exposure for 48 h did not increase mortality, if compared to non-dechorionated embryos. In contrast, prolongation of exposure up to 96 h (total age: 120 hpf) resulted in LC_{50} values similar to

those found in adult and eleutheroembryo tests (KCl: 1.85 g/L; K_2SO_4: 0.89 g/L; $KHCO_3$: 1.38 g/L; Tab. 6). Thus, the differences between early embryo toxicity and adult fish toxicity are not a consequence of a barrier function of the chorion.

In contrast to the potassium salts, testing of the analogous sodium salts (NaCl, Na_2SO_4, and $NaHCO_3$) gave a different picture: for $NaHCO_3$ – according to Mount *et al.* (1997), HCO_3^- is the least toxic ion next to Cl^- – there was also a difference between standard and prolonged as well as eleutheroembryo toxicity; however, by far not as pronounced as with the potassium salts (48 h LC_{50}: 8.83 g/L; 120 h LC_{50}: 2.14 g/L; eleutheroembryo LC_{50}: 2.54 g/L). Furthermore, the 48 h LC_{50} was comparable to adult toxicity (8.61 g/L). Likewise, for sodium sulfate (Na_2SO_4), the deviation between embryo toxicity (48 h LC_{50}: 19.35 g/L) and adult toxicity (96 h LC_{50}: 10.38 g/L) was comparatively small. Similar LC_{50} values were found for prolonged embryo toxicity (12.35 g/L) and eleutheroembryo toxicity (15.6 g/L), showing only a weak increase of mortality for larvae.

For sodium chloride, the differences between 48 h (LC_{50}: 7.09 g/L) and 120 h (LC_{50}: 6.06 g/L) toxicity were even smaller, but correlated well with adult fish toxicity (96 h LC_{50}: 7.95 g/L). In contrast, eleutheroembryo toxicity deviated by a factor of about 2 (LC_{50}: 12.14 g/L). Therefore, in contrast to the potassium salts, enhanced toxicity for the embryos exposed for 120 h to sodium salts seemed to be rather a consequence of prolonged exposure than a consequence of toxicity increasing with age.

Results thus suggest that embryonic zebrafish handle potassium exposure differently to adult fish. In adult fish, the gills play – in addition to their function in gas exchange and elimination of nitrogenous waste – a central role in ion exchange and osmoregulation as well as acid-balance (McDonald *et al.*, 1991). For transepithelial ion exchange the chloride cells (mitochondria-rich cells) were identified to be the responsible site (Flik *et al.*, 1995; Foskett and Scheffey, 1982; Kaneko *et al.*, 2002; Marshall, 1995; Towle, 1990): Their basolateral membranes contain membrane channels through which ions are pumped selectively and against concentration gradients by the activity of a Na^+, K^+-ATPase, whereas their apical membrane channels are involved in acid-base regulation. The electrolytes Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, and HCO_3^- play a major role in the osmotic and ionic regulation of extra- and intracellular fluids in fish. The principal role of these ions is connected with the regulation of cellular osmotic pressure and the maintenance of acid-base balance. Na^+ and Cl^- are the principal cation and anion, respectively, in the extracellular fluids of the body, whereas K^+ is the major cation of the intracellular space. The most important functions of K^+ are the

regulation of intracellular fluids, solubilization of proteins, operating of nerve impulses and contracting of the muscles.

In most cases, fish gills as a respiratory and osmoregulatory organ start functioning after hatching, however, the precise timing may differ in different species (Varsamos *et al.*, 2005). Nevertheless, pre-hatch teleost embryos are able to maintain the osmotic balance of the body fluids (Alderdice, 1988; Guggino, 1980; Kaneko *et al.*, 1995; Varsamos *et al.*, 2005): During early embryonic development, chloride cells have been shown to be present in the yolk-sac membrane and other body surfaces for embryos of a numerous fish species (Dépêche, 1973; Hwang, 1989; Hwang *et al.* 1985; Kaneko *et al.*, 1995, 2002, 2008; Lasker and Threadgold, 1968; Lin *et al.*, 2006;; Shen and Leatherland, 1978). Density and abundance of chloride cells vary between different species, most likely in relation to the differential osmoregulatory requirements depending on their natural habitats. Furthermore, in contrast to chloride cells of adult fish, functional differentiation of the chloride cells in the yolk-sac membrane is autonomous and independent of the endocrine and nerve systems (Shiraishi *et al.*, 2001). In several teleost species, a shift of chloride cell distribution from the skin to the gills was demonstrated to coincide with the yolk absorption and beginning of exogenous feeding (Gonzalez *et al.*, 1996, Rojo *et al.*, 1997, Rombough, 1999). Rombough (2002), who forced zebrafish larvae (3 to 21 days post fertilization) to rely on cutaneous processes, was able to show that 50 % physiological saline had no significant effect on larvae 3 days post fertilization (~72 hpf) suggesting that at this stage cutaneous exchange was sufficient to satisfy ion regulatory requirements. In contrast, at 7 days post fertilization, physiological saline significantly improved survival. Furthermore, dimethylamino-styryl-ethylpyridiniumiodine (DASPEI) staining for mitochondria-rich cells indicates that large numbers of ionocytes begin to appear on the gill of zebrafish larvae at approx. 5 to 7 days post-fertilization (Rombough, 2002). Examination of the Na^+ metabolism in zebrafish larvae showed indirectly that mitochondria-rich cells are distributed on the yolk surface of 55 hpf larvae and on the gills of larvae at 7 days post-fertilization (Esaki *et al.*, 2007). These results suggest that in case of zebrafish, ion regulation functions shift from the skin to the gills at about 5 days post fertilization. Interestingly, this point of time coincides with our findings of enhanced potassium toxicity at 120 hpf, leading to the assumption that in the case of potassium a change in body function processes takes a direct influence on toxicity.

5 Conclusion

Results document that for all potassium salts tested the standard 48 h FET results in an underestimation of toxicity, if the adult fish toxicity is accepted as a “golden standard”. However, prolongation of the exposure period eliminates the difference between FET and the conventional acute fish toxicity test. For potassium salts, prolongation of exposure *per se* does not seem responsible for enhanced toxicity; rather, the sensitivity of zebrafish embryos to potassium salts significantly increases with age. Basically, results from toxicity tests with sodium salts confirm the conclusion that for chemical testing, the FET should be extended to 96 h, i.e. beyond hatch. In contrast to the potassium salts, however, enhanced toxicity for the embryos exposed for 120 h to sodium salts seemed to be rather a consequence of prolonged exposure. In all cases, tests with eleutheroembryos yield toxicity data comparable to those obtained with adult fish.

Chapter 6 – Overall discussion and conclusion

In 2007, with the new European chemical policy REACH (registration, evaluation and authorization of chemicals), for the first time the aspect of protection of laboratory animals was incorporated into EU chemicals legislation (REACH, 2006): animal testing should - whenever possible - be reduced or replaced by alternative methods (according to the concept of 3Rs by Russell and Burch, 1959). With regard to the aquatic environments, acute toxicity tests with fish (as vertebrates) are of particular interest to be replaced by alternative methods. Such a candidate is the fish embryo toxicity test (Nagel, 2002; Braunbeck *et al*., 2005), submitted by the German Federal Environment Agency as a draft guideline (Braunbeck *et al*., 2006). A good correlation between acute fish toxicity tests and embryo toxicity tests could be demonstrated (Ratte and Hammers-Wirtz, 2003; Braunbeck *et a*l., 2005; Lammer *et al*., 2009) and a potential restricted availability of the test substances due to the static exposure scenario of the standard fish embryo test compared to the flow-through system used in acute fish toxicity testing was investigated by Lammer (2009). Nevertheless, some questions regarding sensitivity differences between fish embryos and adult fish remained: the influence of (1) differences in uptake, accumulation and metabolization of the test compounds between embryonic and adult stages (Barry et al., 1995; Ensenbach, 1987; Van Leeuwen, 1985), and (2) protection of the embryo by the chorion (Barry et al., 1995; Braunbeck et al., 2005; Cheng et al., 2007, Creton et al., 2004, Gellert and Heinrichsdorff, 2001).

A main focus of this thesis was therefore to determine the influence of the chorion on chemical toxicity for zebrafish embryos. For this end, the available information on the genesis and morphology, chemical composition, biological function and permeability of the fish chorion was reviewed under toxicological aspects to understand possible chemical interactions between chorion components and xenobiotics potentially preventing the transfer of external contaminants into the embryos. Apart from known stage-specific alterations of chorion permeability as well as a certain “natural” influence of environmental conditions on the permeability, it could be demonstrated that the permeability can be manipulated e.g. by use of DMSO. However, distinct physico-chemical properties of either the chorion itself or the chemicals to be uptaken by the embryo could only be identified in exceptional cases: Heavy metals are known to be blocked by the chorion via binding, most likely via complexation by anionic charged groups, possibly thiol-groups, which are abundantly present in the chorion (Pullella *et al*., 2006). However, it is not ascertained yet if this accounts only for heavy metals with positive standard electrode potentials, since studies are contradictory and binding and permeability are dependent on salinity, as well as on water hardness,

temperature and the presence of dissolved organic matter. Furthermore it is suspected, that the chorion pores potentially restrict the uptake of compounds depending on their size (Cheng *et al.*, 2007; Creton, 2004; Kashiwada, 2006; Léonard *et al*., 2005; Lillicrap, 2010), which was investigated further at a later stage within this thesis. For lipophilic substances, on the other hand, which in most instances can penetrate membranes easily, it seems rather applicable that with increasing lipophilicity the substance is accumulated in the yolk and becomes available slowly at the beginning of yolk consumption, thus delayed in their effectiveness (Broyles and Noveck, 1979; Calamari et al., 1981; Ensenbach, 1987; Galassi et al. 1982; Klaverkamp et al. 1977; Korn and Rice 1981; Marchetti 1965; Skidmore, 1966; Van Leeuwen et al., 1985; Wiegand et al., 2000). For differences in the sensitivity of early embryos, the ontogenetic stage at begin of exposure could therefore be more important than differences in chorion permeability, a presumption which also likewise was subjected to further investigations within this thesis. With regard to possible chemical interactions between chorion components and xenobiotics, there is still a need for supplementary work on understanding the chemical composition of the chorion and in particular the function and properties of the pores which disperse across fish chorions.

To follow up on evidences, that the chorion may restrict the uptake of compounds (Braunbeck et al., 2005; Cheng *et al.*, 2007; Creton, 2004; Kashiwada, 2006; Léonard et al., 2005; Lillicrap, 2010; Rudolf 2000; Scholz et al., 2008; Wendler, 2006, as a first step, dechorionation methods were evaluated for application in the fish embryo test with the zebrafish (*Danio rerio*). Several protocols exist for fish embryo dechorionation, using either enzyme solutions to digest the chorion (Collodi *et al*., 1992; Stuart *et al*., 1990; Westerfield, 2007) or forceps to remove the chorion mechanically (Westerfield, 2007). Since for recovering embryos for genetic engineering or generating embryonic cells for cell cultures, it seemed sufficient to select undamaged embryos or embryonic cells for further operations, the actual survival rates of dechorionated embryos following all procedures mentioned above have never been given special attention to. For chemical toxicity assessment, however, a reproducibly high survival rate is of fundamental importance. In the present thesis, it was demonstrated that a modified fish embryo test can be conducted with zebrafish embryos dechorionated at 24 hpf with reliably high survival rates (Henn and Braunbeck, 2011). However, the standard positive control test substance, 3,4-dichloroaniline, must be replaced, e.g. by acetone, since this substance exerts its toxicity during the first 24 h of development.

Dechorionation of younger stages is basically possible, but with decreasing survival rates; in zebrafish, 8 hpf seems to be the lower limit for dechorionation.

The successful test of the dechorionation procedure with the cationic polymer Luviquat HM 552 which has been suspected to be blocked by the chorion (Léonard *et al*., 2005) contributes to the assumptions that chorion pores might restrict the uptake of compounds depending on their size (Creton, 2004; Cheng *et al.*, 2007; Léonard *et al*., 2005): Luviquat HM 522 was demonstrated to be blocked by the chorion, but to exert full toxicity (1) in embryos that are allowed to hatch normally (prolonged fish embryo test), (2) in embryos dechorionated at the of 24 hpf, as well as (3) in eleutheroembryos exposed from beyond hatch for up to 48 h (Henn and Braunbeck, 2011). Likewise the results of experiments with polyethylene glycols of different molecular size (between 3 000 and 12 000 Da) provide evidence that the size of a molecule can be a limiting factor regarding the uptake of a molecule through the chorion. Freshly fertilized zebrafish eggs were placed in relatively high concentrated solutions of polyethylene glycols of different molecular weight. For exposure to polyethylene glycols with ≥ 3000 g/mol, a depression of the chorion was observed, which is suspected to be an osmotic reaction of the egg: assuming that the chorion is not permeable for polyethylene glycols ≥ 3000 Da, exposure to relatively high concentrated solutions of polyethylene glycols would generate osmotic pressure, and, since polyethylene glycols cannot pass the chorion and get into the egg, the water molecules would pass through the chorion from an area of low solute concentration (inside the egg) to one of high solute concentration (outside the egg), resulting in a reduction of the egg internal “pressure” and chorion shrinkage. A “critical” molecular size might thus lie between 2000 and 3000 Da for polyethylene glycols, which would be consistent with findings from Creton (2004). Contradictory observations of Lilicrap (2010), suggesting that the uptake of PEG4000 is reduced whereas PEG40000 (average Mn: 40000 Da) is able to cross the chorion and enter the embryos show that, show, however, that the actual size of a molecule not only depends on its molecular size. For example interactions within a molecule as well as with the environment (e.g. formation of hydrate coverings) could play a crucial role. An actual molecular size cut off value can therefore not reasonably be set.

The differential uptake and metabolization of the chemicals in the embryos and the adults may be another reason for the differences in toxicity. Busquet *et al*. (2008) could demonstrate 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. Combining the fish embryo toxicity test with a Metabolic Activation System (MAS; microsomes from cytochrome P450-activated mammalian systems) during exposed to proteratogens (cyclophosphamide and ethanol), resulted in the formation of significant lethal or teratogenic effects in exposed fish embryos. 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.

In the present work, a known outlier, KCl, was investigated in comparison with other salts (K_2SO_4, $KHCO_3$, NaCl, Na_2SO_4, and $NaHCO_3$) in the standard as well as in the prolonged FET, in tests with eleutheroembryos and dechorionated embryos. Results were compared to acute fish toxicity data retrieved from the US EPA Ecotoxicity database and other data on larval toxicity available from the open literature. Findings clearly show that only for the standard 48 h fish embryo test potassium salt results differ strongly from adult toxicity values. Prolongation of the exposure period to 96 or 120 h leads to embryo results very similar to adult toxicity data. In contrast to the potassium salts, testing of the analogous sodium salts showed that there is also a difference between standard and prolonged as well as eleutheroembryo toxicity; however, by far not as pronounced as with the potassium salts. These differences seem to be rather a consequence of prolonged exposure than a consequence of toxicity increasing with age. As an explanation for the differences between sodium and potassium salts, different functionalities of the different ions in the osmotic balance are assumed (Mount *et al.*, 1997). The stage-specific toxicities of the potassium salts on the other hand may be ascribed to different sites of osmoregulatory processes of embryonic and adult fish. The chloride cells (mitochondria-rich cells) play a central role in ion exchange and osmoregulation (Flik et al., 1995; Foskett and Scheffey, 1982; Kaneko et al., 2002; Marshall, 1995; Towle, 1990). In adult fish, the gills were identified as the responsible side for transepithelial ion exchange (McDonald et al., 1991). In contrast, pre-hatch teleost embryos are able to maintain the osmotic balance of the body fluids (Alderdice, 1988; Guggino, 1980; Kaneko et al., 1995; Varsamos et al., 2005) via chloride cells present in the yolk-sac membrane and other body surfaces (Dépêche, 1973; Hwang, 1989; Hwang et al. 1985; Kaneko et al., 1995, 2002, 2008; Lasker and Threadgold, 1968; Lin et al., 2006; Shen and Leatherland, 1978). These results of a couple of studies (Esaki *et al.*, 2007; Rombough, 2002) suggest that in case of zebrafish, ion regulation functions shift from the skin to the gills at about 5 days post fertilization. This point of time coincides with the findings of enhanced potassium toxicity at 120 hpf, leading to the assumption that in the case of potassium a change in body function processes takes a direct influence on toxicity.

These results confirm that the period around hatching is from a physiological and toxicological point of view a critical stage during embryogenesis (Van Leeuwen *et al*., 1985), which should be included in the testing procedure for chemical testing. Based upon data found by Belanger *et al*. (2010) it is recommended that testing of developing zebrafish embryos should be terminated between 24 and 48 hr after hatching in order to be compliant with above mentioned animal welfare legislation within Europe.

In terms of potential limitations for the use of zebrafish embryos in ecotoxicity testing as an alternative for the standard fish toxicity test, the following conclusions can be drawn from the present thesis:

With regard to possible chemical interactions between chorion components and xenobiotics, there is still a need for supplementary work on understanding the chemical composition of the chorion and in particular the function and properties of the pores which disperse across fish chorions. Nevertheless, it seems that the chorion is not a major barrier for simple chemicals, however, there are exceptions: (1) Substances like cationic polymers, which form complex or large structures with other similar molecules through chemical interactions such as ionic bonding, and (2) substances which likely interact with SH-groups, e.g. heavy metals, should not be assessed for their ecotoxicity using fish embryos, since the chorion may act as a barrier here. Likewise, (3) large molecules (e.g. polymers) could be blocked by the chorion, however, a reasonable molecular size cut off value for fish embryo testing cannot be set. The results from tests with a known outlier confirm that the period around hatching is a critical stage during embryogenesis, which should be included in the testing procedure for chemical testing. The FET should therefore at least in preliminary test be extended to 96 h, i.e. beyond hatch, which would be compliant with animal welfare legislation within Europe.

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