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Development of an OECD Guidance Document for the Application of OECD Test Guideline 236 (Acute Fish Embryo Toxicity Test)

Final report

by

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Abstract: The chorion structure and biotransformation capacities of zebrafish as boundary conditions for OECD Test Guideline 236 (Acute Fish Embryo Toxicity Test)

In July 2013, the Fish Embryo Acute Toxicity Test (FET) was adopted by OECD as Test Guideline 236. The FET thus represents the first fully validated alternative method within the OECD Test Guidelines Programme in the ecotoxicology sector. However, the practical applicability of the FET has ever since been discussed with respect to a series of aspects, which are addressed in this report: With respect to the potential barrier function of the zebrafish chorion, the molecular size for a free passage of non-charged chemical substances across the chorion of the zebrafish could be set at 3,000 - 4,000 Da. The limit for the passage of charged molecules is lower than that of non-charged ones. DMSO concentrations of ≥ 0.1 % decrease the barrier function of the chorion. Zebrafish embryos, juveniles and adults have been studied to a different extent with respect to their biotransformation capacities. Our knowledge on biotransformation capacities in zebrafish is fragmentary. On this basis, transfer of biotransformation data from juvenile to adult fish is not possible. In zebrafish embryos, biotransformation could be documented whenever studied in more detail; only for rare exceptions, lack of biotransformation was demonstrated. Gene activation cannot be translated into biochemical functionality; mammalian data cannot be extrapolated to fish. The analysis of historical short-term (acute) Fish Toxicity Test (AFT) data produced according to OECD TG 203 within a dataset used for a corresponding FET study by ECHA started with 2936 studies on a total of 1842 substances. Applications of filters identical to those used for the FET analysis resulted in exclusion of 62.3 % of the AFT studies and 63.0 % of the substances. Data of the present report will be integrated into OECD project no. 2.54: „Guidance Document on an Integrated Approach on Testing and Assessment for Fish Acute Toxicity Testing“ – Integration of the Fish Embryo Test into the Threshold Approach”.

Kurzbeschreibung: Chorionstruktur und Biotransformationskapazität des Zebrabärblings als Randparameter für die OECD-Richtlinie 236 (Akuter Fischembryotest)

Im Juli 2013 wurde der akute Fischembryotest (FET) als OECD-Prüfrichtlinie 236 anerkannt. Der FET stellt damit die erste voll validierte Alternativmethode im Rahmen des OECD-Prüfrichtlinienprogramms im Bereich der Ökotoxikologie dar. Die praktische Anwendbarkeit wurde jedoch seitdem hinsichtlich mehrerer Aspekte diskutiert, die im vorliegenden Bericht adressiert werden: Im Hinblick auf die potentielle Barrierefunktion der Eihülle des Zebrabärblings konnte eine Molekulargröße von 3000 – 4000 Da für die freie Passage ungeladener Moleküle identifiziert werden. Das Limit für die Passage geladener Moleküle ist geringer. DMSO-Konzentrationen ≥ 0,1 % reduzieren die Barrierefunktion weiter. Embryonen, juvenile und erwachsene Zebrabärblinge sind im Hinblick auf ihre Biotransformation unterschiedlich gut untersucht; insgesamt ist unser Wissen fragmentiert. Auf dieser Basis ist die Übertragung von Daten von juvenilen Fischen auf erwachsene und umgekehrt nicht möglich. Wann immer die Biotransformation in Embryonen genauer betrachtet wurde, konnte sie zumindest qualitativ nachgewiesen werden. Nur für seltene Ausnahmen, konnte eine nicht vorhandene Biotransformation im Embryo demonstriert werden. Genaktivierung kann nicht in biologische Funktionalität übersetzt werden; Säugerdaten können nicht auf Fisch extrapoliert werden. Die Analyse eines historischen Datensatzes zur akuten Fischtoxizität, der bereits als Grundlage für die Bewertung der Eignung des FET gedient hatte, ging von 2936 Studien mit Daten zu 1842 Substanzen aus. Die Anwendung der gleichen Filter, die von der ECHA für den FET angesetzt wurden, führte zum Ausschluss von 62,3 % der Studien bzw. 63,0 % der Substanzen. Die Daten des vorliegenden Berichts werden in das OECD-Projekt Nr. 254 („Guidance Document on an Integrated Approach on Testing and Assessment for Fish Acute Toxicity Testing“ – Integration of the Fish Embryo Test into the Threshold Approach”) integriert.

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AFT	Acute Fish Toxicity (OECD TG 203)
CLSM	Confocal Laser Scanning Microscopy
COS	Center for Organismal Studies (University of Heidelberg)
DMSO	Dimethyl sulfoxide
CYP	Cytochrome P450
3D	Three-dimensional
Da	Dalton
ECHA	European Chemicals Agency, Helsinki
EC_{50}	Test concentration with 50 % effect
ECOD	7-Ethoxycoumarin-O-deethylase
EDTA	Ethylene diamine tetraacetic acid
EROD	7-Ethoxyresorufin-O-deethylase
FET	Fish Embryo Acute Toxicity Test (OECD TG 236)
GD	(OECD) Guidance Document
hpf	hours post-fertilization
h	Hours
LC_{50}	Test concentration with 50 % mortality
LOEC	Lowest Observed Effect Concentration
NOEC	No Observed Effect Concentration
mRNA	Messenger ribonucleic acid
OECD	Organization for Economic Co-operation and Development
P450	Cytochrome P450
PEG	Polyethylene glycol
POW	Octanol/water partitioning coefficient
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
TG	(OECD) Test Guideline
TI	Teratogenicity index
UBA	German Environment Agency, Dessau
VMG-eco	(OECD) Validation Management Group for Ecotoxicity Testing
WNT	Working Group of National Coordinators for the OECD Test Guidelines Programme
For abbreviations in figures, see figures legends.	

Summary

In July 2013, the Fish Embryo Acute Toxicity Test (FET; OECD 2013) validated by Germany together with the OECD Ad hoc Expert Group on the Fish Embryo Toxicity Test and the OECD Validation Management Group for Ecotoxicity Testing (VMG-eco) was adopted by the Working Group of National Coordinators for the OECD Test Guidelines Programme (WNT) as OECD Test Guideline (TG) 236 under the title "Fish Embryo Acute Toxicity (FET) Test". Given that non-feeding stages of zebrafish (*Danio rerio*) development do not fall under the auspices of the current EU Directive 2010/63/EU on the protection of animals used for scientific purposes (EU, 2010) until the age of 5 days (Strähle et al., 2011), the procedure of the Fish Embryo Acute Toxicity (FET) Test represents the first fully validated alternative method within the OECD Test Guideline Programme in the ecotoxicology section. However, since its adoption by OECD in 2013, the practical applicability of the FET has been discussed with respect to a series of aspects including (1) the suspected, but poorly understood barrier function of the chorion and (2) the speculated, but hardly documented biotransformation potential of zebrafish embryos. With respect to the applicability of the FET within the REACH regulation, (3) the adequateness of the FET has been much debated. Only recently, Sobanska et al. (2018) published an analysis of the FET protocol and database, which, however, gave rise to criticism and will, thus, be critically questioned in the present publication. Finally, the suitability of the FET (4) to contribute to other OECD projects such as OECD project no. 2.54 ("Guidance Document on an Integrated Approach on Testing and Assessment for Fish Acute Toxicity Testing" – Integration of the Fish Embryo Test into the Threshold Approach; OECD Guidance Document 126) is being discussed. All of these issues are addressed in the present UBA report.

The potential barrier function of the zebrafish chorion

For long, a major objection to the acceptance of the Fish Embryo Test (FET) according to OECD TG 236 has been the putative protecting role of the chorion (eggshell) of the embryo. Whenever fish embryo toxicity tests produced results different from data published for the conventional Acute Fish Toxicity test (AFT) according to OECD TG 203, impaired permeability of the zebrafish chorion has been speculated. Starting as early as in 2008, the Federal Environmental Protection Agency (UBA) has funded investigations into the permeability of the zebrafish chorion at the University of Heidelberg (cf. UBA report FKZ 37080650400). Since then, more-in-depth experiments have been carried out. The present report provides an integration of all investigations conducted so far at the Aquatic Ecology and Toxicology Group in the Center for Organismal Studies at the University of Heidelberg and elsewhere.

With respect to the potential barrier function of the zebrafish chorion, further evidence was provided by means of fluorescent markers and differently sized polyethylene glycols to conclude that the molecular size for a free passage of non-charged chemical substances across the chorion of the zebrafish could be set at approx. 3,000 - 4,000 Da. The rate of passage of charged molecules across the chorion of zebrafish is substantially lower than that of non-charged molecules. As a conclusion, the passage of most uncharged industrial chemicals across the zebrafish molecule can be assumed to not be impaired by a systematic size limitation.

Dimethyl sulfoxide (DMSO) concentrations of ≥ 0.1 % decrease the barrier function of the chorion. Since dimethyl sulfoxide (DMSO) does not affect zebrafish embryo survival up to concentrations well above 2 %, DMSO may be used as a detergent to facilitate the passage of substances that might be critical in terms of their molecular size to pass the zebrafish chorion. Since DMSO does not affect zebrafish embryo survival up to concentrations well above 2 %, DMSO may be used as a solvent to facilitate the passage of substances that might be critical for handling in aqueous solutions due to their high lipophilicity. So far, there is no convincing

evidence that – after appropriate solution by the aid of solvents – highly lipophilic substances are not taken up by zebrafish embryos. See also advice given in OECD Guidance Document (GD) no. 23 on aqueous-phase aquatic toxicity testing of difficult test chemicals.

For highly lipophilic substances, neither the Acute Fish Toxicity test (AFT, OCED TG 203) nor the Fish Embryo Toxicity test (FET, OECD TG 236) appear to be suitable protocols, since highly lipophilic substances might accumulate over extended periods of time and might not have reached a steady-state after an exposure time restricted to 96 h. Rather, for highly lipophilic substances, longer-term tests such as a Fish Early Life-Stage test according to OECD TG 210 should be conducted.

In case of substances, the passage of which across the zebrafish chorion might be critical, dechorionation of zebrafish embryos may be applied: With appropriate modifications of the FET protocol, embryos can reproducibly be dechorionated at 24 h post-fertilization (hpf) with reliably high survival rates of ≥ 90 %. Dechorionation of younger stages (< 24 hpf) is generally possible, however with lower survival rates.

Biotransformation and bioactivation capacities in early life stages of zebrafish (*Danio rerio*)

Major objections to the use of the fish embryo test (FET) with the zebrafish (Danio rerio) have been based on the assumption of limited biotransformation capacities in early life stages of fish. Repeatedly, doubts have been expressed concerning the comparability of metabolic capacity between early juvenile and adult life stages of zebrafish. In fact, a limited biotransformation or bioactivation capacity could lead to underestimations of toxic or teratogenic potency of a xenobiotic, which would be bioactivated in other life stages. On the other hand, given the *ex utero* development, early-life stages of (zebra)fish depend on their intrinsic metabolic capacity for elimination or transformation of xenobiotics; therefore, it seems reasonable to assume at least qualitatively appropriate biotransformation capacities.

As a consequence, in order to strengthen the acceptance of the Fish Embryo Toxicity (FET) test, it seemed essential to attempt to better understand the metabolic capacities of zebrafish embryos across different life-stages. However, while literature concerning spatial and temporal expression patterns of xenobiotic metabolizing enzymes has been growing fast at the genome and RNA levels, only few investigations addressed protein function and catalytic activities, although these are ecologically much more relevant parameters than DNA- and RNA-based characteristics. The database concerning zebrafish metabolism, especially in early-life stages, is much less complete than that available for other model organisms (e.g. rainbow trout) and is scattered over more than 100 studies.

So far, zebrafish embryos, juveniles and adults have been studied to a very different extent with respect to their biotransformation capacities. Overall, our knowledge about xenobiotic transformation capacities in zebrafish can – at best – be called fragmentary. Since especially juveniles have largely been neglected, transfer of biotransformation data from juvenile fish to adult fish does not appear justified. In zebrafish embryos, however, biotransformation could be documented whenever studied in more detail. So far, only for rare exceptions such as allyl alcohol and albendazol, a lack of biotransformation could be demonstrated. Studies on biotransformation gene expression (induction) is not as conclusive as studies in enzyme biosynthesis (transcriptomics, proteomics) or – even better – biochemical activity of enzymes. Since our knowledge on the spectrum of substances accepted as substrate(s) by various cytochrome P450 isoforms is quite limited, there is no way to extrapolate gene activation to biochemical functionality.

There is no adequate database to extrapolate observations from mammalian studies to fish and fish embryos. For many P450 isoforms, we do not even have conclusive evidence of congruence in cytochrome P450 terminology between mammalian studies and studies in lower vertebrates. Therefore, transfer of a link between nomenclature and metabolic function from mammals to fish ("same nomenclature means same function") or vice versa is not necessarily justified. With respect to biotransformation phase II reactions, our knowledge is even more fragmentary than it is for phase I metabolization.

As a gold standard for studies into biotransformation capacities in (zebra)fish embryos, juveniles and adults, gene expression studies need to be linked to proteome analysis data and chemical analytical analyses into the metabolites formed by cytochrome P450 enzymatic activity. Such studies, however, are most challenging in terms of expertise, resources, time and funding. Given the limitations of resources and the multitude of potential substrates for cytochrome P450 conversion, studies providing indirect evidence of the existence of active biotransformation seem more promising for screening purposes. Such indirect evidence may come from (1) studies designed to visualize the formation of fluorescent or colored metabolites, (2) studies based on chemical analyses of emerging metabolites, and (3) studies based on the detection of biological effects by substances activated by cytochrome P450 action.

An analysis of the relevance and adequateness of the Fish Acute Toxicity Test (AFT) according to OECD TG 203 to fulfil the information requirements and addressing concerns under REACH

The analysis of historical short-term (acute) Fish Toxicity Test (AFT) data produced according to OECD TG 203 within a dataset already used for the ECHA FET study (Scholz et al., 2016) started with 2936 studies on a total of 1842 substances from the ECHA database. Exclusion of studies with not precisely defined LC_{50} entries (e.g. only approximate information on LC_{50} range given) eliminated 54 % of all studies and 50 % of the test substances originally contained in the database. Exclusion of studies on substances that have only been tested in a single species only resulted in a remainder of 34.6 % of the original dataset and 18.8 % of substances, respectively.

Both intra- and interspecies comparisons of AFT data revealed ranges for a given substance between 1- and 1000fold. For the majority of studies, the deviation was minor, i.e. between a factor of 1 and 10. In the interspecies comparison, 72.4 % of the AFT studies produced results with deviations within a factor of 10. However, deviations by a factor between 10 and 100 (22 %) or even a factor between 100 and 1000 (5.6 %) could also be observed. Finally, only 5.8 % of the studies/substances of the intraspecies comparison gave exactly the same LC_{50} value.

Given that approx. 60 % of the existing AFT data would not fulfil the requirements set in the ECHA FET study (Scholz et al., 2016; Sobanska et al., 2018), massive re-testing of acute fish toxicity would be required. Especially study results for substances tested at concentrations close or above their water solubility, but without analytical verification should be interpreted with care or considered for revision.

Data on the AFT analysis are preliminary; analyses into effects of the application of further filtering criteria are ongoing.

Contributions to OECD project no. 2.54: "Guidance Document on an Integrated Approach on Testing and Assessment for Fish Acute Toxicity Testing" – Integration of the Fish Embryo Test into the Threshold Approach (Revision of OECD short Guidance no. 126)"

Both the literature review on biotransformation capacities in various developmental stages of zebrafish (chapter 2) and the analysis of the relevance and adequateness AFT data according to OECD TG 203 to fulfil the information requirements and addressing concerns under REACH

(chapter 3) will be integrated as major contributions to OECD project no. 2.54: „Guidance Document on an Integrated Approach on Testing and Assessment for Fish Acute Toxicity Testing" – Integration of the Fish Embryo Test into the Threshold Approach (OECD Short Guidance Document 126).

Zusammenfassung

Im Juli 2013 wurde der mit der OECD Ad hoc Expert Group on the Fish Embryo Toxicity Test und der und OECD Validation Management Group for Ecotoxicity Testing (VMG-eco) validierte akute Fischembryotoxizitätstest (FET; OECD 2013) von der Working Group of National Coordinators for the OECD Test Guidelines Programme (WNT) als OECD Prüfrichtline (TG) 236 mit dem Titel "Fish Embryo Acute Toxicity (FET) Test" verabschiedet. Da nach allgemeinem Rechtsverständnis zumindest in der Bundesrepublik Deutschland nicht selbständig fressende Entwicklungsstadien des Zebrabärblings (*Danio rerio*) bis zu einem Alter von 5 Tagen (Strähle et al., 2011) nicht unter den Schutz von Organsimen im Sinne der gültigen EU Directive 2010/63/EU on the protection of animals used for scientific purposes (EU, 2010) fallen, stellt das Protokoll für den Fischembryotest (FET) das erste vollständig validierte Alternativverfahren innerhalb des OECD Testrichtlinienprogramms im Bereich der Ökotoxikolgie dar. Seit seiner Akzeptanz durch die OED im Jahr 2013 ist die praktische Anwendbarkeit des Fischembryotoxizitätstests im Hinblick auf eine Reihe von Gesichtspunkten jedoch immer wieder diskutiert worden: zunächst (1) die immer wieder vermutete, aber ausgesprochen schlecht verstandene potentielle Barrierefunktion des Chorions sowie (2) die kaum untersuchte Biotransformationskapazität junger Entwicklungsstadien des Zebrabärblings. Im Hinblick auf die Anwendbarkeit des Fischembryotests z.B. im Rahmen der REACH-Richtlinien der EU wurde (3) die Eignung des FET intensiv diskutiert. Erst 5 Jahre nach der Akzeptanz des Fischembryotests als OECD-Richtlinie publizierten Sobanska et al. (2018) eine Analyse des FET-Protokolls und der zugrundeliegenden FET-Datenbank, die Anlass zur Kritik auslöste und daher in der vorliegenden Studie kritisch beleuchtet wird. Schließlich wird (4) die Eignung des Fischembryotests zur Integration in andere laufende OECD-Aktivitäten wie Projekt Nr. 2.54 ("Guidance Document on an Integrated Approach on Testing and Assessment for Fish Acute Toxicity Testing" – Integration of the Fish Embryo Test into the Threshold Approach; OECD Guidance Document 126) diskutiert. Diese vier Aspekte im Zusammenhang mit dem Fischembryotest werden in der vorliegenden Zusammenfassung adressiert.

Die potentielle Barrierefunktion des Chorions des Zebrabärblings

Seit langem bildet die Existenz des Chorions, einer extrazellulären Eischale um den Embryo des Zebrabärblings, die den Embryo auch gegen den adversen Einfluss von Chemikalien schützen soll, die Grundlage für eine Ablehnung des Fischembryotests nach OECD TG 236. Wann immer Fischembryotests Daten lieferten, die sich von jenen korrespondierender konventioneller akuter Fischtoxizitätsdaten (OECD TG 203) unterschieden, wurde der Verdacht auf eine eingeschränkte Permeabilität des Chorions des Zebrabärblings geäußert. Bereits seit dem Jahr 2008 hat das Umweltbundesamt Untersuchungen an der Universität Heidelberg zur Permeabilität des Chorions gefördert (siehe UBA-Bericht zu Projekt FKZ 37080650400); Seitdem wurden zahlreiche weitere Studien durchgeführt. Der vorliegende Bericht fasst alle Untersuchungen zusammen, die seitdem von der Arbeitsgruppe Aquatische Ökologie und Toxikologie an der Universität Heidelberg und anderen Arbeitsgruppen fertig gestellt wurden.

Im Hinblick auf die potentielle Barrierefunktion des Chorions des Zebrabärblings erbrachten Untersuchungen mit fluoreszierenden Markersubstanzen und unterschiedlich großen Polyethylenglykolen weitere Hinweise, die den Schluss zulassen, dass ein Molekulargewicht von 3000 – 4000 Dalton als Grenze für die freie Passage von ungeladenen Substanzen über das Chorion des Zebrabärblings angesehen werden kann. Die Transferrate bzw. -geschwindigkeit von geladenen Molekülen ist deutlich geringer als der Transfer von ungeladenen Molekülen. Es darf der Schluss gezogen werden, dass das Chorion des Zebrabärblings schon aufgrund der Molekulargröße für die meisten (ungeladenen) Industriechemikalien keine Barriere darstellt.

In Konzentrationen ab 0,1 % reduziert Dimethylsulfoxid (DMSO) die Barrierefunktion des Chorions weiter. Da DMSO bis zu einer Konzentration von deutlich > 2 % die Überlebensrate von Embryonen des Zebrabärblings nicht beeinflusst, kann DMSO als Detergenz eingesetzt werden, um die Transferrate von Substanzen zu erhöhen, die sonst aufgrund ihrer Molekülgröße das Chorion nur langsam überwinden würden. Des Weiteren kann DMSO als Lösungsvermittler für Substanzen empfohlen werden, die aufgrund ihrer Lipophilität als in wässrigen Lösungen als schlecht löslich eingestuft werden müssten. Bei adäquater Nutzung geeigneter Lösungsvermittler gibt es bisher keine Hinweise darauf, dass hoch lipophile Substanzen nicht im Fischembryotest (FET) getestet werden könnten. Siehe hierzu auch die Empfehlungen des OECD Guidance Document (GD) Nr. 23 für schwierige Prüfsubstanzen (GD on aqueous-phase aquatic toxicity testing of difficult test chemicals).

Grundsätzlich erscheinen jedoch wegen der geringen Testdauer weder der Akute Fischtest (AFT; OECD Guideline Nr. 203) noch der Fischembryotest (FET; OECD TG 236) als geeignete Protokolle zur Prüfung hoch lipophiler Substanzen, da diese aufgrund ihrer geringen Wasserlöslichkeit und langsamen, jedoch lange anhaltenden Bioakkumulation innerhalb eines Expositionszeitraums von 4 Tagen keinen Gleichgewichtszustand erreichen dürften. Vielmehr sollte für hoch lipophile Substanzen gleich ein länger andauernder Test wie ein Early Life-Stage Test (OECD TG 210) durchgeführt werden.

Für Substanzen, die hinsichtlich ihres Durchtritts durch das Chorion des Zebrabärblings als kritisch betrachtet werden, kann die Dechorionierung in das Protokoll des Fischembryotests integriert werden: Mit nur geringen Modifikationen des FET-Protokolls können Embryonen des Zebrabärblings reproduzierbar und mit hoher Überlebenswahrscheinlichkeit (> 90 %) ab einem Alter von 24 h nach der Befruchtung dechorioniert werden. Die Dechorionierung jüngerer Entwicklungsstadien (< 24 h) ist grundsätzlich möglich, führt jedoch zu geringeren Überlebensraten.

Biotransformation und Bioaktivierungskapazität früher Entwicklungsstadien des Zebrabärblings (*Danio rerio*)

Viele Einsprüche gegen die Anwendbarkeit des Fischembryotests mit dem Zebrabärbling basieren auf der Annahme, dass frühe Entwicklungsstadien des Fisches nur über eine verminderte Biotransformationskapazität verfügen. Immer wieder wurden Zweifel geäußert, dass die metabolische Kapazität von frühen juvenilen und adulten Stadien des Zebrabärblings vergleichbar sei. Tatsächlich könnte eine begrenzte Biotransformationskapazität theoretisch zu einer Unterschätzung von Toxizität und Teratogenität von Substanzen führen, die der Bioaktivierung bedürfen. Andererseits hängen frühe Lebensstadien von Fischen in Anbetracht ihrer *Ex utero*-Entwicklung von einer intrinsischen Befähigung zur Transformation und Elimination von (exogenen und endogenen) toxischen Stoffen ab. Es scheint daher plausibel, zumindest qualitativ vom Vorhandensein einer entsprechenden Biotransformationskapazität auszugehen.

Um weiter Argumente für die Akzeptanz des Fischembryotests (FET) zu liefern, erschien zumindest der Versuch unabdingbar, die metabolischen Kapazitäten unterschiedlich alter Entwicklungsstadien des Zebrabärblings besser zu verstehen. Während unser Wissen über örtliche und zeitliche Expressionsmuster von Biotransformationsenzymen auf der Ebene der mRNA in den letzten Jahren stark zugenommen hat, befassten sich nur relativ wenige Studien mit Proteinfunktion und enzymatischer Aktivität, obwohl diese von ungleich größerer ökologischer Bedeutung als die Expression auf mRNA-Ebene sind. Grundsätzlich ist die Datenbank zum Metabolismus des Zebrabärblings auch heute noch insbesondere im Hinblick auf

frühe Entwicklungsstadien deutlich unvollständiger als für andere Modellorganismen wie z.B. die Regenbogenforelle und auf über 100 wissenschaftliche Publikationen verstreut.

Im Hinblick auf ihre Biotransformationskapazitäten sind Embryonen, Juvenilstadien und Adulte des Zebrabärblings sehr unterschiedlich gut untersucht; trotz der rasanten Entwicklung des Zebrabärblings als Modellorganismus in den letzten Jahren kann unser Wissen über den Metabolismus von Xenobiotika beim Zebrabärbling bestenfalls als fragmentarisch bezeichnet werden. Da insbesondere Juvenilstadien weitgehend vernachlässigt wurden, erscheint der Transfer vor allem von Erkenntnissen bei juvenilen auf adulte Zebrabärblinge nicht statthaft. In Embryonen des Zebrabärblings konnte Biotransformation jedoch in allen Studien, die genauer ins Detail gingen, zumindest qualitativ belegt werden. Bislang konnte das Fehlen einer Biotransformation nur für seltene Ausnahmen wie Allylalkohol und Albendazol belegt werden. Grundsätzlich sind Studien zur Expression (Induktion) von Biotransformationsgenen weniger aufschlussreich als Untersuchungen zur Biosynthese der aktiven Enzyme (Transkriptomics, Proteomics) oder – noch besser – Untersuchungen zur biochemischen Aktivität der Enzyme. Da unsere Kenntnisse zum Spektrum der Substanzen, die als potentielle Substrate verschiedener Isoformen von Cytochrom P450 dienen können, sehr beschränkt sind, besteht auch nicht die Möglichkeit, von der Genaktivierung auf biochemische Funktionalität zu schließen.

Auch für den Transfer von Erkenntnissen zu Säugetieren auf Fische bzw. Fischembryonen fehlt die entsprechende Datenbasis. Für viele Isoformen von Cytochrom P450 gibt es keine ausreichenden Hinweise auf eine Kongruenz wenigstens der Terminologie von Isoformen von Cytochrom P450 bei Säugetieren und niederen Wirbeltieren. Aus diesem Grund ist ein Transfer von Nomenklatur und metabolischer Funktion („gleicher Name bedeutet gleiche Funktion“) von Säugetieren auf Fische (oder umgekehrt) nicht zulässig. Im Hinblick auf Enzyme der Biotransformation Phase II ist unser Wissen mit noch größeren Lücken behaftet als für Enzyme der Phase I.

Als Goldstandard für Biotransformationsstudien mit Embryonen, Juvenilstadien und Adulten von Fischen müssen Genexpressionsstudien mit Daten der Proteomanalyse und Analysen der gebildeten Metaboliten, die bei den biochemischen Reaktionen gebildet werden, verlinkt werden. Solche Studien stellen jedoch im Hinblick auf Expertise, Ressourcen, Zeit und Finanzierung eine erhebliche Herausforderung dar. In Anbetracht der Beschränkungen von Ressourcen und der Vielzahl potentieller Substrate für Cytochrom P450 erscheinen Studien zum indirekten Nachweis der Existenz aktiver Biotransformation für Screenings mehr zu versprechen und schneller zum Ziel zu führen als der Nachweis von Genaktivierung, Proteinsynthese und -aktivität für eine Vielzahl einzelner Isoformen von Cytochrom P450. Derartige indirekte Hinweise können sich ergeben aus (1) dem optischen (mikroskopischen) Nachweis der Bildung fluoreszierender oder gefärbter Metaboliten, (2) dem chemisch-analytischen Nachweis der gebildeten Metaboliten sowie (3) dem Nachweis der gebildeten Metaboliten über ihre biologische Wirkung nach entsprechender Bioaktivierung von zunächst untoxischen Substraten. Die vorliegende Studie stellt Beispiele für diese indirekten Methoden vor.

Analyse der Relevanz und Eignung von Daten aus akuten Fischtests (AFT) nach OECD TG 203 für die Erfüllung der Informationsanforderungen unter REACH

Die Analyse eines historischen Datensatzes zur akuten Fischtoxizität (AFT) auf der Basis von Tests nach der OECD TG Nr. 203, der bereits die Grundlage für die Analyse in einer von der Europäischen Chemikalienagentur (ECHA) in Auftrag gegebenen Studie zur Eignung des Fischembryotests (OECD TG 236) gebildet hatte (Scholz et al., 2016), ging ursprünglich von 2936 Studien mit insgesamt 1842 Substanzen aus. Der Ausschluss von Studien, die keine

präzisen Angaben zum LC_{50} (z.B. nur näherungsweise Angaben zu einem ungefähren LC_{50}-Bereich) machten, führte zu einer Elimination von 54 % aller Studien sowie 50 % der Testsubstanzen, die ursprünglich im Datensatz enthalten waren. Der Ausschluss von Studien zu Substanzen, die nur in einer einzelnen Fischspezies durchgeführt worden waren, beließ noch 34,6 % bzw. 18.8 % der ursprünglich im Datensatz enthaltenen Studien bzw. Substanzen in der weiteren Untersuchung.

Sowohl Intra- als auch Interspeziesvergleiche von AFT-Daten ergaben für bestimmte Substanzen Schwankungen der Daten um Faktoren bis zu 1000. Für die Mehrheit der Substanzen waren die Abweichungen marginal, d.h. im Bereich von Faktoren zwischen 1 und 10. So ergab der Interspeziesvergleich für 72,4 % der Fälle Abweichungen mit einem Faktor bis zu 10. Jedoch konnten für 22 bzw. 5,6 % auch Abweichungen um Faktoren zwischen 10 und 100 bzw. zwischen 1000 und 10000 beobachtet werden. Letztendlich konnte im Rahmen des Intraspeziesvergleichs für nur 5,8 % der Studien bzw. Substanzen der gleiche LC_{50}-Wert ermittelt werden.

Die Tatsache, dass ca. 60 % der existierenden AFT-Daten die Anforderungen nicht erfüllen, die von Scholz et al. (2016) sowie Sobanska et al. (2018) vor dem Hintergrund von REACH an den Fischembryotest (FET) gestellt wurden, scheint eine umfangreiche Neutestung von Substanzen für REACH – auch mit dem akuten Fischtest nach OECD TG 203 – erforderlich zu machen. Insbesondere Studien im Bereich von Substanzkonzentrationen nahe oder über der Löslichkeitsgrenze der Substanzen erfordern besondere Aufmerksamkeit und sollten notfalls erneut überprüft werden.

Die Daten zur Analyse der AFT-Daten sind vorläufig; die Studie wird weitergeführt.

Beiträge des Fischembryotests (FET) zu OECD-Projekt Nr. 2.54: „Guidance Document on an Integrated Approach on Testing and Assessment for Fish Acute Toxicity Testing" – Integration of the Fish Embryo Test into the Threshold Approach (OECD Guidance Document 126)"

Sowohl die Literaturstudie zur Biotransformationskapazität in verschiedenen Entwicklungsstadien des Zebrabärblings (Kapitel 2) als auch die Analyse der Relevanz und Eignung des akuten Fischtests (AFT) nach OECD TG 203 für die Erbringung der notwendigen Informationen und die Behandlung von Bedenken unter REACH (Kapitel 3) werden als wichtiger Beitrag in das OECD-Projekt „Guidance Document on an Integrated Approach on Testing and Assessment for Fish Acute Toxicity Testing" – Integration of the Fish Embryo Test into the Threshold Approach (OECD Guidance Document 126)" integriert.

1 The zebrafish (*Danio rerio*) chorion as a potential barrier for the uptake of xenobiotics into the zebrafish embryo

1.1 Introduction

For long, a major objection to the acceptance of the Fish Embryo Test (FET) according to Organization for Economic Co-operation and Development (OECD) Test Guideline (TG) 236 has been the putative protecting role of the chorion (eggshell) of the embryo. Whenever fish embryo toxicity tests produced results different from data published for the conventional Acute Fish Toxicity test (AFT) according to OECD TG 203, impaired permeability of the zebrafish chorion has been speculated. Starting as early as in 2008, the German Environmental Agency (UBA) has funded investigations into the permeability of the zebrafish chorion at the University of Heidelberg (cf. UBA report FKZ 37080650400). Since then, more-in-depth experiments have been carried out. The present report provides an integration of all investigations conducted so far at the Aquatic Ecology and Toxicology Group in the Center for Organismal Studies at the University of Heidelberg and elsewhere.

1.2 Development of the zebrafish chorion

Since in most teleosts fertilization of eggs takes place in the free water body, fish eggs are shed during the spawning act into the free water spaces, where they are fertilized by simultaneously released sperm (Fedderwitz, 2008; Hisaoka, 1958; Kais, 2009). As a consequence, the eggs of non-live-bearing fish are protected by an outer shell, the chorion, against mechanical damage, infection by parasites and potentially also against chemical insult. Genetically and developmentally important, the chorion protects against polyspermy (Donovan and Hart, 1983). In zebrafish, sperm lack acrosomal structures to penetrate the chorion; in contrast, the egg chorion has a preformed permeation site named micropyle (Mold et al., 2001). In unfertilized zebrafish eggs, the micropyle area has an average diameter of 2.1 - 2.5 µm (Donovan and Hart, 1983), and the area for penetration of the sperm is limited to approx. 100 µm, where cortical granules have accumulated under the plasmalemma.

So far, the chorion of teleosts is thought to be a primary sheath (Brivio et al., 1991), i.e. the primary chorion is formed by the oocyte itself (Yamagami et al., 1992). Secondary shells are produced by follicular cells, and tertiary shells are produced by other reproductive organs such as the oviduct (Yamagami et al., 1992). As mentioned, the teleost chorion prevents multiple fertilization (Gilkey et al., 1978). The morphological structure and chemical composition of the fish chorion significantly varies with species (Brivio et al., 1991), most likely as a consequence to adaption to various environments (Hart et al., 1984).

In the zebrafish, the chorion has an average diameter of 1.5 - 2.5 µm (Rawson et al., 2000) and consists of three layers: an external electron-dense layer (Zona radiata externa), an intermediate fibrillary layer (upper Zona radiate interna) and an inner layer made up of 16 horizontal electron-dense sublayers interspersed with 15 sublayers of lower electron-density (deep Zona radiate interna) (Hart et al., 1984). However, the innermost layer of the eggshell has also been given various synonymous names: translucent membrane, vitelline membrane, vitelline sheath, zona pellucida, chorion, primary, secondary and tertiary shell (Arukwe and Goksoyer, 2003). The outer, intermediate and inner layers of the zebrafish chorion measures about 0.2 - 0.3, 0.3 - 0.6 and 1.0 - 1.6 µm in thickness, respectively (Rawson et al., 2000).

The chorion of fish is produced during oocyte maturation within the female gonads (Selman et al., 1993):

(1) In oocyte maturation stage IA (pre-follicular, primary growth stage), oocytes are arranged in nests of oocytes and measure 7 - 20 µm in diameter; a chorion cannot yet be discerned. Stage IB is characterized by interdigitating microvilli formed by both the oocytes and the follicular cells as well as secretion of electron-dense materials secreted from either side. This first manifestation of a chorion shows only one single layer and reaches a thickness of approx. 0.15 µm (Fig. 1a).

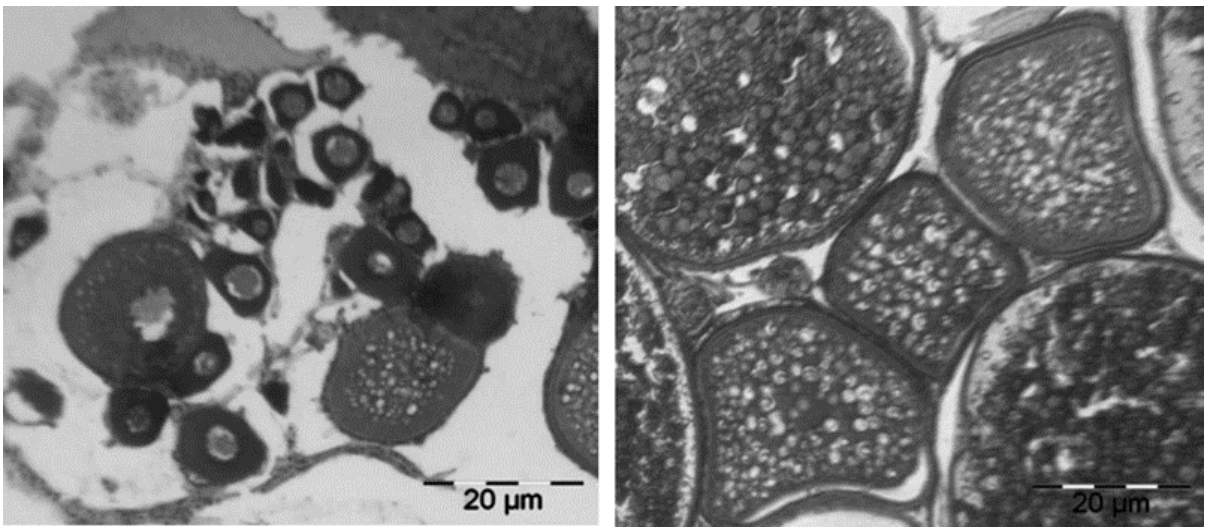
(2) In stage II (cortical alveolus stage), the oocyte diameter reaches 0.14 - 0.34 µm and is surrounded by a translucent layer of flattened to cubic follicular cells extending long microvilli towards the oocytes (Fig. 1b). The chorion itself has reached almost 6 µm in thickness and consists of 2 - 3 layers built up by several sublayers. The second layer has formed between the primary layer and the oolemma (cell membrane of the oocyte), whereas the third layer is being formed alongside the oolemma. Pores across all of these layers are kept open by microvilli from both the oocyte and the follicular cells.

(3) During oocyte maturation stage III (vitellogenesis), zebrafish oocytes may reach diameters of 340 – 690 µm (Fig. 2). The cubic translucent follicular cells surround a chorion of at least three layers; however, the chorion starts to attenuate, but microvilli still keep the chorion pore canals open.

(4) During stage IV (maturation), the follicular cells withdraw their microvilli from the pore canals, which then close (Donovan and Hart, 1983). The chorion diameter further declines, and oocyte have reached 690 – 730 µm in diameter.

(5) In stage V (mature oocyte), the chorion has reduced its diameter further to < 300 µm. Under the electron microscope, the pores appear empty; however, as can be visualized under the light microscope, the distal endings of the pore canals are obstructed by some dense materials. Follicular cells can no longer be discerned, and the eggshell has become translucent. Upon spawning and contact to the external medium water, the vitelline membrane detaches from the oocyte plasma membrane and forms the final inner chorion layer, the perivitelline space (Coward et al., 2002).

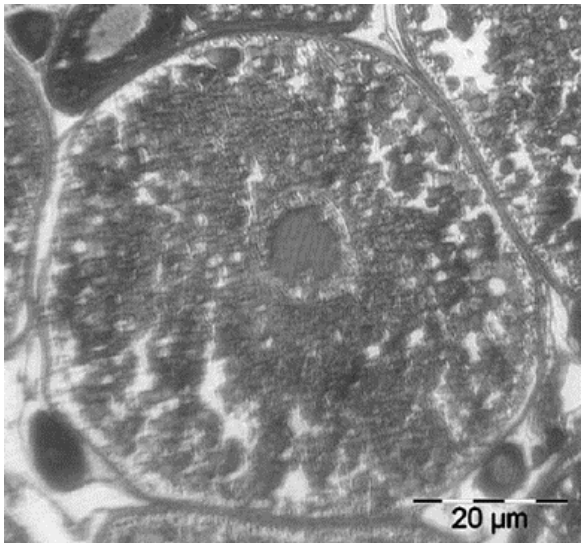
Figure 1: Oocytes of zebrafish (*Danio rerio*)



Non-fertilized oocytes of zebrafish (*Danio rerio*) in stage I (primary growth; left) and stage II (cortical alveolus; right).

Given its structure, the zebrafish chorion should be permeable to water, electrolytes and smaller hydrophilic organic substances (Hisaoka, 1958; Hisaoka and Firlit, 1960). In fact, water permeability strongly depends on the age of the oocytes: After fertilization and a subsequent phase of water hardening, the permeability for water rapidly declines (Hagedorn et al., 1997a). Correspondingly, as early as 1 h after fertilization, zebrafish eggs show a significantly reduced sensitivity to effluents (Gellert and Heinrichsdorff, 2001). Using deuterium-labelled water, embryos up to the 2-cell stage were found to take up water much more slowly than subsequent stages, i.e. water permeability is significantly increased after water hardening of the chorion (Adams et al., 2005).

Figure 2: Zebrafish (*Danio rerio*) oocytes in maturation



Non-fertilized zebrafish (*Danio rerio*) oocyte in maturation stage III (vitellogenesis). The chorion consists of three layers and starts to attenuate.

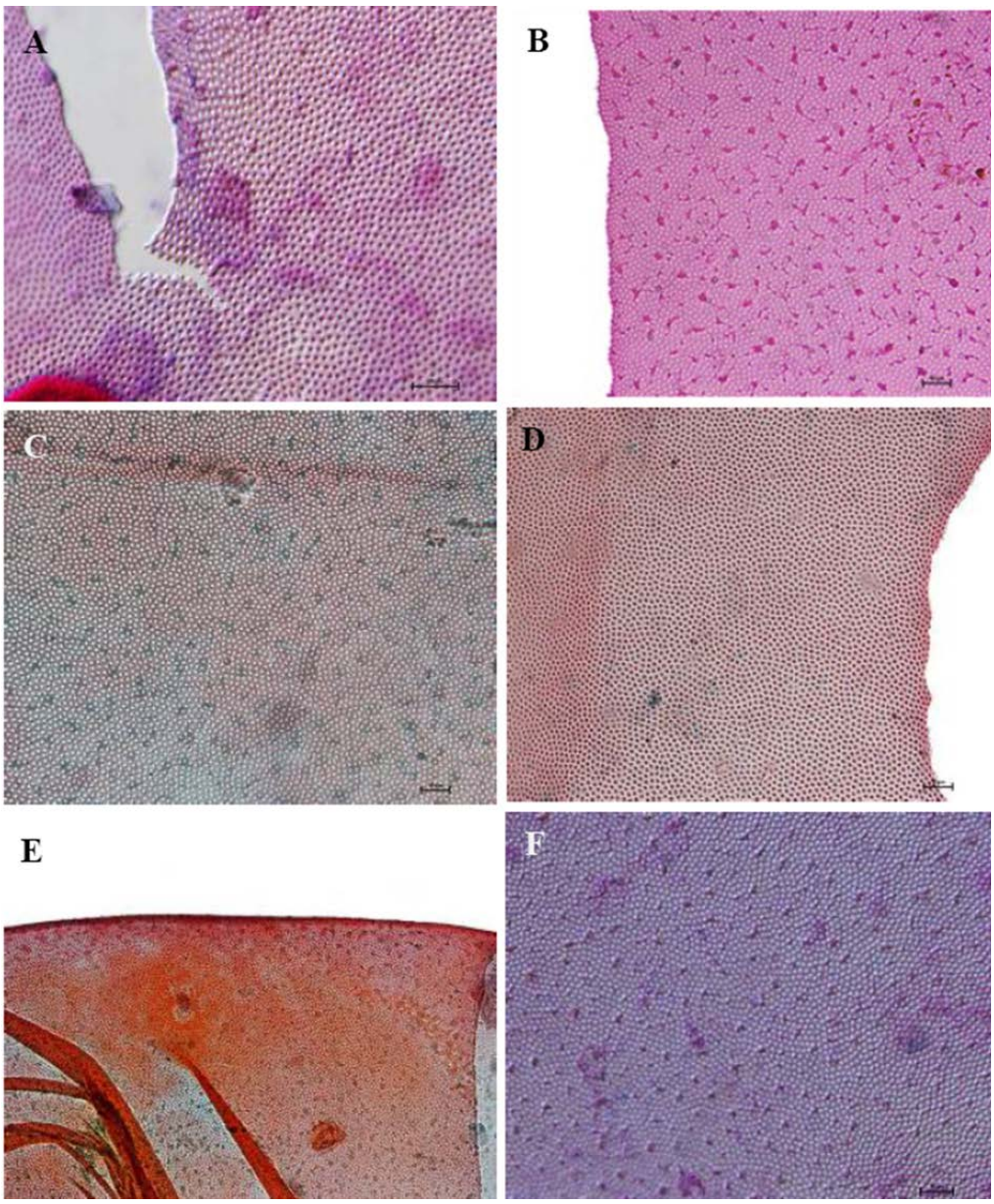
As a consequence of hardening, the mechanical stability of zebrafish eggs also increases: Penetration of the chorion by means of a micropipette requires much higher forces in the pharyngula stage than in the gastrula stage, and as a blastula, the egg is more difficult to penetrate than before spawning. (Kim et al., 2005). Overall, the mechanical resistance of the zebrafish egg declines with development. Artificial softening of the chorion can easily be effected by application of proteolytic pronase (chorionase), an enzyme released immediately before the natural onset of the hatching process (Kim et al., 2005). During normal embryo development, chorionase is secreted by hatching gland cells located in a belt-like arrangement along the anterior side of the yolk sac (Inohaya et al., 1997; Schoots et al., 1982). Since chorionase does not digest the outermost layers of the chorion, premature hatching in consequence of chorionase secretion by adjacent oocytes is prevented.

There is a controversial discussion about the permeability of the zebrafish chorion for organic compounds: Whereas Zhang und Rawson (1996) documented the chorion to be freely permeable for water and “small” molecules, the free permeability has been questioned for later developmental stages (Adams et al., 2005; Harvey et al., 1983). Likewise, the relative contribution to a potential barrier function of the chorion itself and the perivitelline space is under discussion (Harvey et al., 1983; Zhang and Rawson, 1996). Finally, the syncytial layer of the yolk has also been discussed with respect to its contribution to a barrier function (Hagedorn et al., 1998; Hagedorn et al., 1997b).

1.3 Structure of the zebrafish chorion

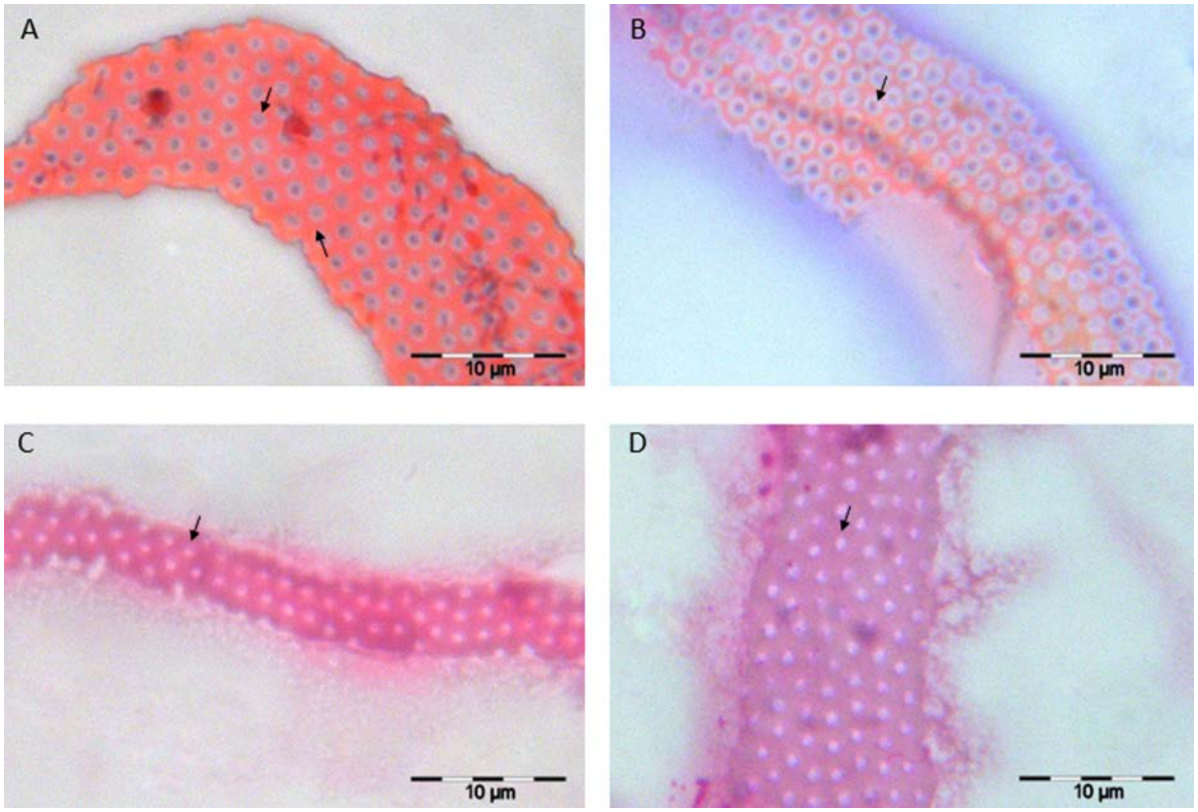
Whether or not the pores are exclusively responsible for the transfer of materials (chemicals) across the chorion is not clear. The spacing of pores on the zebrafish chorion is quite regular (Figs. 3, 4). In the electron microscope, the pores can be visualized as prominent structures across the multi-layered chorion (Fig. 5). In the scanning electron microscope, the surface of the zebrafish chorion displays various depressions and protrusions (Fig. 6). In the scanning electron microscope, broken edges and inner surface views of the chorions document that the pore canals are obstructed close to the outer surface of the chorion (Figs. 6 - 9). For more details, see Fedderwitz (2008).

Figure 3: The zebrafish chorion



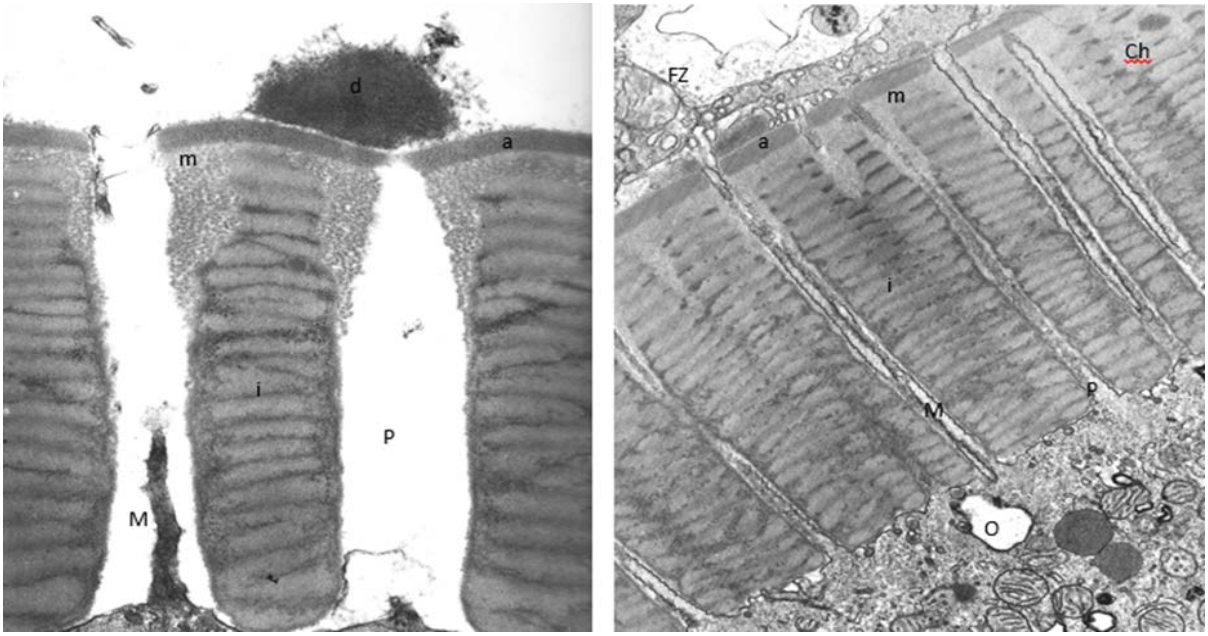
Preparation of the zebrafish chorion: A) the chorion of a 48 hpf embryo was torn apart along the pores (Alcian blue- Periodic acid-Schiff (PAS) stain, 40×); B) outer surface of the chorion with distinctive projections brightly colored (hematoxylin eosin (HE) stain, 60×); C) outer chorion surface of a 72 hpf zebrafish embryo (Masson-Goldner, 60×); D) inner chorion surface of a 72 hpf zebrafish embryo (Masson-Goldner, 60×); E) micropyle (Masson-Goldner, 40×); F) outer chorion surface (Alcian blue-PAS-stain, 60×). From Böhler (2012).

Figure 4: Surface of the chorion of zebrafish (*Danio rerio*) eggs



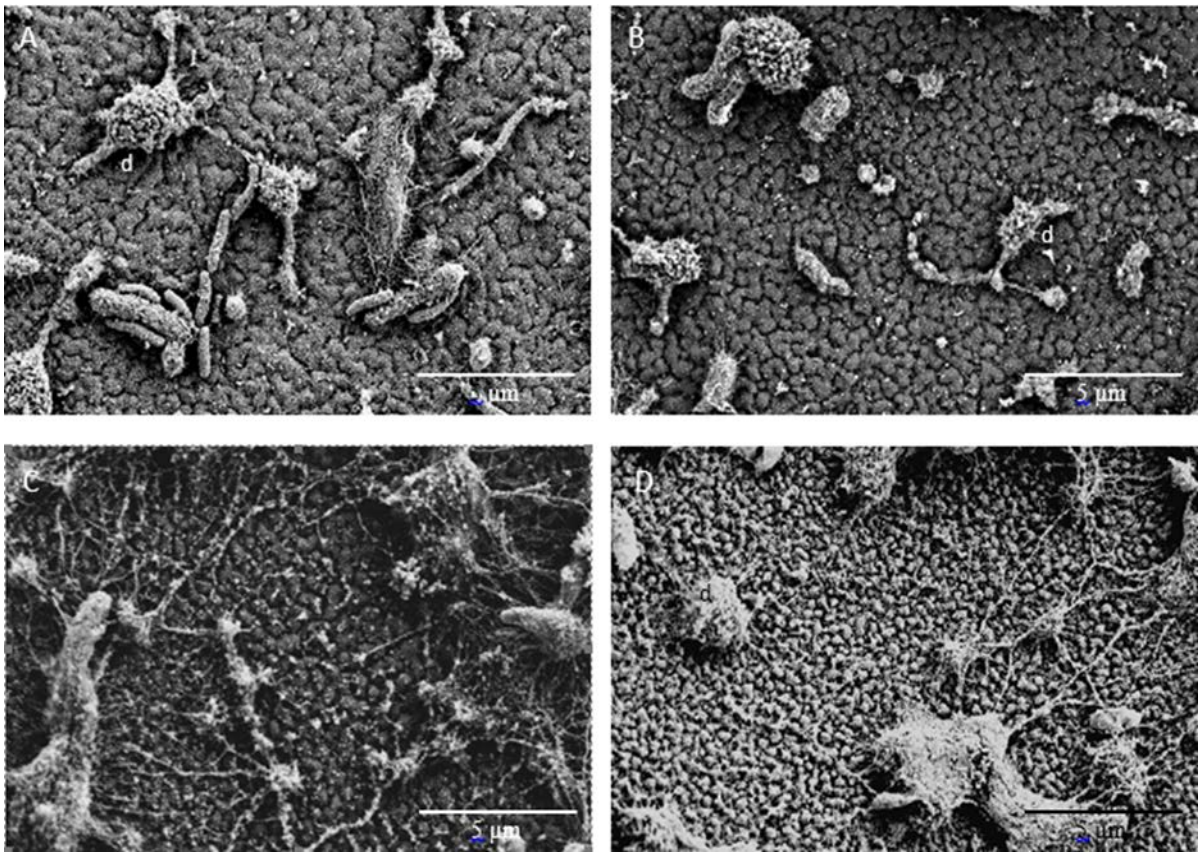
Surface views of the chorion of zebrafish (*Danio rerio*) eggs at 24 h after fertilization clearly show the regular dispersion of the chorion pores (→). Staining techniques: A: Goldner, B. Azan, C: HE, D: PAS. From Fedderwitz (2008) and Braunbeck et al. (2012).

Figure 5: Layers of the zebrafish (*Danio rerio*) chorion



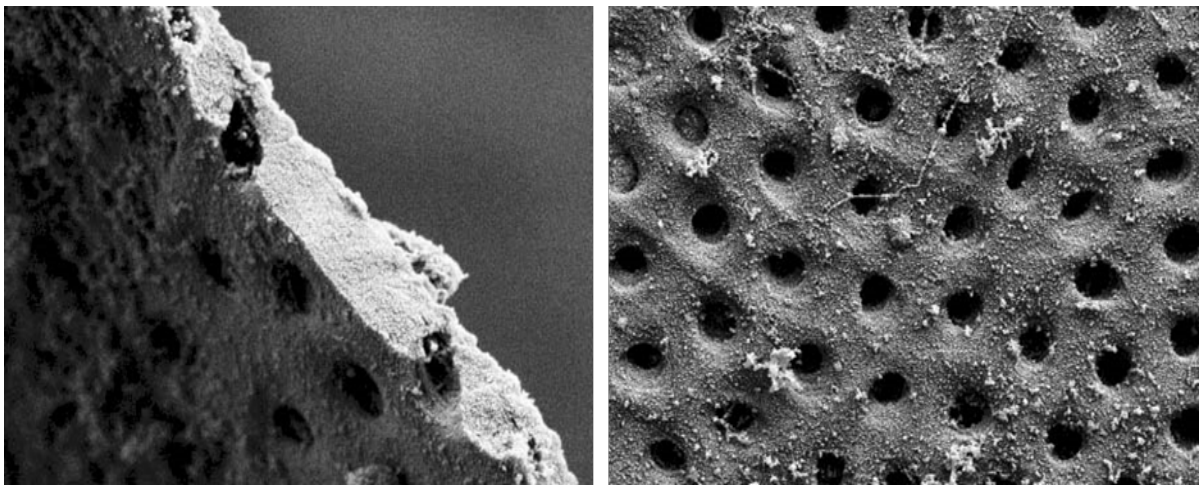
The multi-layered zebrafish (*Danio rerio*) chorion (Ch) shows multiples pores (P), which are penetrated by microvilli (M) both from the follicular cells (FZ) and the oocyte (O). a: outer apical layer; m: intermediate layer; i: inner layer; d: dense material. Magnification left: 20,000×; right: 6,600×. From Fedderwitz (2008) and Braunbeck et al. (2012).

Figure 6: Surface of the zebrafish (*Danio rerio*) chorion



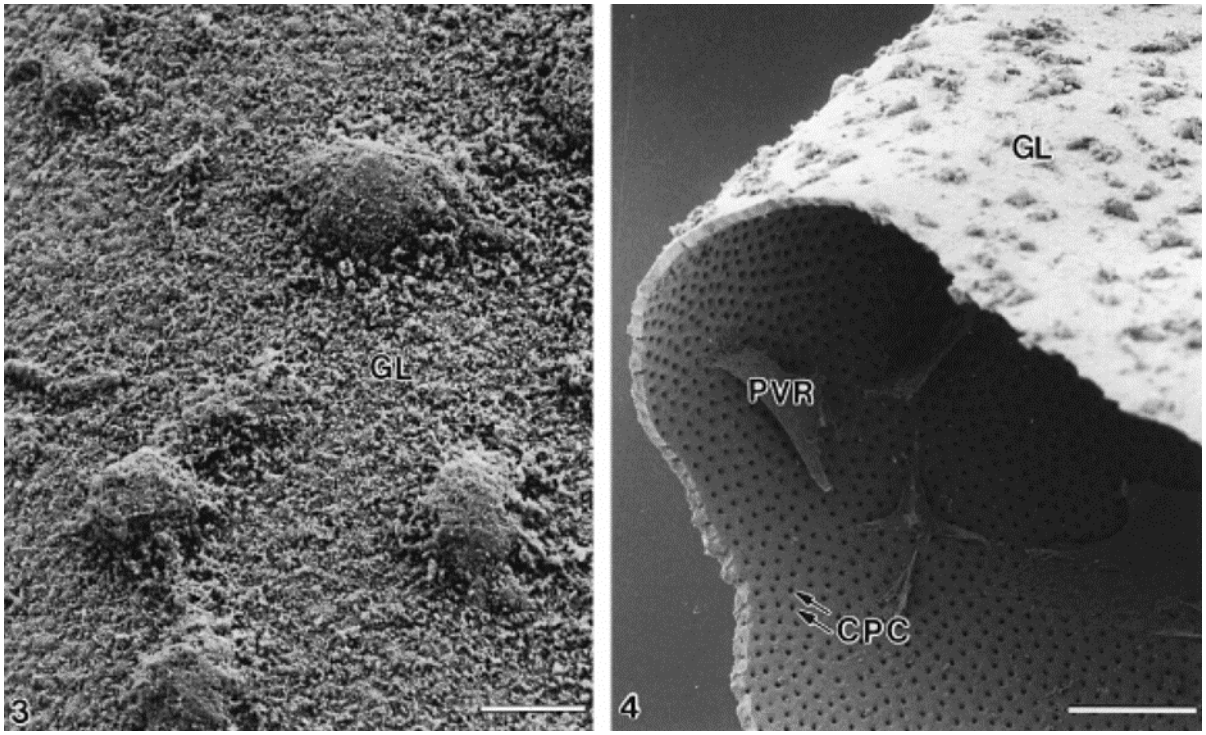
Outer surface of the zebrafish (*Danio rerio*) chorion in the scanning electron microscope: 12 h after fertilization, the surface displays shallow indentations as well as larger shallow protrusions. 60 h after fertilization, small bubble-like protrusions are visible. A: 12 h; B: 24 h; C: 48 h; D: 60 h. d: electron-dense materials. From Fedderwitz (2008) and Braunbeck et al. (2012).

Figure 7: Inner surface of the zebrafish (*Danio rerio*) chorion



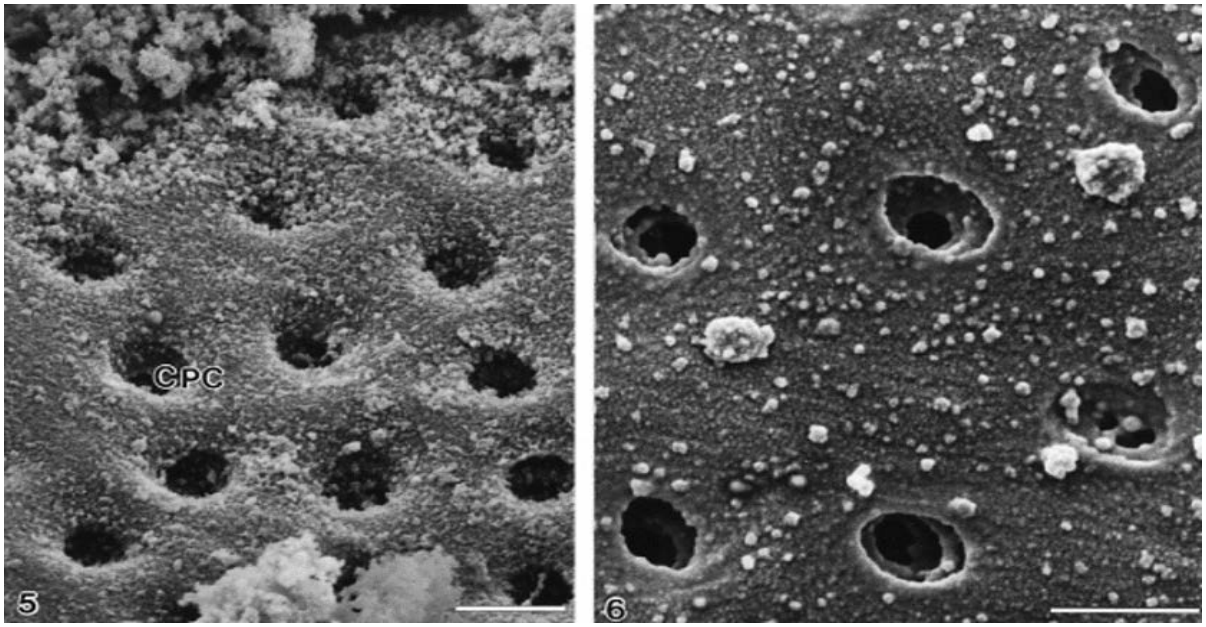
Along broken edges (left) and from the inner surface (right) of the zebrafish (*Danio rerio*) chorion, chorion pores become evident at regular spacing as canals that do not completely penetrate the chorion, but are plugged close to the outer surface. From Fedderwitz (2008).

Figure 8: Granular layer of the zebrafish (*Danio rerio*) chorion



Left: Field-emission scanning electron microscopy of the outermost granular layer (GL) of the zebrafish (*Danio rerio*) chorion with distinct projections (2.0 – 3.0 µm in diameter) at intervals of 2 – 10 µm. Bar: 2 µm. Right: Field-emission scanning electron microscopy view of the outer chorion surface, with the granular layer (GL) and inner surface with openings of the chorion pore canals (CPC). At a few places, remnants of materials from the perivitelline space (PVR) are attached to the inner side of the chorion. Bars: 10 µm. From Rawson et al. (2000).

Figure 9: Pore canals in the chorion of zebrafish (*Danio rerio*)

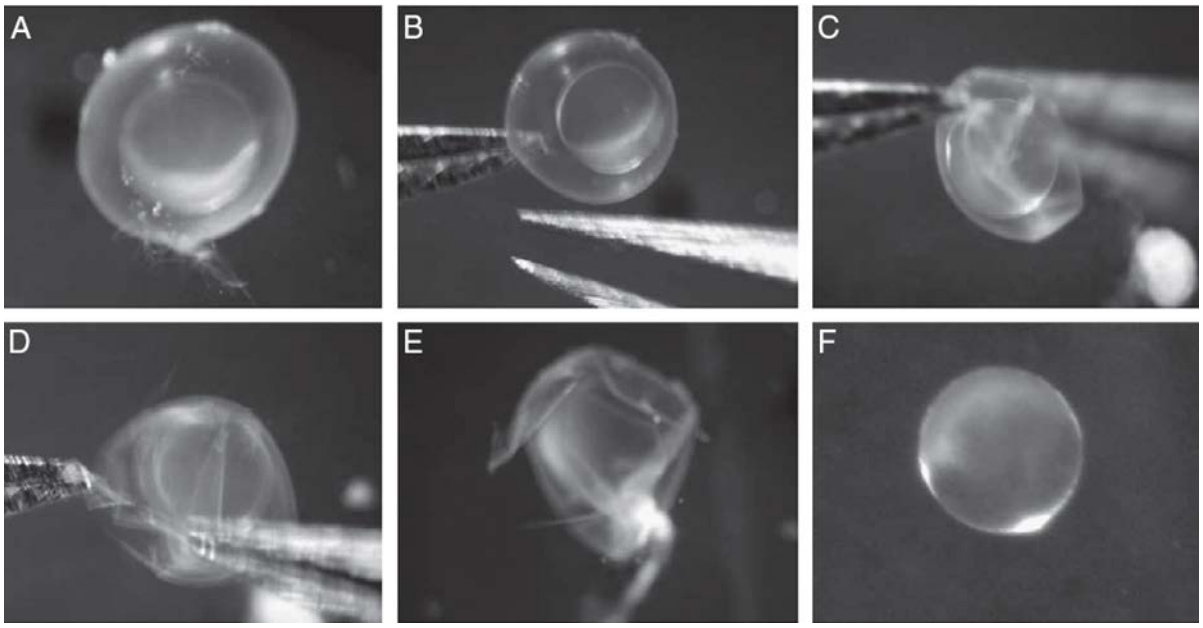


Field-emission scanning electron microscopy of the openings of the pore canals in the chorion of zebrafish (*Danio rerio*). Bars: 1 µm. From: Rawson et al. (2000).

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

The most obvious approach to investigate the role of the chorion as a barrier for chemical toxicity is to experimentally 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 (Fig. 10). For the specific purposes of these studies, it seemed sufficient to select undamaged embryos or embryonic cells for further operations. In none of the studies mentioned above, however, the actual survival rates of dechorionated embryos were given special attention and 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 (Leonard et al., 2005).

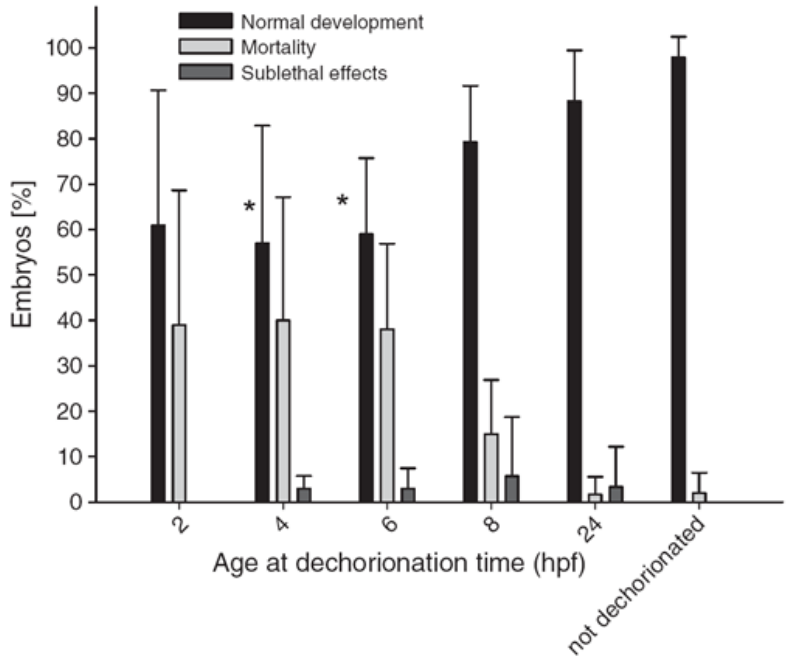
Figure 10: Dechorionation of zebrafish (*Danio rerio*) embryos



Mechanical dechorionation of zebrafish (*Danio rerio*) embryos using Dumont™ 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; and F: dechorionated embryo. Average diameter of the embryo with chorion: 0.7 mm.

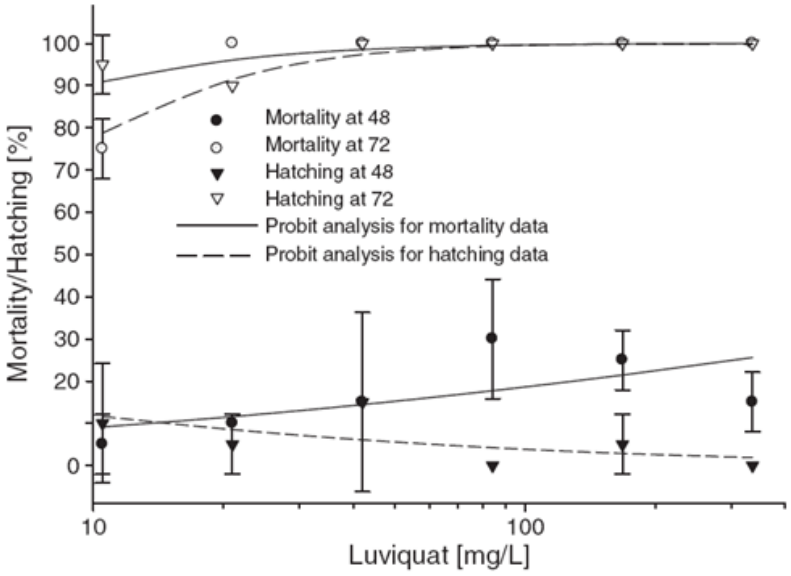
After some optimization, zebrafish embryos could be dechorionated from an age of 24 h after fertilization (Henn and Braunbeck, 2011) with satisfactorily high survival rates (Fig. 11). For the model high molecular weight substance Luviquat, it could be demonstrated that access of high molecular weight molecules could be given access to the zebrafish embryo by means of dechorionation (Figs. 12, 13).

Figure 11: Correlation of zebrafish (*Danio rerio*) age and survival after dechorionation



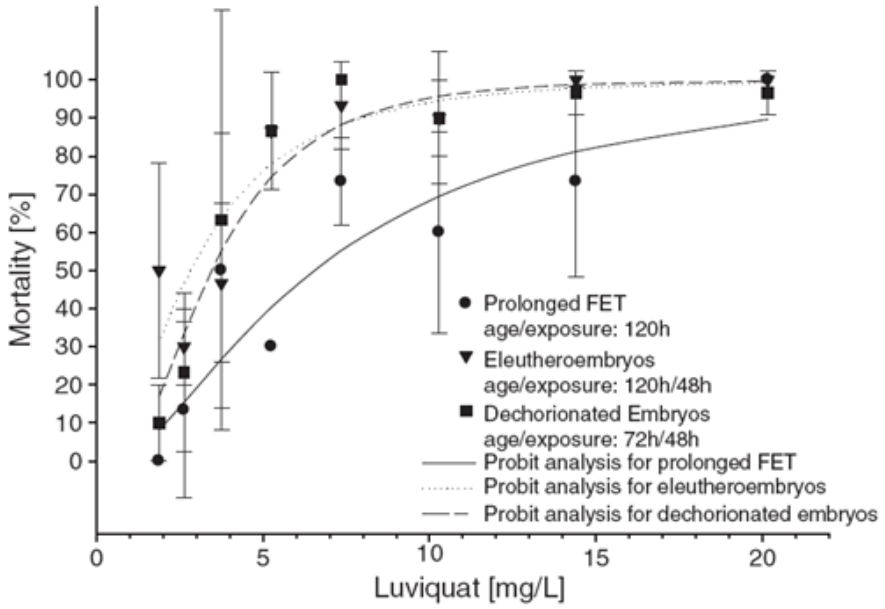
Correlation between the age of zebrafish embryos at time of dechorionation and the ratio of normal development versus mortality as well as sublethal effect rates on the second day after dechorionation. For comparison, data for non-dechorionated embryos have been added.*Results statistically different compared to the group dechorionated 24 hpf (Dunn's method, $p < 0.05$). From Henn and Braunbeck (2011) and Braunbeck et al. (2012).

Figure 12: Mortalities of non-dechorionated zebrafish (*Danio rerio*) embryos after exposure to Luviquat



Mortalities of non-dechorionated zebrafish (*Danio rerio*) embryos (—) after 48 (●) and 72h (○) and corresponding hatching rates (- - -) after 48 h (▼) and 72 h (▽) of exposure to Luviquat. From Henn and Braunbeck (2011) and Braunbeck et al. (2012).

Figure 13: Mortalities of dechorionated and non-dechorionated zebrafish (*Danio rerio*) embryos after exposure to Luviquat



Comparison of mortalities after 120 h of exposure to Luviquat in non-dechorionated zebrafish (*Danio rerio*) 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; ■). From Henn and Braunbeck (2011) and Braunbeck et al. (2012).

For more details and a detailed protocol for zebrafish dechorionation with reproducibly high survival rates, see Henn and Braunbeck (2011).

1.5 Fluorescent dyes as markers for the permeability of the chorion in zebrafish (*Danio rerio*) embryos

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 el al. 2001) and might then function as a barrier for even smaller molecules. Ozoh (1980) found dechorionated zebrafish embryos to be more sensitive against copper intoxication than non-dechorionated embryos, which corroborated several other studies with different teleost species showing accumulation of different heavy metals on the outside or within the chorion (Stouthart et al., 1994; Van Leeuwen et al., 1985; Wedemeyer, 1968). Only few studies indicated a weak barrier function of the zebrafish chorion for lipophilic substances such as γ-hexachlorocyclohexane (lindane) (Braunbeck et al., 2005) and small substances such as dimethyl sulfoxide (DMSO) (Harvey et al., 1983). In other teleost species like the medaka (*Oryzias latipes*), the same seems to hold true for substances like cypermethrin and thiobencarb (Gonzalez-Doncel et al., 2004; Villalobos et al., 2000).

An essential aspect of the permeability of the fish egg chorion is the molecular weight. Polymers and high molecular weight surfactants (~ 40,000 – 100,000 g/mol) were suspected to be blocked by the chorion, since comparison between embryo, eleutheroembryo toxicities (Leonard et al., 2005) and dechorionated embryos resulted in considerably higher sensitivity of the latter both (Henn and Braunbeck, 2011). Creton (2004) investigated the inhibition of the endoplasmic reticulum (ER) Ca2+ pump in zebrafish embryos and established that the chorion is permeable to fluorescent dextrans of 3000 Da, but is not permeable to fluorescent dextrans of 10,000 Da. To identify a “critical molecular size” for the passage across the chorion, polymers are ideal test

substances: 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 that might interact with the chorion are introduced. Due to the size evidences of between 3 kDa and 10 kDa (Creton, 2004), Kais (2009) tested fluorescent dextran between 3 and 40 kDa with respect to their ability to pass the chorion.

The use of fluorescence dyes allows the visualization of test substances and, thus, their uptake into the egg as well as its accumulation can be followed. Wavelength and amount of the emitted energy depend on both the fluorophore and the chemical environment of the fluorophore (Tsien et al., (eds) 1995). The two well-known classes of highly fluorescent dyes, rhodamines and fluoresceins, are derivatives of xanthenes and belong to heterocyclic compounds. In addition to their similarity to PAHs, rhodamine and fluorescein are ecologically relevant by themselves. Uranin is the water-soluble sodium salt of fluorescein and has been used in river systems, most notably in the case of the Chicago River, where fluorescein was the first substance used to dye a river green on St. Patrick's Day in 1962. In industrial applications, it is used to color automotive coolants (anti-freeze), for dying wool and silk, as an Air-Sea rescue marker, as ground water tracing dye, soap solutions and for the coloring of agrochemicals and fertilizers. Rhodamines are used as dyes for paper and textiles, in luminous pigments, in dye lasers, as well as in cell biology, where they have served as markers in fluorescence microscopy.

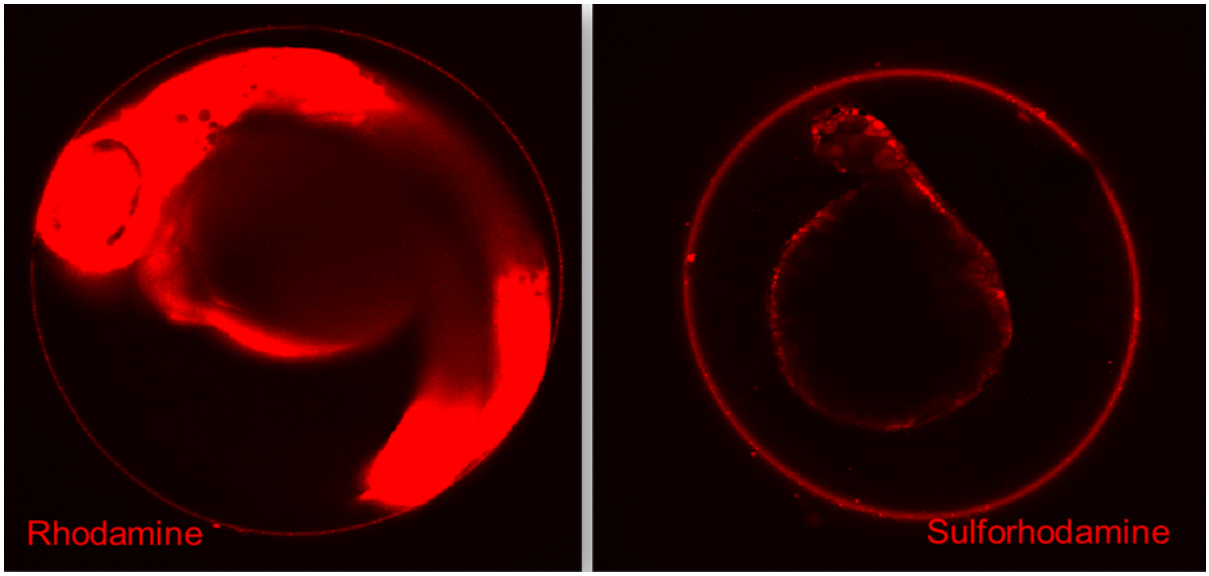
Apart from water solubility and partition coefficient, other parameters have to be considered, e.g. Topological Polar Surface Area (TPSA), which is an estimation of the polar fraction of the molecular surface area. Similar to the physicochemical parameters such as the influence of the particular structure and its complexity, it affects the environmental impact and toxicological potential. The complexity rating of the compounds is a rough estimate of how complicated a structure is in terms of both elements and the displayed structural features including symmetry. Generally, larger compounds are more complex than smaller ones, but highly symmetrical compounds or compounds with few distinct atom types or elements are downgraded.

The diploma thesis by Kais (2009) was designed to investigate the mechanism(s) of uptake and distribution of fluorescent dyes with different chemical characteristics in the zebrafish embryo. Lipophilicity, molecular weight, different substituents, as well as different charging are important issues for ecotoxicological questions. Rhodamine b and sulforhodamine b were representatives for good water solubility and low log POW. Fluorescein is a small molecule and is regarded as a basic molecule. This study investigates how the chemical behavior of fluorescein might change with the insertion of two chlorine atoms or with an additional carboxyl group. Embryos were studied after 24 h and 48 h in the standard fish embryo toxicity test (FET). The time between 26 h and 48 h (called pre-hatching time) is especially important for the question of distribution due the fact that the structure of the chorion profoundly changes during this developmental period. Experimental results provide evidence of massive quantitative changes in the uptake and distribution following the chorion softening process, which is mostly due to the proteolytic activity at the pre-hatching stage (Kim et al., 2005).

In fact, fairly water-soluble molecules of lower molecular weight such as rhodamine rapidly penetrate the zebrafish chorion and accumulate within the embryo (Fig. 14). Although only of slightly higher molecular weight (580 versus 479) and an even lower KOW value (1.44 versus 2.28), sulforhodamine is transferred much more slowly than rhodamine, most likely due to (1) the overall negative charge of sulforhodamine and (2) the bulky nature of the two carboxyl groups (Fig. 14). In addition to molecular size and charge, exposure time is a critical factor for the transfer of substances across the zebrafish chorion (Fig. 15). Most importantly, solvents with detergent-like properties such as dimethyl sulfoxide massively facilitate the transfer of

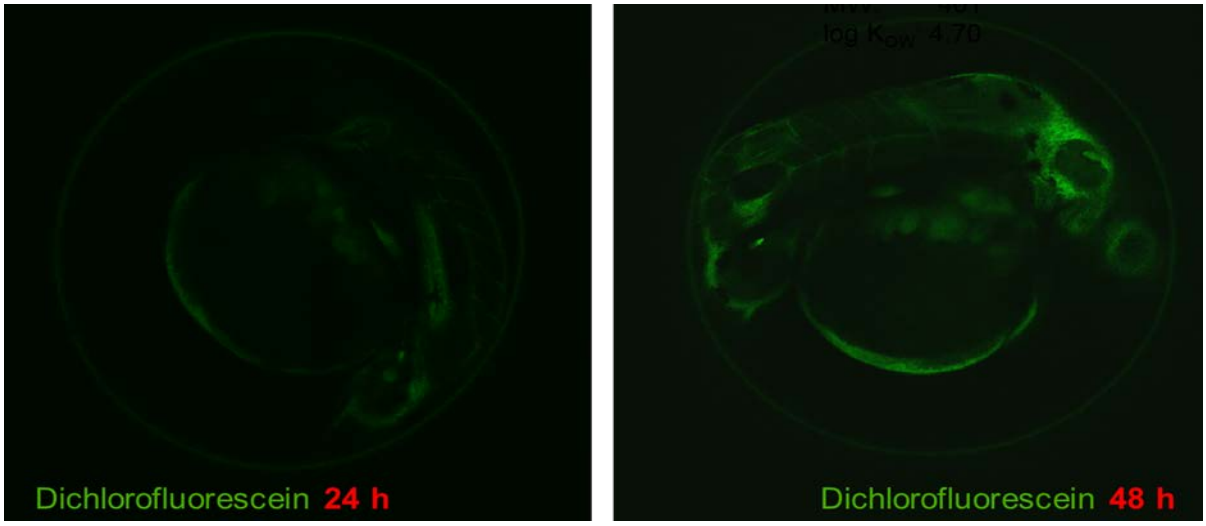
substances with moderate to higher lipophilicity such as fluorescein (Fig. 16). However, it should be noted that DMSO also supports the uptake of markers into dechorionated embryos, i.e. the solvent not only modifies the properties of the chorion, but also of the zebrafish embryo itself (Fig. 17). For more details, see Kais (2009) and Kais et al. (2013).

Figure 14: Rhodamine and sulforhodamine transport across the zebrafish (*Danio rerio*) chorion



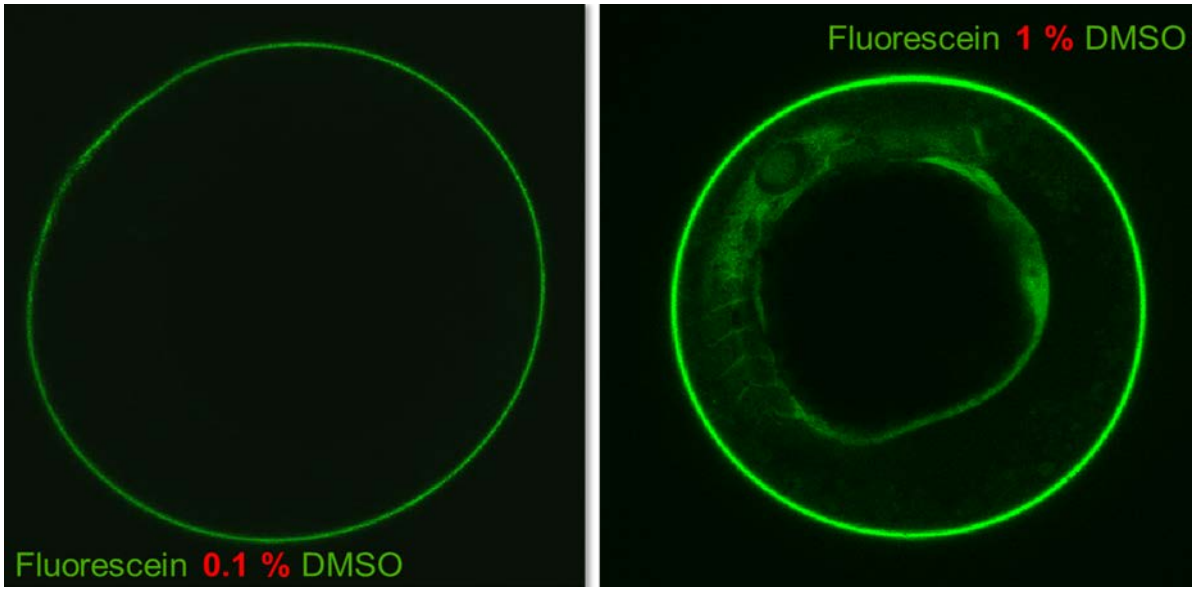
Rhodamine and sulforhodamine transport across the zebrafish (*Danio rerio*) chorion after 48 h water-borne exposure: Since the accumulation of rhodamine inside the embryo by far exceeds that of sulforhodamine, there is evidence that positively charged molecules show lower penetration rates across the zebrafish chorion. From Kais (2009).

Figure 15: Time-dependent passage of the fluorescent marker dichlorofluorescein across the zebrafish (*Danio rerio*) chorion



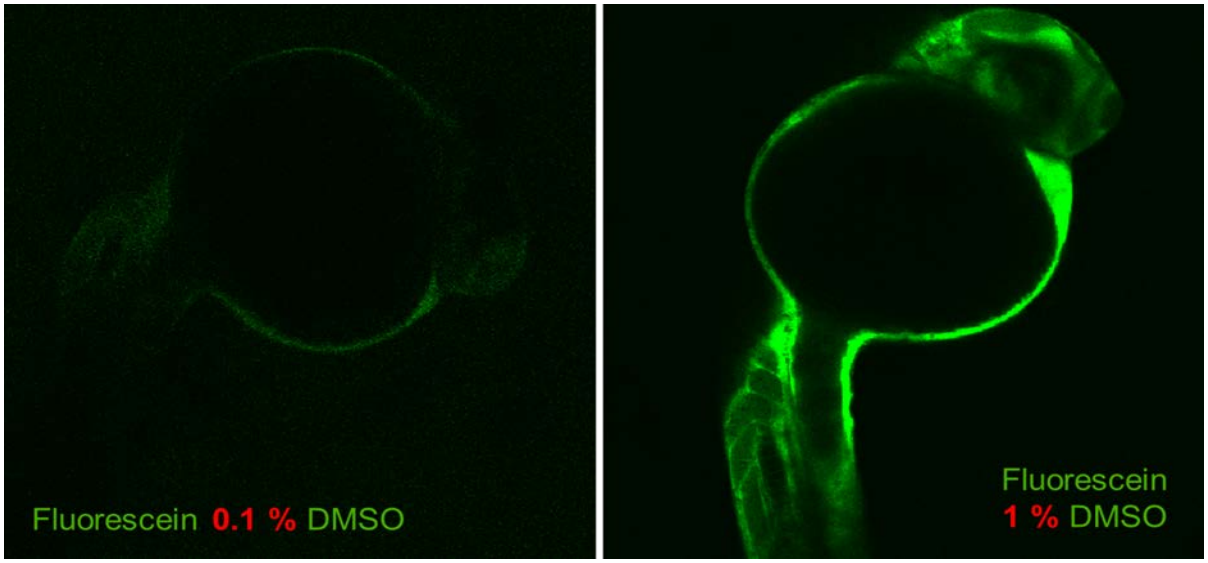
Prolonged exposure to the fluorescent marker dichlorofluorescein increases the transfer across the zebrafish (*Danio rerio*) chorion and the accumulation inside the embryo, thus documenting the time-dependence of the passage of materials across the chorion. From Kais (2009) and Braunbeck et al. (2012).

Figure 16: Impact of DMSO on substance transfer across the chorion of zebrafish (*Danio rerio*)



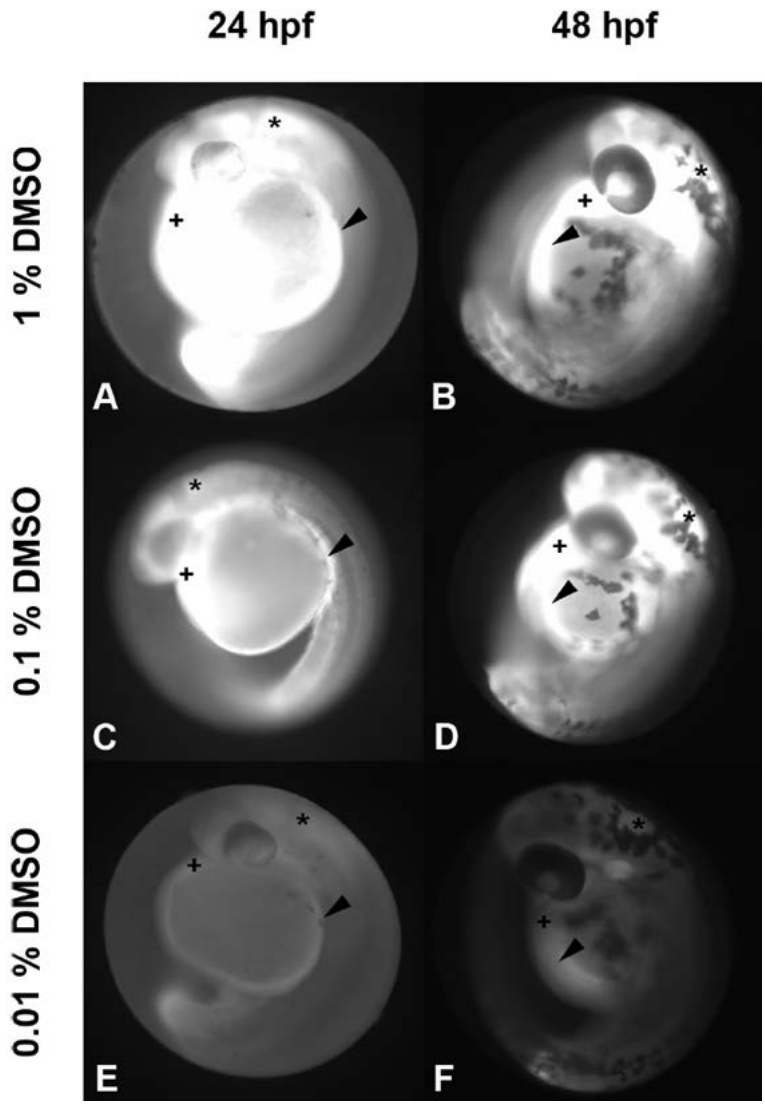
Increased concentrations of the solvent (detergent) dimethyl sulfoxide (DMSO) facilitate the uptake of fluorescent markers such as fluorescein across the chorion of the zebrafish (*Danio rerio*) embryo. The use of detergent may thus be a tool to facilitate / improve the passage of materials across the fish chorion. From Kais (2009) and Braunbeck et al. (2012).

Figure 17: Impact of DMSO on substance uptake into zebrafish (*Danio rerio*) embryos



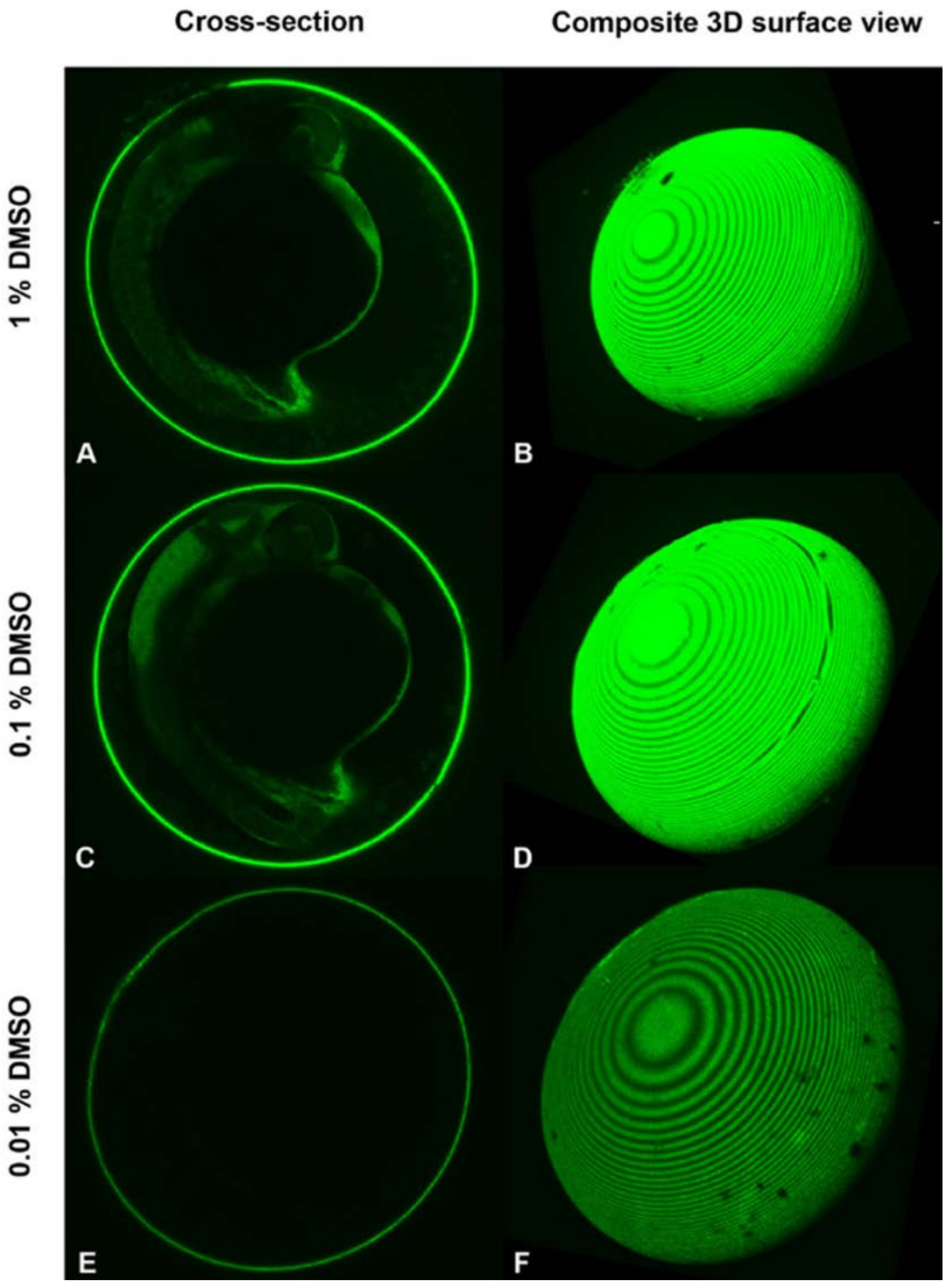
DMSO strongly increases the uptake of the fluorescent marker fluorescein into dechorionated zebrafish (*Danio rerio*) embryos, ie. the solvent not only modifies the properties of the chorion (cf. Fig. 16), but also of the zebrafish embryo itself. From Kais (2009) and Braunbeck et al. (2012).

Figure 18: Epifluorescence images of zebrafish (*Danio rerio*) embryos



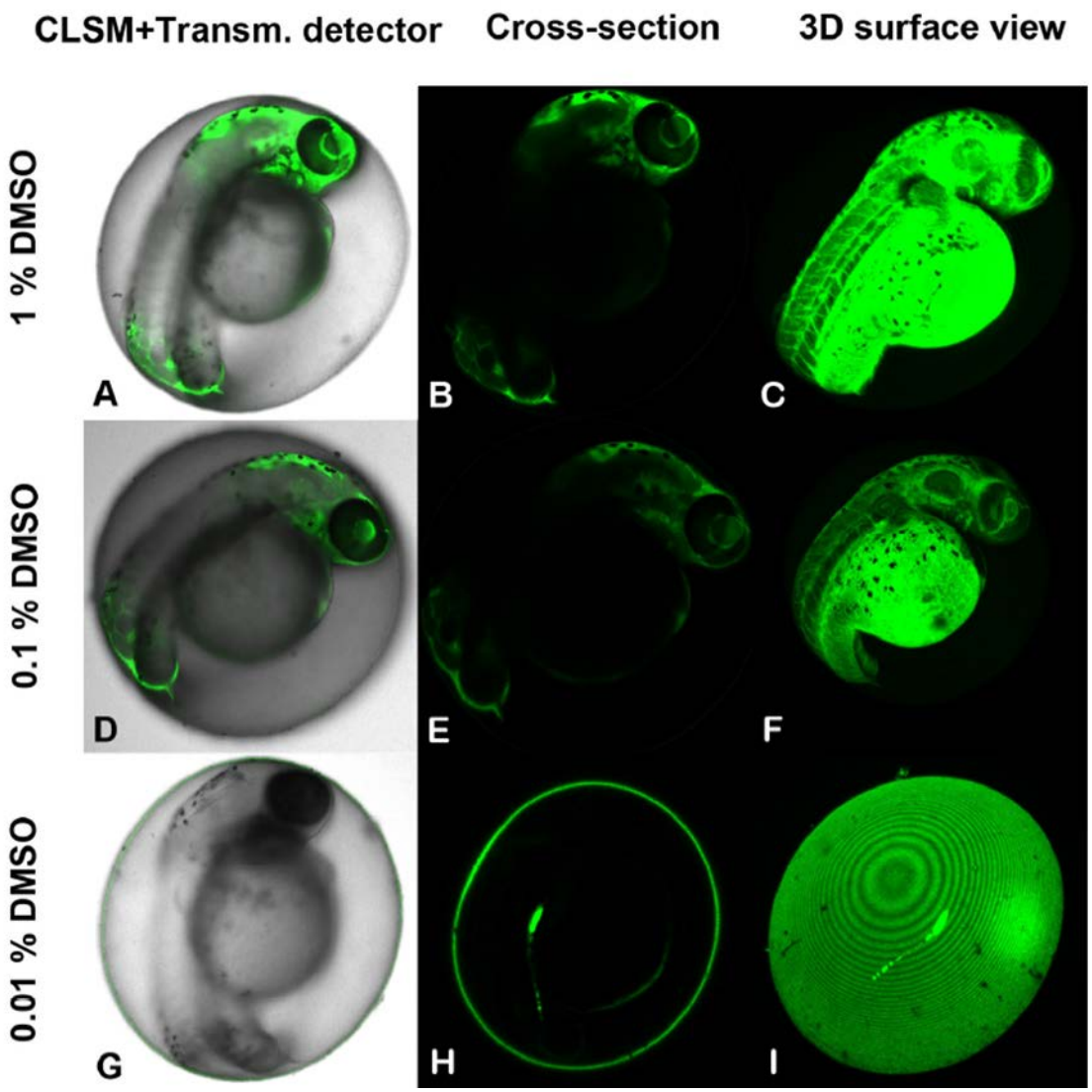
Epifluorescence images of zebrafish (*Danio rerio*) embryos at the age of 24 hpf (A, C, E) and 48 hpf (B, D, F) exposed to 100 mg/L fluorescein dissolved in 1% (A, B), 0.1% (C, D) and 0.01% DMSO (E, F): With 0.1 and 1% DMSO, fluorescein showed a strong signal inside the embryo (A–D); especially in the common cardiac vein (=duct of Cuvier) (►) including the heart (+) and vessels inside the brain (*). In contrast, with 0.01% DMSO, fluorescein fluorescence inside the embryo was weak, but still quite high (E and F). In the embryo, fluorescence intensity increased with DMSO concentration (A > C > E). Exposure time: 30 ms (24 hpf) and 2 ms (48 hpf) (Kais et al., 2013).

Figure 19: Cross-sections and surface images of zebrafish (*Danio rerio*) embryos



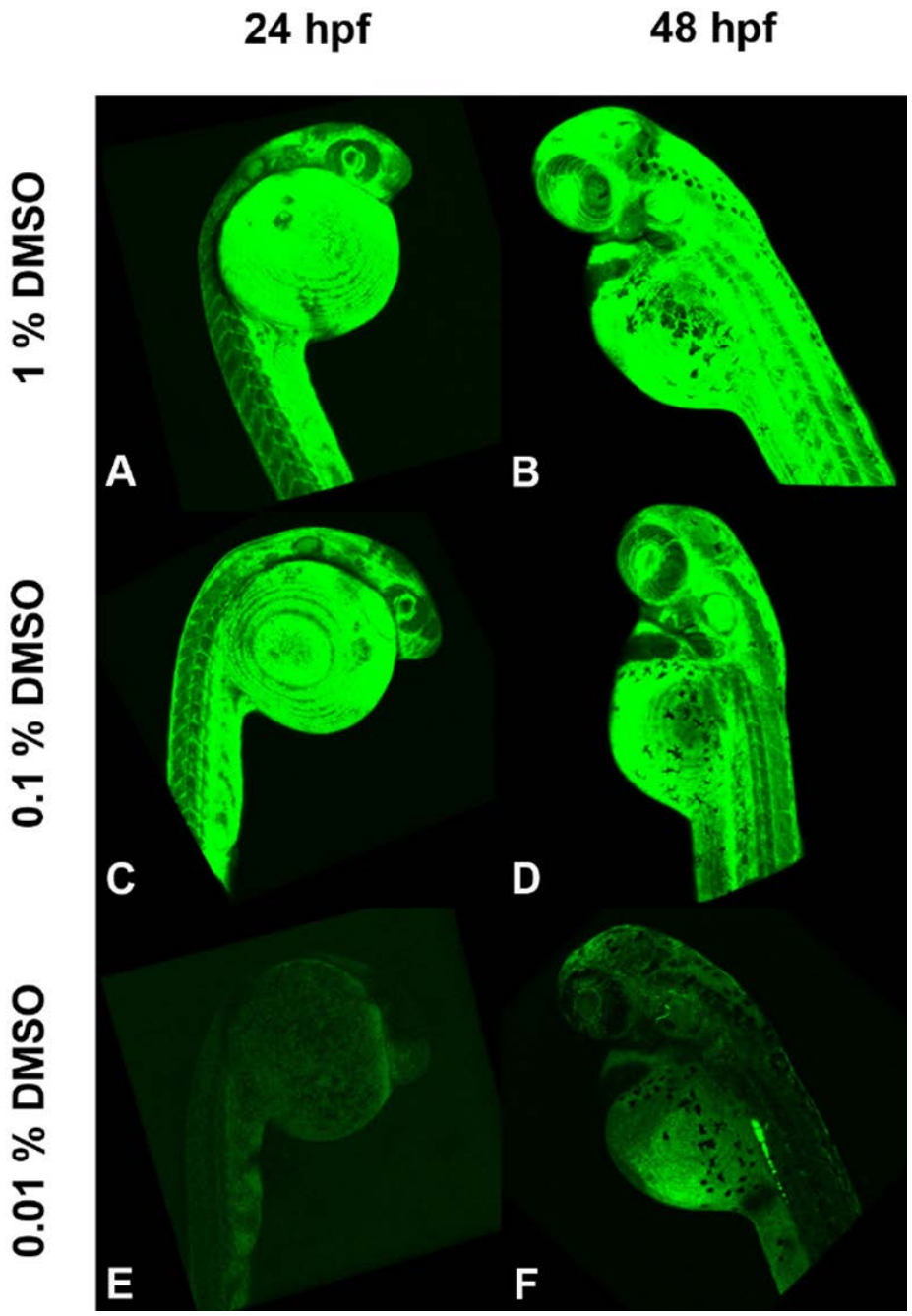
Cross-sections (A, C, E) and composite three-dimensional surface (B, D, F) confocal laser scanning images of zebrafish (*Danio rerio*) embryos at the age of 24 hpf after exposure to 100 mg/L fluorescein dissolved in 1% (A and B), 0.1% (C and D) and 0.01% DMSO (E and F): With 0.1 and 1% DMSO, fluorescein passed the chorion and accumulated within the embryo (A and C); in contrast, with 0.01% DMSO, fluorescein did not pass the chorion, and no signal in the embryo was detectable (E). All images were taken at 100% laser power and a high voltage setting of 20. From Kais et al. (2013) and Braunbeck et al. (2012).

Figure 20: Transmission, cross-section and 3D images of zebrafish (*Danio rerio*) embryos



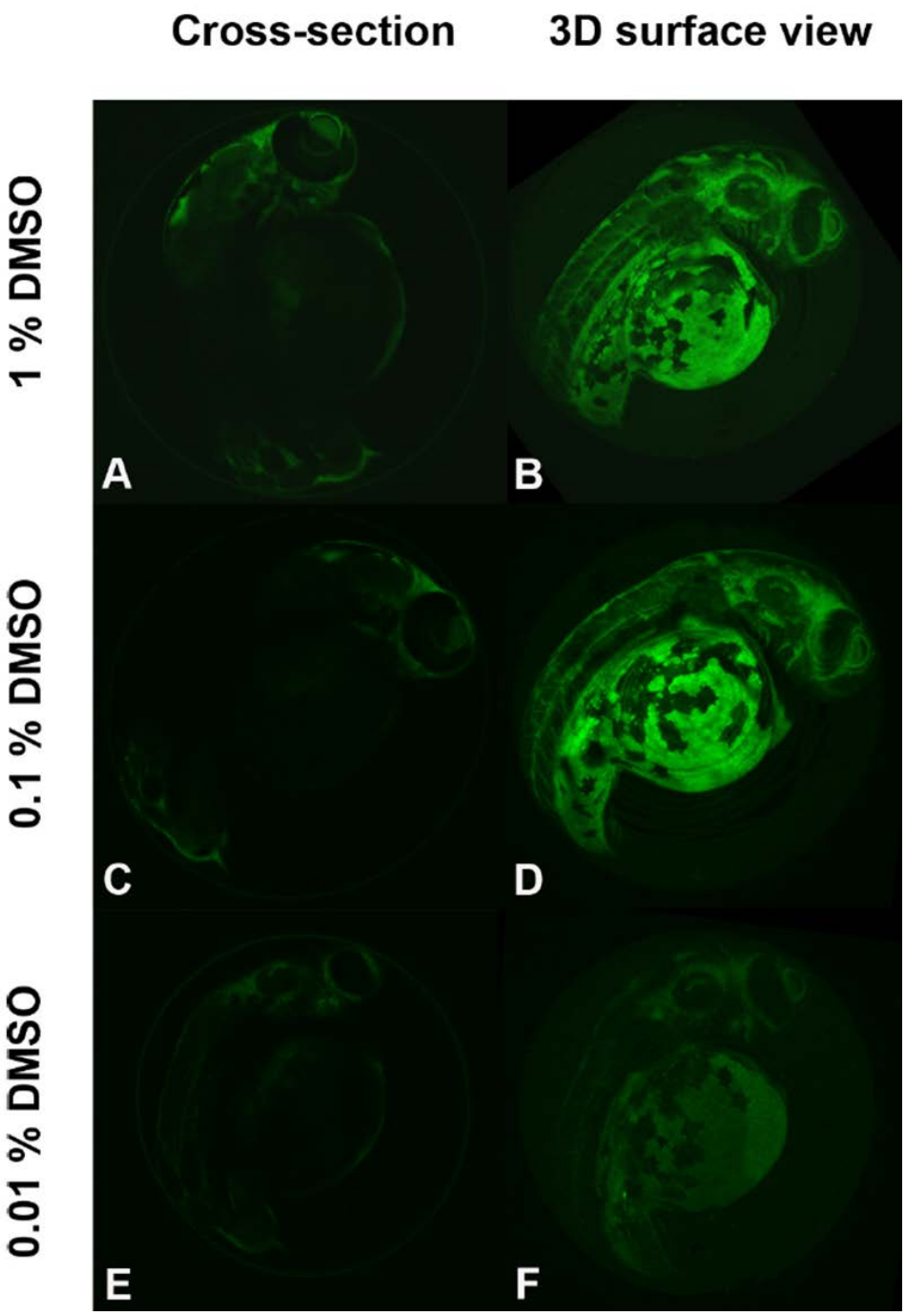
Transmission detector (A, D, G) as well as cross-section (B, E, H) and 3D maximum intensity rendering (C, F, I) confocal laser scanning images of zebrafish (*Danio rerio*) embryos (48 hpf) after exposure to 100 mg/L fluorescein dissolved in 1 % (A - C), 0.1% (D - F) and 0.01 % DMSO (G - I): In 0.1 and 1 % DMSO, showed a strong signal inside in the embryo (A–F), which clearly outshined the signal of the chorion and a small portion of fluorescein in the intestinal lumen (G - I). Fluorescence intensity inside the embryo decreased with DMSO concentrations (C > F > I). Fluorescence images were taken at 100 % laser power and a high voltage setting of 1 (B, C, E, F) and 20 (H and I). From Kais et al. (2013) and Braunbeck et al. (2012).

Figure 21: Composite 3D surface confocal laser images of zebrafish (*Danio rerio*) embryos



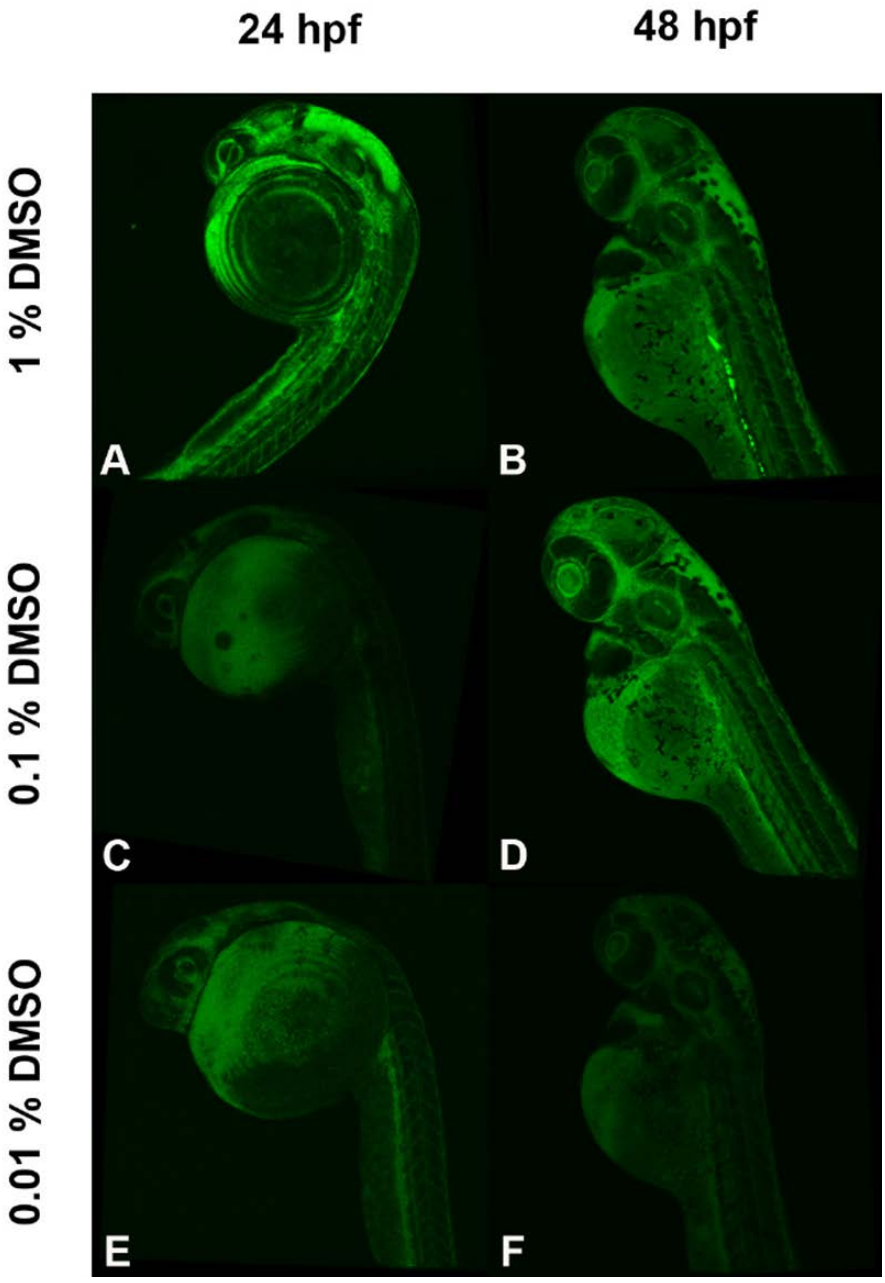
Composite three-dimensional surface (maximum intensity rendering) confocal laser images of zebrafish (*Danio rerio*) embryos, dechorionated after 24 h (A, C, E) and 48 h (B, D, F) exposure to 100 mg/L fluorescein dissolved in 1% (A and B), 0.1 % (C and D) and 0.01 % DMSO (E and F): With 0.1 and 1 % DMSO, fluorescein gave a strong signal inside the embryo (A - D); with 0.01 % DMSO, fluorescein fluorescence inside the embryo (E and F) and in a small section of the intestinal lumen (F) was weak. Fluorescence images of 24 hpf embryos were taken at full laser power and a high voltage setting of 20 (A), 30 (C) and 150 (E), whereas 48 hpf embryo images were taken at laser power of 5 (B), 3 (D) and 100 (F) and a high voltage setting of 15 (B and D) and 100 (F). From Kais et al. (2013) and Braunbeck et al. (2012).

Figure 22: Cross-section and composite 3D surface images of zebrafish (*Danio rerio*) embryos after exposure to 2,7-dichlorofluorescein I



Cross-section (A and C) and composite 3D surface (B, D - F) confocal laser images of zebrafish (Danio rerio) embryos at the age of 48 hpf after exposure to 50 mg/L 2,7-dichlorofluorescein in 1 % (A and B), 0.1 % (C and D) and 0.01 % DMSO (E and F): In 0.1 and 1 % DMSO, there was an increased fluorescence intensity of 2,7-dichlorofluorescein inside the embryo (A - D); 0.01 % DMSO, however, did not show a definite change in the signal strength (F). All images were taken at full laser power and a high voltage setting of 10. From Kais et al. (2013) and Braunbeck et al. (2012).

Figure 23: Cross-section and composite 3D surface images of zebrafish (*Danio rerio*) embryos after exposure to 2,7-dichlorofluorescein II



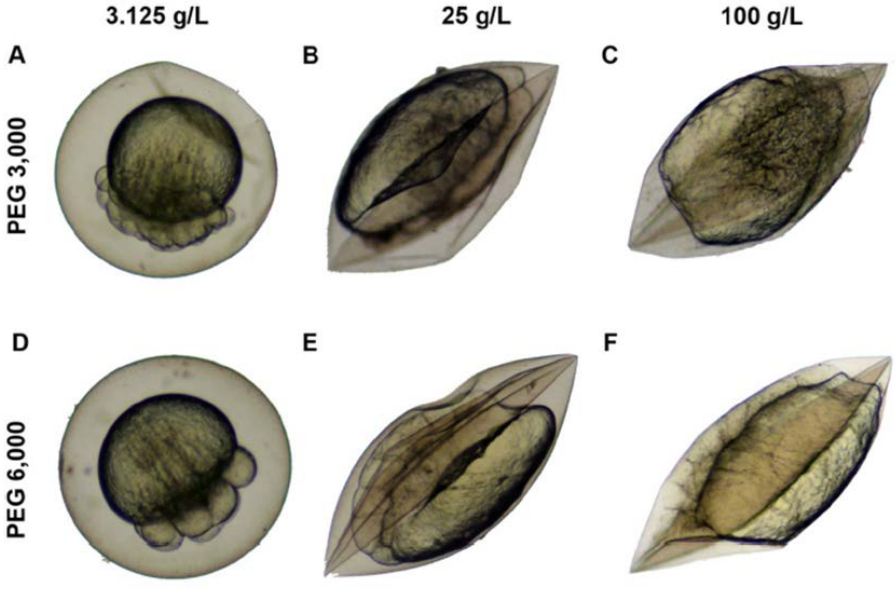
Composite three-dimensional surface confocal laser images of zebrafish (*Danio rerio*) embryos dechorionated after 24 h (A, C, E) and 48 h (B, D, F) exposure to 50 mg/L 2,7-dichlorofluorescein dissolved in 1 % (A and B), 0.1 % (C and D) and 0.01 % DMSO (E and F): Following exposure in 0.1 and 1 % DMSO, 2,7-dichlorofluorescein gave an increased signal inside the embryo (A - D), especially in the intestinal lumen. In contrast, with 0.01 % DMSO, fluorescence intensity was much lower (E and F). All fluorescence images were taken at full laser power and a high voltage setting of 50 (24 hpf) and 5 (48 hpf) (Kais et al., 2013) and Braunbeck et al. (2012).

1.6 Polyethylene glycols of different molecular size as markers of chorion permeability in the zebrafish (*Danio rerio*) embryo

In order to more closely identify the upper limits of the molecular size of chemicals to cross the chorion of zebrafish, differently sized, non-toxic and chemically inert polyethylene glycols (PEGs; 2000 - 12,000 Da) were applied at concentrations (9.76 mM) high enough to provoke osmotic pressure. Whereas small PEGs were expected to be able to cross the chorion, restricted uptake of large PEGs was hypothesized to result in shrinkage of the chorion (Fig. 24). Due to a slow, but gradual uptake of PEGs over time, molecular size-dependent equilibration in conjunction with a regain of the spherical chorion shape was observed. Thus, the size of molecules able to cross the chorion could be narrowed down precisely to ≤ 4000 Da, and the time-dependency of the movement across the chorion could be described. To account for associated alterations in embryonic development, fish embryo toxicity tests (FETs) according to OECD TG 236 (OECD, 2013) were performed with special emphasis to changes in chorion shape.

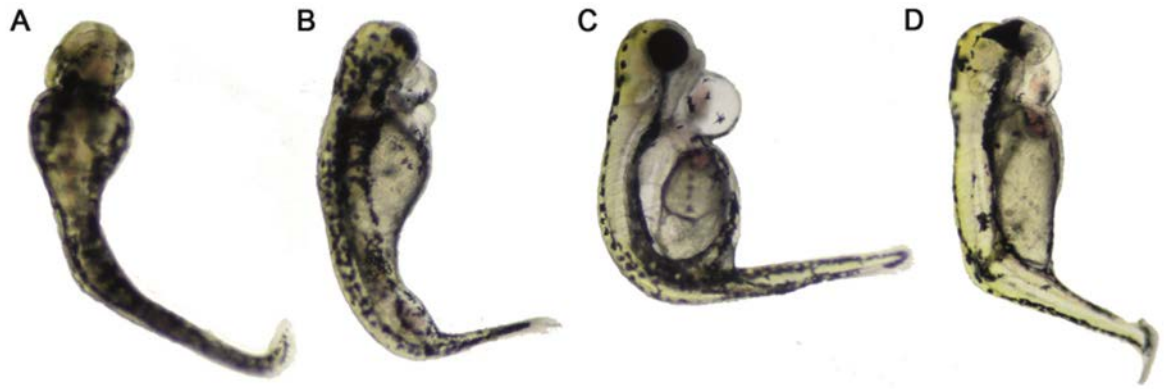
FETs revealed clear-cut size-effects: the higher the actual molecular weight (= size) of the PEG, the more effects (both acutely toxic and sublethal) were found (Fig. 25). No effects were seen with PEGs of 2000 and 3000 Da. In contrast, PEG 8000 and PEG 12,000 were found to be most toxic with LC_{50} values of 16.05 and 16.40 g/L, respectively. Likewise, the extent of chorion shrinkage due to increased osmotic pressure strictly depended on PEG molecular weight and duration of exposure. A reflux of water and PEG molecules into the chorion and a resulting re-shaping of the chorion could only be observed for eggs exposed to PEGs ≤ 4000 Da. Results clearly indicate a barrier function of the zebrafish chorion for molecules larger than 3000 to 4000 Da.

Figure 24: Shrinkage of the chorion of zebrafish (*Danio rerio*) embryos after transfer into polyethylene glycols of different molecular mass



After transfer into PEG test solutes, the chorion of zebrafish (*Danio rerio*) embryos showed a concentration-dependent shrinkage due to effects by PEG 3000 (A - C) and PEG 6000 (D - F). Only minor depression of the chorion were found at 3.125 g/L PEG 3000 (A) and PEG 6000 (D), whereas at 25 g/L (B, E) and 100 g/L (C, F) stronger depressions were evident, finally leading to a tightly wrapped chorion around yolk and cells (C and F). From Pelka et al. (2017).

Figure 25: Sublethal effects of PEG 6000 in zebrafish (*Danio rerio*) embryos



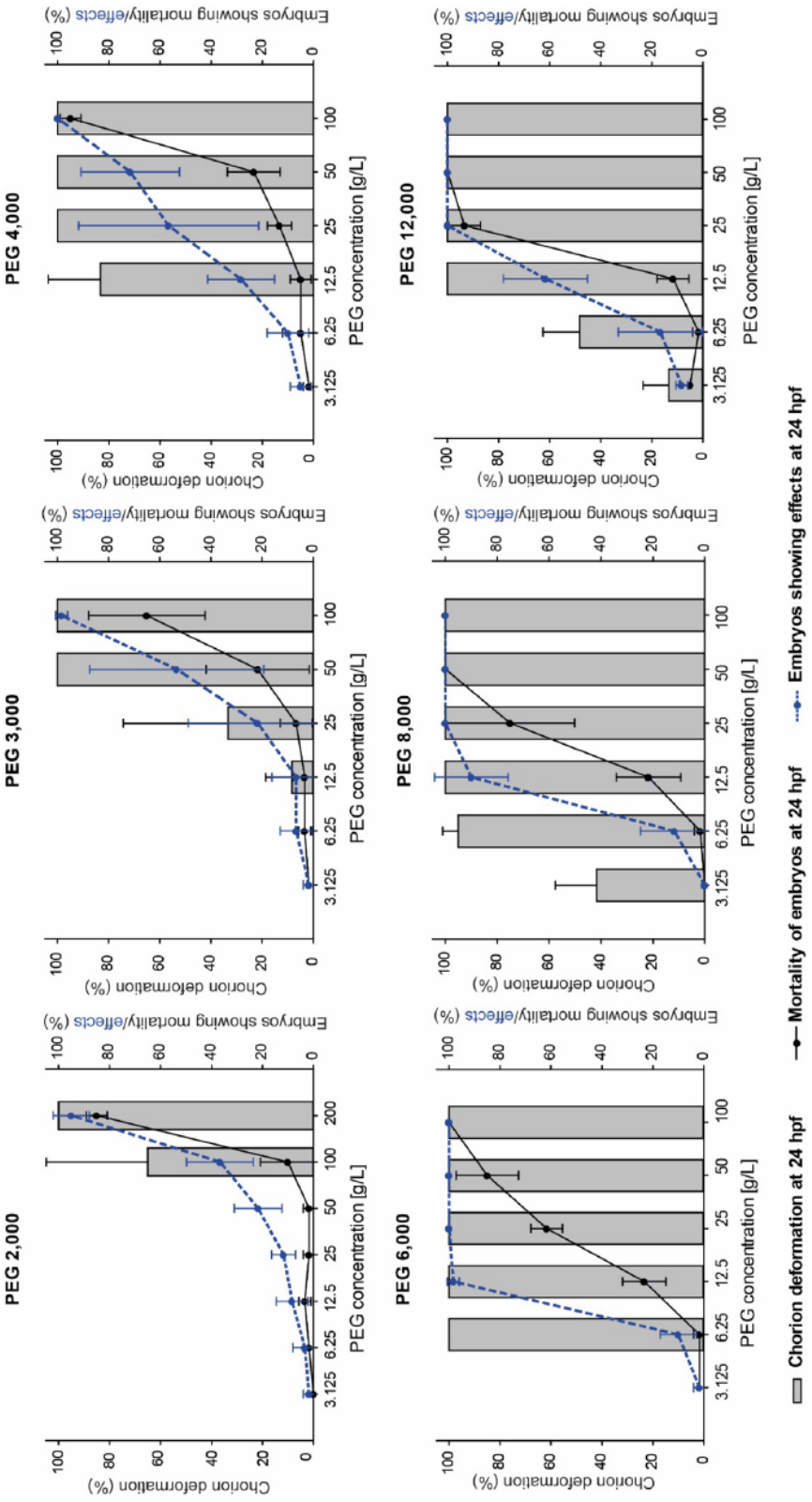
Sublethal effects in zebrafish (*Danio rerio*) embryos after 96 h exposure to 12.5 g/L (A, B), 25 g/L (C) and 50 g/L PEG 6000 (D). Note the absence (A) or deformation of the eyes (B - C), sharply bent tails, formation of edemata as well as yolk deformations of PEG-exposed embryos. From Pelka et al. (2017).

In any case, the PEG-size-dependent deformation of the chorion showed a clear-cut dose-response relationship. In addition, this could be correlated with a concentration-dependent increase of acute lethality and sublethal effects (Fig. 26).

The minor (PEG 2000 and PEG 3000) to strong deformations of the chorions (PEGs ≥ 4000 Da) observed immediately after the onset of exposure gradually changed with progressing exposure. Whereas eggs exposed to PEG 2000 quickly regained the spherical shape of their chorions (Fig. 27), the recovery of chorion shape of embryos exposed to PEG 3000 took 48 h. For PEG 4000, minor recovery from chorion deformation could be observed. Since the space between the chorion and the embryo slightly increased within 48 h, the embryos were able to resume their movements, even within the deformed chorions. For PEGs ≥ 6000 Da, however, the deformations of the chorion observed at the very start of exposure seemed to even increase after 24 and 48 h, indicating a continuation of the shrinkage process. Finally, embryos exposed to PEGs 8000 and 12,000 did not develop further and quickly coagulated (Fig. 27).

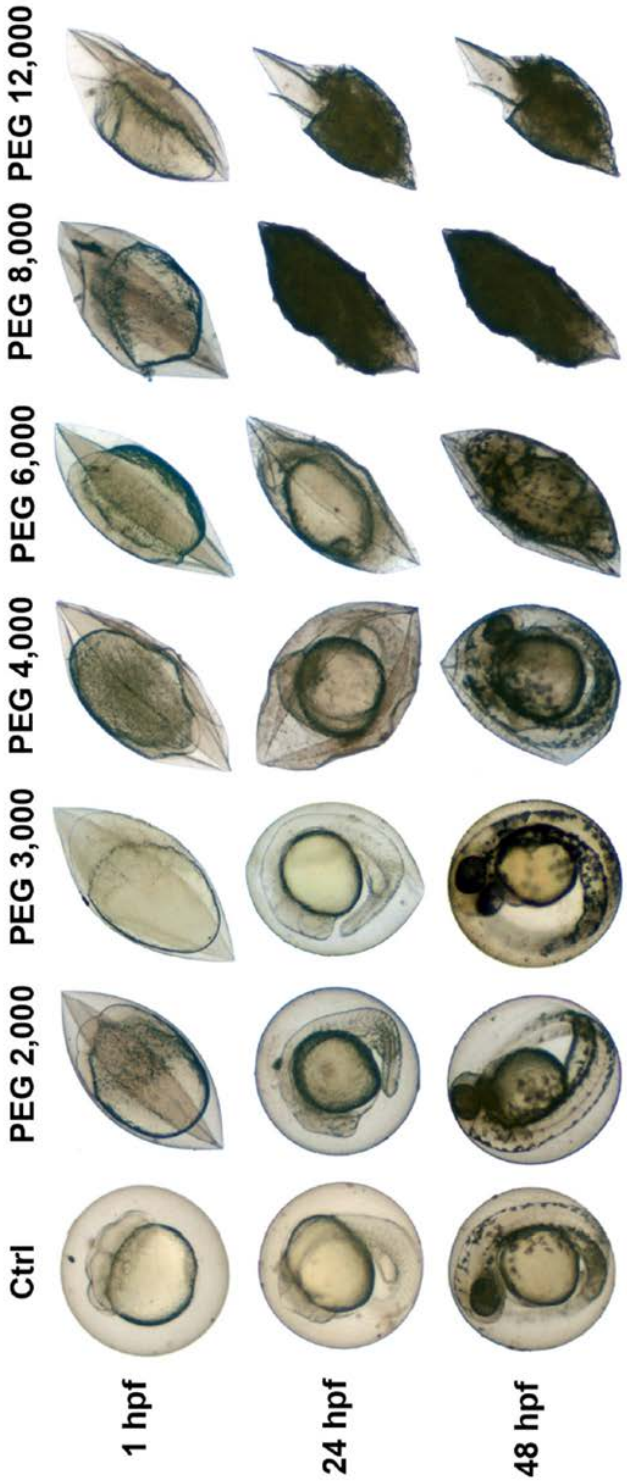
In order to graphically illustrate the PEG size- and time-dependence of the equilibration process, the average difference of the chorion area at different time points in relation to the area at the start of exposure was determined (Fig. 28). Again, two groups of PEG size ranges could be distinguished: For PEGs ≤ 4000, an increase of the chorion area could be observed, indicating a recovery from the deformation due to gradual influx of external medium. The biggest difference compared to the start of exposure was found for PEG 3000 with a difference in chorion area of 13.16, 14.44 and 16.52 % after 24, 36 and 48 hpf, respectively. In contrast, for PEGs ≥ 6000 a decrease of chorion area was evident. For embryos exposed to PEGs 8000 and 12,000, the chorion area even decreased by 1.54 and 2.18 %, respectively. For more details, see Pelka et al. (2017).

Figure 26: Chorion deformation of zebrafish (*Danio rerio*) eggs after exposure to PEGs



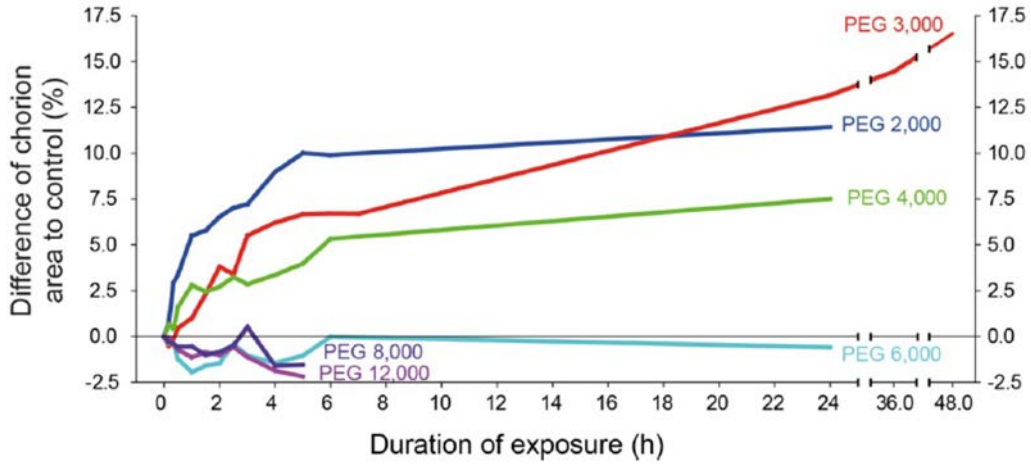
Exposure of zebrafish (*Danio rerio*) eggs for 24 h to different polyethylene glycols (PEGs) reveals chorion deformation (grey columns) at almost all concentrations of the differently sized PEGs. Likewise, for all tested PEGs, there was a concentration-dependent increase of acute lethality (solid black line) and sublethal effects (dotted blue line). From Pelka et al. (2017).

Figure 27: Chronological sequence of deformation of the zebrafish (*Danio rerio*) chorion following exposure to differently sized polyethylene glycols (PEGs)



Chronological sequence of deformation of the zebrafish (*Danio rerio*) chorion following exposure to differently sized polyethylene glycols (PEGs). Each column shows the same egg exposed to dilution water (ctrl) or 9.67 mM of the different PEGs at start of exposure (approx. 1 hpf) as well as at 24 and 48 hpf. Whereas the chorions of embryos exposed to PEG 2000 and 3000 regained a spherical shape, the chorions of PEGs ≥ 4000 Da remained deformed. Note coagulation of embryos (darkening, intransparency) exposed to PEGs ≥ 8000 Da. From Pelka et al. (2017).

Figure 28: Changes in the area of the chorion of zebrafish (*Danio rerio*) embryos exposed to polyethylene glycols (PEGs) of different molecular weight



Changes in the area of micrographs of the chorion of zebrafish (*Danio rerio*) embryos exposed to polyethylene glycols (PEGs) of different sizes (molecular weight) in relation to the areas at the onset of exposure. The exposure covers the time-period from the onset of exposure (approx. 5 min) to the time point of no further shrinkage of the chorion (approx. 24 h). For PEG 3000, additional measurements were made after 36 and 48 h. Note that maximum shrinkage is already reached after approx. 7 h for most PEGs. Data are given as percent change over negative controls at the beginning of the exposure for three independent runs with 20 embryos (n = 3). Coagulated eggs at PEGs 8000 and 12,000 were excluded from evaluation. From Pelka et al. (2017).

1.7 Conclusions: Barrier function of the zebrafish chorion

From multiple experiments with both fluorescent markers and differently sized polyethylene glycols, the following conclusions can be drawn:

- With appropriate modifications of the FET protocol, embryos can reproducibly be dechorionated at 24 h post-fertilization (hpf) with reliably high survival rates of ≥ 90 %. Dechorionation of younger stages (< 24 hpf) is generally possible, however with lower survival rates.
- By means of fluorescent marker molecules, the molecular size for the passage of non-charged chemical substances across the chorion of the zebrafish could be set at approx. 3000 Da.
- More in-depth studies with differently sized polyethylene glycols allowed narrowing down the limit for free passage across the chorion of the zebrafish between 3000 and 4000 Da. Depending on time, polyethylene glycols with a molecular weight ≤ 3000 Da show a more or less free passage.
- The passage of most uncharged industrial chemicals is not impaired by a systematic size limitation.
- Dechorionation of zebrafish eggs eliminates problems associated with the passage of the zebrafish chorion by high molecular weight substances.
- The passage of charged molecules across the chorion of zebrafish is substantially lower than that of non-charged molecules. Given the complex interaction of three-dimensional

molecular configuration and molecular charge, a precise prediction of the penetration barrier for charged molecules is not possible without a complex model based on a comprehensive database (which are not yet available).

- Dimethyl sulfoxide (DMSO) concentrations of 0.1 % and 1 % decrease the barrier function of the chorion, whereas a concentration of 0.01 % DMSO remains ineffective. As a consequence, DMSO may be used without complications as a solvent, however, only at a maximum concentration of 0.01 % (0.1 ml/L; 100 mg/L), which is in line with the recommendations of the OECD GD 23 on difficult substances (OECD, 2018).
- Since dimethyl sulfoxide (DMSO) does not impact zebrafish embryo survival up to concentrations well above 2 %, DMSO may be used as a detergent to facilitate the passage of substances that might be critical (1) in terms of their molecular size to pass the zebrafish chorion (2) for handling in aqueous solutions due to their high lipophilicity. So far, there is no convincing evidence that – after appropriate solution by the aid of solvents – highly lipophilic substances are not taken up by zebrafish embryos.
- For highly lipophilic substances, neither the Acute Fish Toxicity test (AFT, OCED TG 203) nor the Fish Embryo Acute Toxicity test (FET, OECD TG 236) appear to be suitable protocols, since highly lipophilic substances might accumulate over extended periods of time and might not have reached a steady-state after an exposure time restricted to 96 h. Rather, for highly lipophilic substances, longer-term tests such as a Fish Early Life-Stage test according to OECD TG 210 (OECD, 2013b) should be conducted (Oris et al., 2012).

2 Biotransformation and bioactivation capacities in early life stages of zebrafish (*Danio rerio*) – a literature review

2.1 Introduction

Major objections to the use of the FET have mainly been based on the assumption of limited biotransformation capacities in early life stages of fish. Repeatedly, doubts have been expressed concerning the comparability of metabolic capacity between early juvenile and adult life stages of zebrafish (Busquet et al., 2008a; Chng, 2013; Verbueken et al., 2017). In fact, a limited biotransformation or bioactivation capacity could lead to underestimations of toxic or teratogenic potency of a xenobiotic, which would be bioactivated in other life stages (Busquet et al., 2008a; Kluver et al., 2014). On the other hand, given the *ex utero* development, early-life stages of (zebra)fish depend on their intrinsic metabolic capacity for elimination or transformation of xenobiotics (Verbueken et al., 2017); therefore, it seems reasonable to assume at least qualitatively appropriate biotransformation capacities.

As a consequence, in order to strengthen the acceptance of the FET, it seemed essential to attempt to better understand the metabolic capacities of zebrafish embryos across different life-stages. However, while literature concerning spatial and temporal expression patterns of xenobiotic metabolizing enzymes has been growing fast at the genome and RNA levels, only few investigations addressed protein function and catalytic activities, although these are ecologically much more relevant parameters than DNA- and RNA-based characteristics. The database concerning zebrafish metabolism, especially in early-life stages, is much less complete than that available for other model organisms (e.g. rainbow trout) and is scattered over more than 100 studies (Spitsbergen and Kent, 2003).

The following chapter has been designed to provide a state-of-the-art summary aimed at facilitating the identification of remaining gaps in our knowledge on biotransformation in zebrafish in general and zebrafish embryos in specific. The literature study has been initiated as a starting-point for prioritization of further research into biotransformation in zebrafish embryos.

2.2 Metabolism of xenobiotics

The majority of xenobiotics (compounds foreign to the organism) undergo metabolic transformation (biotransformation) in order to be excreted (more) effectively (Donato and Castell, 2003). In vertebrates, xenobiotic metabolism is primarily located in the liver; however, extrahepatic tissues such as kidney, intestine and gills may also make major contributions to xenobiotic metabolism (Buhler and Williams, 1988; Chambers and Yarbrough, 1976).

As a rule, metabolism of xenobiotics is a biphasic process subdivided into phase I and II reactions. Phase I reactions introduce (*de novo* synthesis) or unmask polar groups (e.g. -OH, -COOH, -SH, $-NH_2$) in xenobiotics. These oxidative, reductive, or hydrolytic processes provide functional sites for subsequent conjugation reactions (phase II reactions). The key enzymes for phase I reactions are, among other less important enzyme systems, various isoforms of cytochrome P450-dependent monooxygenases (CYPs). CYP-mediated reactions primarily include hydroxylation and epoxidation of aromatic and aliphatic compounds, ester and ether cleavage, as well as heteroatom dealkylation.

In phase II reactions, functional groups are conjugated with polar endogenous compounds. Phase II reactions include glucuronidation, glutathione and amino acid conjugation as well as acetylation; all of these reactions significantly increase the water solubility of the generated

metabolites, allowing and facilitating their excretion *via* the bile (intestinal route) and/or as urine *via* the kidney route (Buhler and Williams, 1988; Donato and Castell, 2003; Parkinson and Ogilvie, 2001; Schlenk et al., 2008).

2.3 The role of metabolism in activation and detoxification of xenobiotics

Metabolism is well recognized as a critical factor influencing the teratogenic and toxic potency of numerous xenobiotics. In the majority of cases, metabolism leads to detoxification (bioinactivation); however, toxification (bioactivation) of xenobiotics has also been reported for multiple substances (Goldstone et al., 2010; Nebert and Dalton, 2006). For instance, it is assumed that only about one quarter of all carcinogenic xenobiotics are tumorigenic in their non-metabolized form, whereas the remaining xenobiotics require CYP-mediated activation before they can develop full toxicity or teratogenicity (Nebert and Dalton, 2006). Proteratogens and protoxicants can thus be activated enzymatically into electrophilic and reactivate intermediates such as electrophiles, epoxides and free radicals, which readily interact with cellular components such as proteins, DNA or lipids (Blaschke et al., 2010; Miller, 1970; Wells et al., 2004). Typical model xenobiotics that are bioactivated via metabolism include benzo[a]pyrene, allyl alcohol, aflatoxin, cyclophosphamide and carbamazepine (Kluver et al., 2014; Weigt et al., 2011).

Most importantly, toxicity and teratogenicity of metabolically activated substances can be underestimated, if biotransformation capacities are limited (Busquet et al., 2008a; Kluver et al., 2014). In case of bioinactivation, limited biotransformation capacities might lead to a false-negative result. A typical example could be albendazole, which has been reported to be metabolically inactivated in adult zebrafish, but not in embryonic zebrafish (Boix et al., 2015; Carlsson et al., 2013; Mattsson et al., 2012). Supplementation by a mammalian metabolization system resulted in a compensation of the lack of stage-dependent deactivation, i.e. a decrease of developmental toxicity in embryonic zebrafish (Mattsson et al., 2012).

2.4 Cytochrome P450-dependent monooxygenases (CYPs)

It is well established that cytochrome P450-dependent monooxygenases (CYPs) catalyze the oxidative metabolism (phase I metabolism) of many xenobiotics, and approximately 75 % of all xenobiotics are estimated to undergo CYP mediated phase I biotransformation including hydroxylation, dealkylation and/or epoxidation (Guengerich, 2007; Nebert and Dalton, 2006). In general, for zebrafish, much is known about spatial and temporal expression patterns (Otte et al., 2010), but catalytic activities, substrate specificity, inducibility and stereoselectivity of CYPs have only rarely been examined. In general terms, in vertebrates, the number of CYP genes ranges from 40 to > 100, with CYPs distributed among 19 gene families (Nelson et al. 2013).

2.5 Number of CYP isoforms in zebrafish

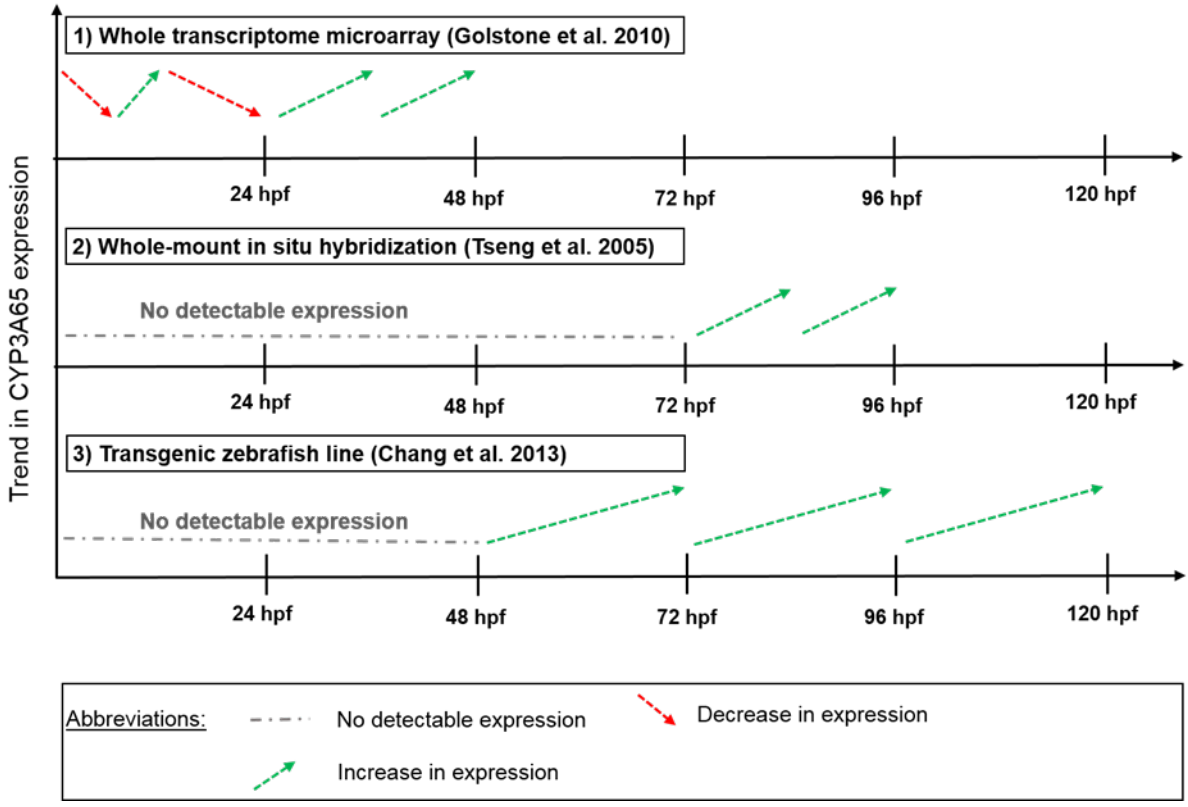
So far, a total of 86 - 96 CYP genes, distributed among 17 - 18 categories of CYP families, have been described for zebrafish (Goldstone et al., 2010; Saad et al., 2016a; Stegeman et al., 2010). CYP families 1, 2, 3 and – to a smaller extent – CYP family 4 are considered to mainly metabolize xenobiotics, while the CYP families 5 - 51 primarily contribute to the metabolism of endogenous substrates (Goldstone et al., 2010). The full number of CYP genes and their family (> 40 % amino acid identity) and subfamily (> 55 % amino acid identity) memberships have already been well documented (Goldstone et al., 2010; Saad et al., 2016a). The following paragraph will review available data of the CYP families 1 - 4 in zebrafish.

In recent years, the focus has been put on genes in CYP family 1, especially on CYP1A as a bioindicator for detecting dioxin-like compounds, polychlorinated biphenyls and polyaromatic hydrocarbons (Jonsson et al., 2009; Kais et al., 2017; Noury et al., 2006). Far less attention has been given to the CYP families 2 to 4.

2.6 Expression patterns of CYPs during zebrafish development

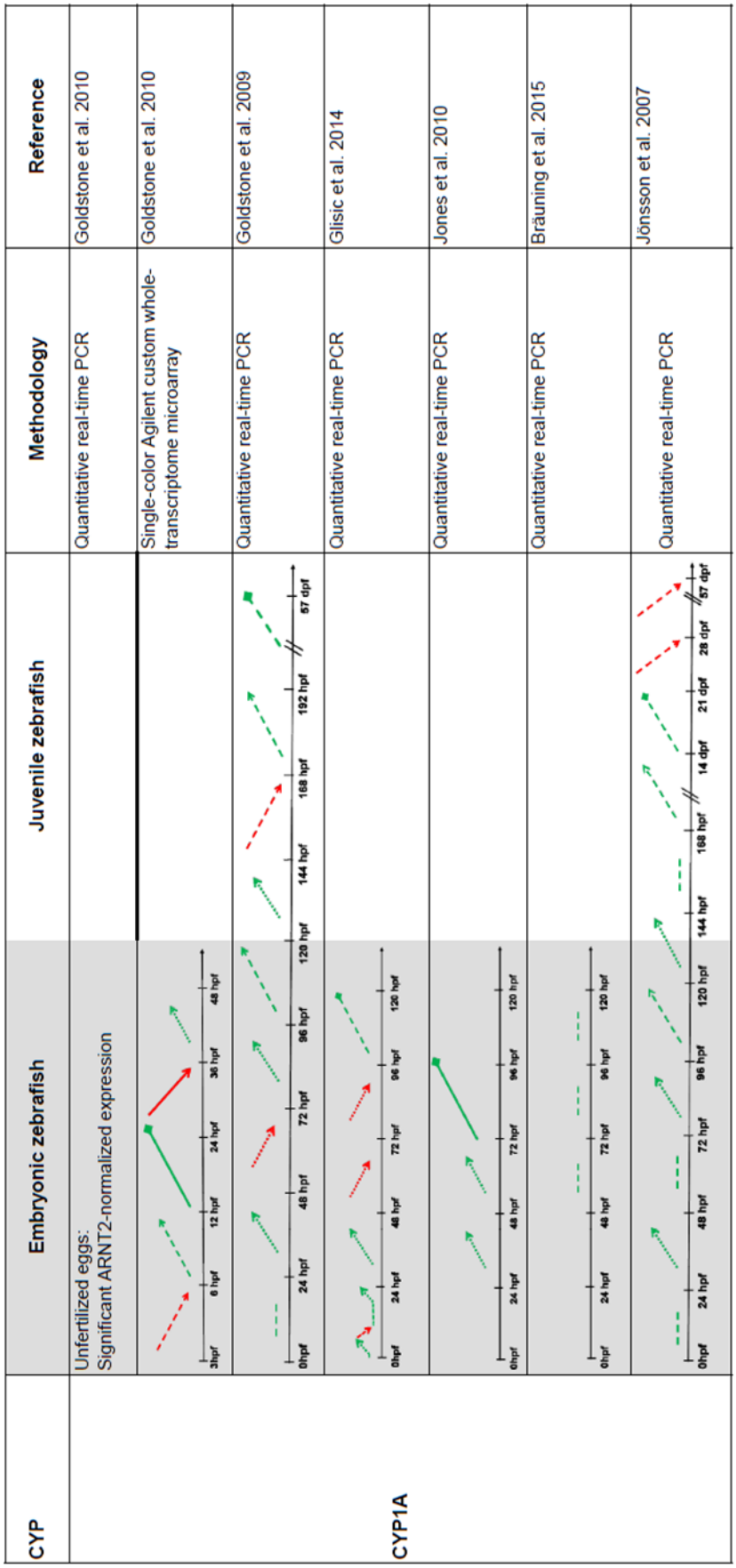
In recent years, most studies have focused on expression patterns of CYPs. They have determined the temporal and spatial expression of CYPs in unchallenged (control) zebrafish by using quantitative real-time PCR (qPCR), microarray techniques, transgenic zebrafish lines, and whole-mount *in situ* hybridization (WISH) technologies. The diversity of methodological approaches, methodology-dependent detection limits and test intervals makes a direct comparison between studies difficult and sometimes leads to conflicting results; as an example, data for CYP3A65 gene expression trends in early-life stages of zebrafish are given in Fig. 29. Fig. 30 summarizes trends in temporal expression patterns for all CYP450 isoforms that could be located in the literature for embryonic and juvenile life-stages zebrafish. Table 1 gives a comprehensive account of spatial and temporal patterns of enzyme expression in zebrafish.

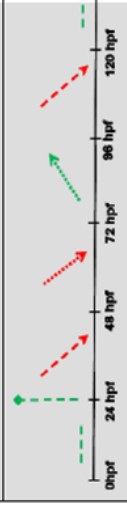
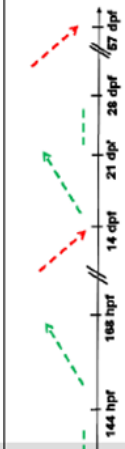
Figure 29: CYP3A65 gene expression trends in early-life stages of zebrafish (*Danio rerio*)



Comparison of CYP3A65 gene expression trends in early-life stages of zebrafish (*Danio rerio*). Data were generated by (1) whole transcriptome microarray (Goldstone et al. 2010), (2) whole-mount in situ hybridization (Tseng et al. 2005), and (3) the use of transgenic zebrafish lines (Chang et al. 2013). From Lörracher & Braunbeck (2019)

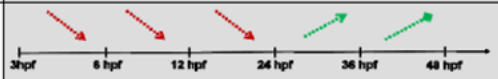
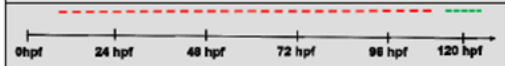
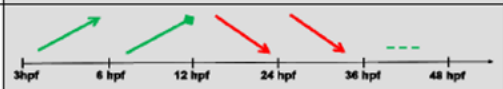
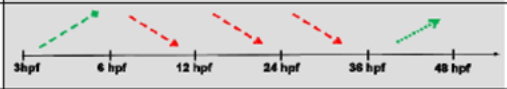
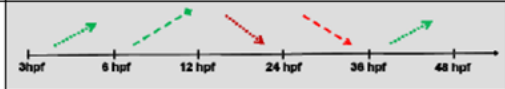
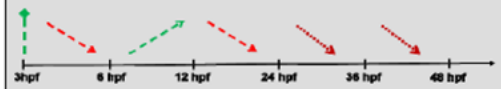
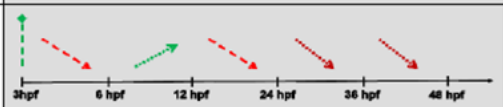
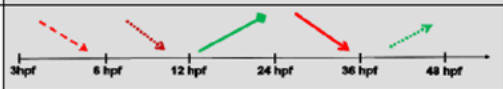
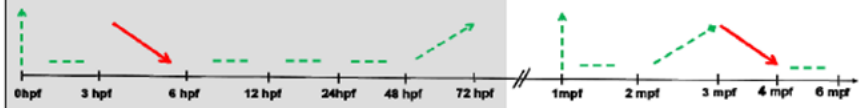
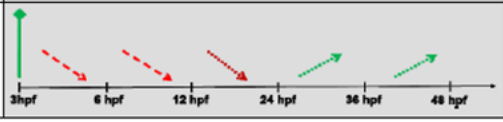
Figure 30: Temporal expression of CYP450 in embryonic and juvenile zebrafish (*Danio rerio*)

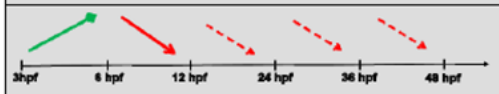
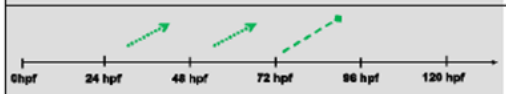
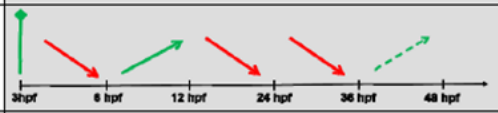
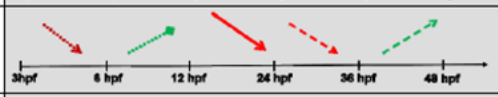
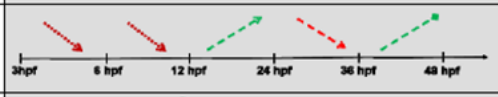
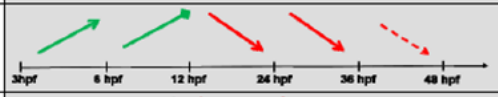
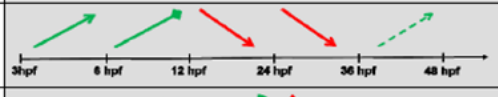
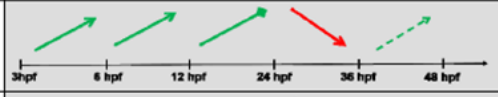
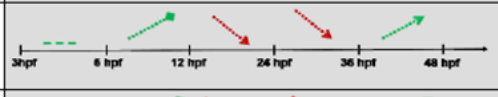
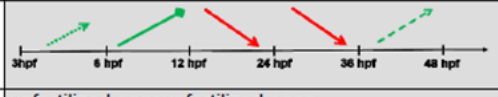
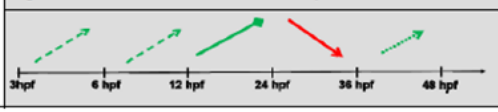


CYP	Embryonic zebrafish	Juvenile zebrafish	Methodology	Reference
CYP1B1			Quantitative real-time PCR Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
			Quantitative real-time PCR	Bräuning et al. 2015
			Quantitative real-time PCR	Jönsson et al. 2007
CYP1C1			Quantitative real-time PCR Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
			Quantitative real-time PCR	Bräuning et al. 2015
			Quantitative real-time PCR	Jönsson et al. 2007
	80 hpf Expression is significantly higher than the expression of CYP1A and CYP1B1		Quantitative real-time PCR	Jönsson et al. 2007

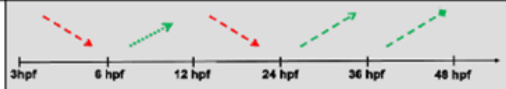
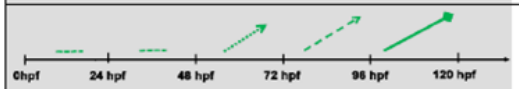
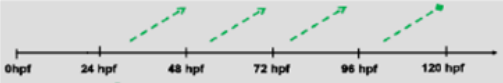
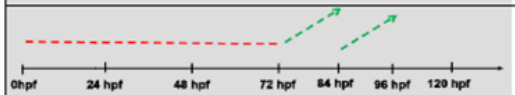
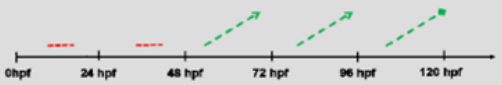
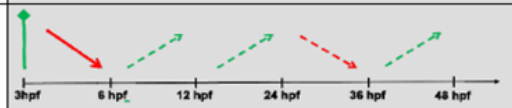
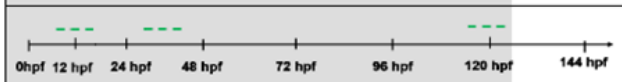
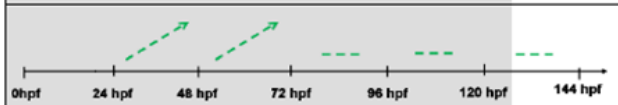
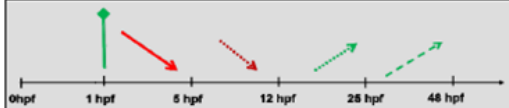
CYP	Embryonic zebrafish	Juvenile zebrafish	Methodology	Reference
CYP1C2			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
			Quantitative real-time PCR	Bräuning et al. 2015
			Quantitative real-time PCR	Jönsson et al. 2007
	80 hpf Expression tends to be higher than the expression of CYP1A or CYP1B1 (Statistically not significant)		Quantitative real-time PCR	Jönsson et al. 2007
CYP1D1			Quantitative real-time PCR Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
			Quantitative real-time PCR	Goldstone et al. 2009
CYP2J1			Whole mount in situ hybridization	Wang et al. 2007
			Quantitative real-time PCR	Wang et al. 2007
CYP2K6			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
			Quantitative real-time PCR	Wang-Buhler et al. 2005

CYP	Embryonic zebrafish	Juvenile zebrafish	Methodology	Reference
CYP2K8			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2K16			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2K17			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2K18			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
			Whole mount in situ hybridization Transgenic line Tg(cyp2k18:egfp)	Poon et al. 2017
CYP2K19			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2K20			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2K21			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2K22 CYP2K7			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2K31			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010

CYP	Embryonic zebrafish	Juvenile zebrafish	Methodology	Reference
CYP2N13			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
			Whole mount in situ hybridization Transgenic line Tg(cyp2n13: egfp)	Poon et al. 2017
CYP2P6			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2P7			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2P9			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2P10			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2P14			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2R1			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
			Quantitative real-time PCR	Peng et al. 2017
CYP2U1			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010

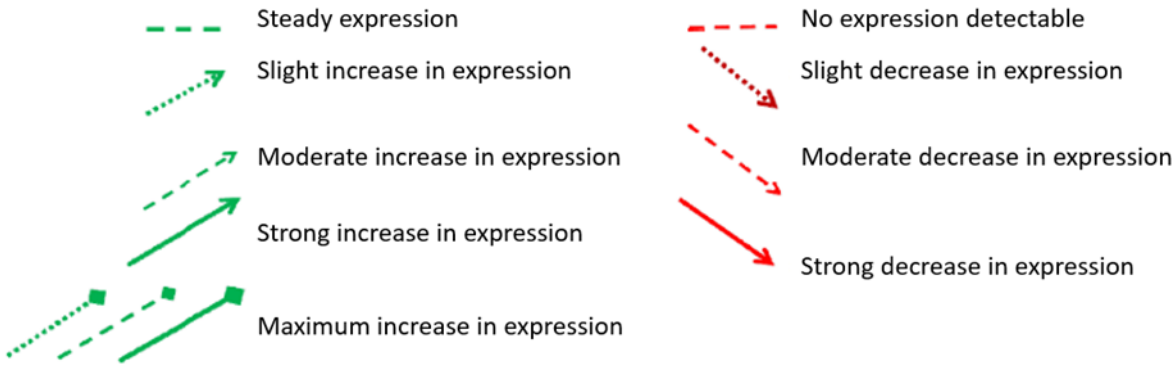
CYP	Embryonic zebrafish	Juvenile zebrafish	Methodology	Reference
CYP2V1 CYP2J26	Unfertilized eggs Significant ARNT2-normalized expression		Quantitative real-time PCR	Goldstone et al. 2010
			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
			Quantitative real-time PCR	Jones et al. 2010
CYP2X7			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2X8			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2X10			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2Y3			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2Y4			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2AA1			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2AA2			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2AA3			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2AA4	unfertilized eggsunfertilized eggs significant ARNT2-normalized expression		Quantitative real-time PCR	Goldstone et al. 2010
			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010

CYP	Embryonic zebrafish	Juvenile zebrafish	Methodology	Reference
CYP2AA7			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2AA8			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2AA9			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2AA11			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2AA12			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2AD2			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2AD3			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2AD6			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP2AE1			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010

CYP	Embryonic zebrafish	Juvenile zebrafish	Methodology	Reference
CYP3A65			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
			Quantitative real-time PCR	Glisic et al. 2014
			Quantitative real-time PCR	Tseng et al. 2005
			Whole-mount in situ hybridization	Tseng et al. 2005
			Transgenic zebrafish line Tg(CYP3A65: EGFP)	Chang et al. 2013
CYP3C1			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
			Whole-mount in situ hybridization	Corley-Smith et al. 2006
			Quantitative real-time PCR	Corley-Smith et al. 2006
			Quantitative real-time PCR	Shaya et al. 2014

CYP	Embryonic zebrafish	Juvenile zebrafish	Methodology	Reference
CYP3C2			Quantitative real-time PCR	Shaya et al. 2014
			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP3C3			Quantitative real-time PCR	Shaya et al. 2014
			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP3C4			Quantitative real-time PCR	Shaya et al. 2014
CYP4F43			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP4V7			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP4V8			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010
CYP4T8			Single-color Agilent custom whole-transcriptome microarray	Goldstone et al. 2010

Comparative trends within the examined CYP450 isoforms (Fig. 30):



Trends in temporal expression patterns for all CYP450 isoforms that could be located in the literature for embryonic and juvenile life-stages zebrafish (*Danio rerio*). From Lörracher & Braunbeck (2019)

Table 1: Spatial and temporal patterns of CYP enzyme expression in zebrafish (*Danio rerio*)

Activity assay	CYP	Embryo		Juvenile		Adult		References
In vivo- EROD assay Substrate: 7-Ethoxyresorufin		8 hpf	Cytoplasm of cells of the envelope layer, yolk syncytial layer, developing germ layers	128 hpf	Intestine, liver, around the anal pore, circulatory system, head kidney, nephric duct, kidney Same spatial distribution as embryo (104 hpf).			Otte et al. 2010, Scornaienchi et al. 2010
		32 hpf	Different parts of the head, straight tube of the heart, dorsal aorta, myotome, envelope of the yolk, pronephric duct, urogenital pore					
		56 hpf	Circulatory system, heart, inner party of the eye, mesencephalon, fourth ventricle, liver primordium, primordia of the pronephric kidney, pronephric duct, urogenital pore					
		80hpf	All vessels, heart, intestine, pancreas, gut, liver, head kidney, pronephric duct					
		104 hpf	Intestine, liver, around the anal / urinary pore, circulatory system, head kidney, nephric duct, kidney					
	Mammals: CYP1 mediated	24-120 hpf	Significant increase in activity					Bräuning et al. 2015
In vitro- EROD assay Substrate: 7-Ethoxyresorufin	Zebrafish: CYP1A > CYP1C2 > CYP1B1 = CYP1C1 > CYP1D1 (Heterologous expressed CYP1s)	2,5 hpf	Activity above the level of detection, but lower than the limit of quantification.					Otte et al. 2017
		48 hpf	Decrease in activity. Activity below the limit of detection					
		96 hpf	Increase in activity. Activity above the limit of quantification					
		120hpf	Activity above the limit of quantification					
		8 - 120 hpf	Activity varies over time Peak in expression at 8 hpf and 104 hpf	128 hpf	Activity approximately 50 - 30% of the level at 104 hpf			Otte et al. 2010, Scornaienchi et al. 2010
		5 hpf	Highest activity with a large inter-batch variation			Liver microsomes	Much higher EROD activity than in the other developmental stages. No gender differences.	Saad et al. 2018
		24 -48 hpf	Negligible activity (significantly lower than at 48 hpf)					
		72 - 96 hpf	Increase in activity, even further at the end of organogenesis					
		120 hpf	Negligible activity					
				2 wpf	Constitutive and significantly inducible EROD activity in the supernatant of body homogenates			Pauka et al. 2011
						Whole gill arch and liver	Constitutive and significantly inducible EROD activity	Jönsson et al. 2009

Activity assay	CYP	Embryo		Juvenile	Adult		References
In *vitro*- BROD assay Substrate: 7-O-Benzylresorufin	Human: CYP1A, CYP2B, CYP3A Zebrafish: CYP1A = CYP1B1 > CYP1Cs > CYP1D1	2.5 -96 hpf 120 hpf	No activity above the limit of detection Activity above the limit of detection but under the limit of quantification				Otte et al. 2017
In vitro-PROD assay Substrate: 7-Pentoxyresorufin	Zebrafish: CYP1A, CYP1Cs > CYP1D	2.5 - 120 hpf	No activity above the limit of detection				Otte et al. 2017
***In vivo*- ECOD assay** Substrate: 7-Ethoxycoumarin	Mammals: CYP2 mediated	96 hpf	Constitutive ECOD activity Incubation time dependence in activity	Relative constitutive ECOD activity: liver > gill > muscle > brain significantly inducible	Liver microsomes	Constitutive ECOD activity ECOD activity does not differ significantly from the activities of *Cyprinus carpio*, *Oryzias latipes* and *Poecilia reticulata*	Jones et al. 2010 Funari et al. 1987 Wu et al. 2014
***In vitro*- ECOD assay** Substrate: 7-Ethoxycoumarin							
***In vivo*- OOMR assay** Substrate: Octyloxymethylresorufin	Mammals: CYP3 mediated	96 hpf	Incubation time dependence in activity significantly inducible				Jones et al. 2010
***In vivo* -luminescence-based Promega P450-Glo™ CYP3A4 assay** Substrate: Luciferin-IPA Substrate: Luciferin-PFBE	Human: CYP3A4	120 hpf	Activity well distinguished from background noise				Chng 2013
***In vitro* -Luminescence-based Promega P450-Glo™ CYP3A4 assay** Substrate: Luciferin-IPA					Liver microsomes	Metabolite concentration below the lower limit of quantification	Verbueken et al. 2017
***In vivo* -luminescence-based Promega P450-Glo™ CYP3A4 assay** Substrate: Luciferin-BE	Human: CYP3A4	48 hpf 72 hpf	Consitutive activity Significant modification by inducers and inhibitors Constitutive activity higher than at 48 hpf				Li et al. 2011

Activity assay	CYP	Embryo		Juvenile	Adult		References
In vivo- BFCOD assay Substrate: 7-Benzyloxy-4-trifluoromethylcoumarin	Human: CYP3A4 >>CYP2B6 Zebrafish: Minimal contribution of CYP1A to the BFCOD activity CYP1A> CYP1C2>CYP1C1, CYP1B1 (Heterologous	96 hpf	Activity consistently measured Significant modification by inducers and inhibitors				Creusot et al. 2014, Renwick et al. 2008
		120 hpf	Constitutive and significantly inducible BFCOD activity				Oziolor et al. 2017
In vitro- BOMR assay Substrate: Benzyloxy-methylresorufin	Human: CYP3A	5 - 120 hpf Microsomes of whole embryo homogenates	BOMR activity only observed at 72 hpf and 96 hpf Activity close to the limit of quantification		Liver microsomes	Activity significantly higher than in microsomes of whole embryo homogenates	Verbueken et al. 2017

Abbreviations:

ECOD	Ethoxycoumarin-O-deethylase
EROD	Ethoxyresorufin-O-deethylase
BFCOD	7-Benzyloxy-4-trifluoromethylcoumarin-O-debenzyloxylase
BOMR	Benzyloxy-methyl-resorufin
BROD	7-benzylresorufin-O- debenzylase
OOMR	Octyloxymethylresorufin
PROD	7-pentoxyresorufin-O-depentylase

Spatial and temporal patterns of CYP enzyme expression in zebrafish (*Danio rerio*). From Lörracher & Braunbeck (2019)

Most CYP genes (51 out of 52 CYP genes investigated for zebrafish) are expressed as mRNA at least once during the embryonic development until < 120 hours post-fertilization (hpf) (Braunig et al., 2015; Chang et al., 2013; Corley-Smith et al., 2006; Glisic et al., 2016; Goldstone et al., 2010; Jones et al., 2010; Peng et al., 2017; Poon et al., 2017a; Shaya et al., 2014; Tseng et al., 2005; Wang-Buhler et al., 2005; Wang et al., 2007). The only exception, so far, is CYP3C4, which, however, has only been investigated from 0 to 48 hpf (Shaya et al., 2014). For most CYPs, expression throughout the early development fluctuates in a wave-like fashion (Fig. 29) peaking at different time points (Goldstone et al., 2010).

For CYP1A, CYP2V1, and CYP2AA4, maternal contribution to mRNA transcript abundance was detected in unfertilized oocytes (Goldstone et al., 2010). CYP1B1 (24 hpf) and CYP1D1 (9 hpf) show their highest expression in early stages of embryonic development (Goldstone et al., 2009; Jonsson et al., 2007a). Other CYPs such as CYP1A, CYP2J26, CYP2K6, and CYP2R1 show an increase in expression after 48 - 72 hpf, which is the period, when zebrafish embryos hatch (Jones et al., 2010; Peng et al., 2017; Wang-Buhler et al., 2005). In the case of CYP1C1, CYP1C2, and CYP1D1, there is a decrease in mRNA transcript abundance towards the end of embryonic development (96 - 120 hpf) (Jonsson et al., 2007a).

Information concerning temporal CYP expression patterns in juvenile life-stages (eleuthero¬-embryos and larvae) of zebrafish (between 120 hpf and 3 months post-fertilization; mpf) are available for the following eight isoforms: CYP1A, CYP1B1, CYP1C1, CYP1C2, CYP1D1, CYP2K6, CYP2R1, and CYP3C1. However, as holds for embryos, the abundance of CYP mRNA transcripts in juvenile life-stages is still fluctuating. CYP1A, CYP1C1, CYP2K6, and CYP2R1 have higher expression levels in juvenile zebrafish than in embryos. For the isoforms CYP1B1, CYP1D1, and CYP3C1, the levels of expression in embryonic life-stages is higher than or equal to the level in juvenile life-stages (Braunig et al., 2015; Corley-Smith et al., 2006; Goldstone et al., 2009; Jonsson et al., 2007a; Peng et al., 2017; Wang-Buhler et al., 2005).

Table 2: CYP isoforms in adult, juvenile, and embryonic life-stages of zebrafish (*Danio rerio*)

Adult		Juvenile	Embryonic
CYP1A	CYP2R1	CYP1A	CYP2K18
CYP1B1	CYP2Y3	CYP2K6	CYP2K7
CYP1C1	CYP2AA1	CYP2K7	CYP2N13
CYP1C2	CYP2AA2	CYP3A65	CYP3A65
CYP1D1	CYP3A65		
CYP2J1	CYP3C1		
CYP2K6	CYP3C2		
CYP2K7	CYP3C3		
CYP2K18	CYP3C4		

CYP isoforms investigated concerning their organ-specific spatial expression patterns in adult, juvenile, and embryonic life-stages of zebrafish (*Danio rerio*). From Lörracher & Braunbeck (2019)

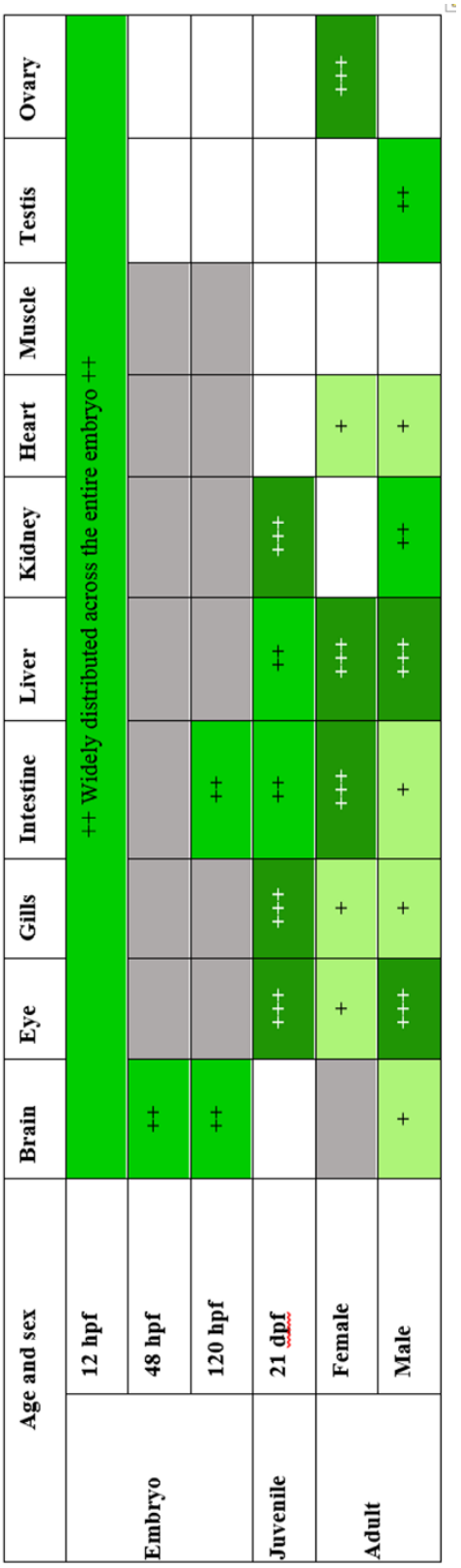
2.7 Spatial expression patterns of CYPs in zebrafish

The overall metabolic capacity is determined by the isoforms and abundance of CYPs expressed in the respective organs. In fish, the liver is considered as the main organ responsible for biotransformation of xenobiotics (Pesonen and Andersson, 1991). CYPs have been described as mainly liver-localized enzymes, but are also expressed in extrahepatic tissues and organs including kidneys, intestine, brain, eyes, heart, muscles, and gills. Using qPCR, antibody staining, transgenic zebrafish lines, and whole mount in situ hybridization, spatial expression patterns of 18 CYPs have been described (Table 2) (Corley-Smith et al., 2006; Fetter et al., 2015; Goldstone et al., 2009; Jonsson et al., 2007b; Kubota et al., 2013; Peng et al., 2017; Poon et al., 2017a; Shaya et al., 2014; Siegenthaler et al., 2017; Taylor, 2005; Tseng et al., 2005; Wang-Buhler et al., 2005; Wang et al., 2007; Yang et al., 2017).

Only for CYP3C1 and CYP3A65, data are available on spatial expression patterns covering all developmental stages. CYP3C1 shows pronounced organ and developmental differences in spatial expression (Table 3). At 12 hpf, CYP3C1 mRNA transcripts are widely distributed through the whole embryo. In later embryonic development (48 and 120 hpf), CYP3C1 mRNA is restricted to the brain and the gastrointestinal tract (Corley-Smith et al., 2006). In juvenile life-stages CYP3C1 is expressed in many organs and tissues including intestine, liver, kidneys, gills, and eyes (Taylor, 2005). In adult zebrafish, expression of CYP3C1 is distributed over multiple organs, with clear sex-differences in the spatial distribution profile (Corley-Smith et al., 2006; Shaya et al., 2014). For a detailed account of spatial expression patterns in embryonic, juvenile and adult zebrafish, see Tables 4 and 5.

Knowledge of spatial and temporal CYP expression is important for a deeper understanding of metabolism, but does not provide a direct insight into functional metabolic activity itself (Sadler et al., 2016), since there is no direct correlation between CYP expression levels, amounts of corresponding proteins, and measurable CYP activities (Bluhm et al., 2014; Goldstone et al., 2010). The CYP activity, for example, can be influenced by changes in protein stability or the efficiency of translation (Bluhm et al., 2014).

Table 3: Spatial expression of CYP3C1 in developmental stages of zebrafish (*Danio rerio*)



The spatial expression profile of CYP3C1 in different developmental stages of zebrafish. White blocks denote non-existence of data, grey blocks denote non-detectable expression, light green low expression, intermediate green blocks denote moderate expression, and dark green blocks high expression (in comparison within the given CYP isoform and life-stage; data from Corley-Smith et al., 2006; Shaya et al., 2014; Taylor, 2005) . From Lörracher & Braunbeck (2019)

Table 4: Spatial expression patterns of CYP 450 in zebrafish (*Danio rerio*)

CYP	Age	Distribution										Applied method	References
		Brain	Eye	Gill	Intestinal tract	Liver	Kidney Pronephros	Heart	Muscle		Others		
CYP1A	21 dpf	×	×	n.s.	●	n.s.	◉ Mesonephric duct	○ Ventricle	×	◉	Pseudobranch Epithelium, Skin of the head Oropharynx	Antibody staining	Taylor 2005
										●	Taste bud Esophagus		
CYP2K6	21 dpf	×	◉ Lens	×	×	×	● Renal tubules	○/◉	×	○	Skin Oropharynx Esophagus	Antibody staining	Taylor 2005
										●	Muscle tunic of the intestine		
CYP2K18	120 hpf	×	×	×	○	×	×	×	×			Transgenic line TG (CYP2K18: egfp)	Poon et al. 2017
	120 hpf	×	×	×	○	○	×	×	×			Whole mount in situ hybridization	
CYP2K22 CYP2K7	96 hpf	×	×	×	×	×	◉	×	×			Whole mount in situ hybridization	Fetter et al. 2015
	21 dpf	n.s.	○	●	●	×	○	n.s.	n.s.	◉	Oropharynx Esophagus Skin of the head	Antibody staining	Taylor 2005
										●	Taste bud Cartilage of the head Skin of the trunk and tail		
CYP2N13	120 hpf	×	×	×	○ Intestinal bulb region	×	×	×	×			Transgenic line (CYP2N13:egfp)	Poon et al. 2017
	120hpf	×	×	×	○	○	×	○	×	○	Olfactory bulb Cloaca Skin	Transgenic line TgFOSMID (CYP2N13:egfp)	
	120 hpf	×	×	×	○	○	×	×	×			Whole mount in situ hybridization	

CYP	Age	Distribution										Applied method	References
		Brain	Eye	Gill	Intestinal tract	Liver	Kidney Pronephros	Heart	Muscle	Others			
CYP3A65	72 hpf	×	×	×	×	○	×	×	×			Whole mount in situ hybridization	Tseng et al. 2005
	84 hpf	×	×	×	● Especially in the foregut	◎	×	×	×			Whole mount in situ hybridization	
	96 hpf	×	×	×	● Especially in the foregut	◎	×	×	×			Whole mount in situ hybridization	
	120 hpf	×	×	×	●	●	×	×	×			Whole mount in situ hybridization	
	21 dpf	×	◎ Cornea	×	○	×	○ Renal tubules	○	○	○	Skin of the trunk Cartilage	Antibody staining	Taylor 2005
CYP3C1	12 hpf	Widely distributed thorugh the whole embryo										Whole mount in situ hybridization	Corley-Smith et al. 2006
	48 hpf	◎	×	×	×	×	×	×	×			Whole mount in situ hybridization	
	120 hpf	◎	×	×	◎	×	×	×	×	◎	Pharynx	Whole mount in situ hybridization	
	21 dpf	n.s.	●	●	◎	○/◎	● Mesonephric duct renal tubes	n.s.	n.s.	◎ ○ ●	Skin of the tail and the trunk Taste bud, skin of the head Oropharynx	Antibody staining	Taylor 2005

Abbreviations:

n.s. not studied

× studied with negative results

Comparison within the given CYP isoform:

○ low expression

◎ moderate expression

● high expression

embryo	< 120 hpf
juvenile	< 3 mpf

From Lörracher & Braunbeck (2019)

Table 5: Spatial expression patterns of cytochrome P450 in adult zebrafish (*Danio rerio*)

Spatial expression patterns of cytochrome P450 in adult zebrafish (*Danio rerio*)

CYP	Sex	Distribution											Applied method	References
		Brain	Eye	Gill	Intestine	Liver	Kidney	Heart	Muscle	Testis	Ovary	Others		
CYP1A	♂ / ♀	○	○	○	○	●	○	○	n.s.	n.s.	n.s.		Quantitative real-time PCR	Goldstone et al. 2008 Cytochrome P450 1D1
	No significant sex differences	○	○	○	●	●	○	○	n.s.	○	○		Quantitative real-time PCR	Jönsson et al. 2007
CYP1B1	No significant sex differences	●	●	○	○	○	○	●	n.s.	○	○		Quantitative real-time PCR	Jönsson et al. 2007
CYP1C1	No significant sex differences	○	●	○	○	○	○	●	n.s.	○	○		Quantitative real-time PCR	Jönsson et al. 2007
CYP1C2	No significant sex differences	○	●	○	○	○	○	●	n.s.	○	○		Quantitative real-time PCR	Jönsson et al. 2007
CYP1D1	♂ / ♀	○	○	○	○	●	○	○	n.s.	n.s.	n.s.		Quantitative real-time PCR	Goldstone et al. 2008
CYP2J1	♂ / ♀	○	n.s.	n.s.	×	○	●	●	○	○	○		Quantitative real-time PCR	Wang et al. 2007
	♂ / ♀	●	n.s.	n.s.	×	×	×	×	n.s.	●	●		Whole mount in situ hybridization	Wang et al. 2007
CYP2K6	♂ / ♀	×	×	×	×	●	n.s.	×	×	×	●		Quantitative real-time PCR	Wang-Buhler et al. 2005
CYP2K7 CYP2K22	♂ / ♀	n.s.	n.s.	n.s.	n.s.	●	n.s.	n.s.	n.s.	○	○		Quantitative real-time PCR	Siegentahler et al. 2017
CYP2K18	♂	n.s.	n.s.	n.s.	n.s.	●	n.s.	n.s.	n.s.	n.s.	n.s.		Quantitative real-time PCR	Yang et al. 2017
CYP2R1	♂ / ♀	×	×	×	×	●	×	×	○	×	○	○ Visceral adipose tissue	Quantitative real-time PCR	Peng et al. 2017
CYP2Y3	♂	n.s.	n.s.	n.s.	n.s.	●	n.s.	n.s.	n.s.	n.s.	n.s.		Quantitative real-time PCR	Yang et al. 2017
CYP2AA1	♀	○	○	n.s.	●	○	○	○	n.s.		○		Quantitative real-time PCR	Kubota et al. 2013
	♂	○	○	n.s.	●	○	○	○	n.s.	○			Quantitative real-time PCR	

CYP	Sex	Distribution											Applied method	References
		Brain	Eye	Gill	Intestine	Liver	Kidney	Heart	Muscle	Testis	Ovary	Others		
CYP2AA2	♀	○	○	n.s.	○	○	●	○	n.s.		○		Quantitative real-time PCR	Kubota et al. 2013
	♂	○	○	n.s.	○	○	●	○	n.s.	○			Quantitative real-time PCR	
CYP3A65	♂ / ♀	○	○	○	●	●	n.s.	○	n.s.	n.s.	n.s.		Quantitative real-time PCR	Tseng et al. 2005
CYP3C1	♀	×	○	○	●	●	n.s.	○	n.s.		●	○ Skin	Quantitative real-time PCR	Corley-Smith et al 2005
	♀	○	○	○	○	○	○	○	n.s.		●	○ Olfactory rosette Spleen	Quantitative real-time PCR	Shaya et al. 2014
	♂	○	●	○	○	●	○	○	n.s.	○		● Olfactory rosette ○ Spleen	Quantitative real-time PCR	Shaya et al. 2014
CYP3C2	♀	○	○	○	●	○	○	○	n.s.		●	○ Olfactory rosette Spleen	Quantitative real-time PCR	Shaya et al. 2014
	♂	○	○	○	○	○	○	○	n.s.	●		○ Olfactory rosette Spleen	Quantitative real-time PCR	Shaya et al. 2014
CYP3C3	♀	○	○	○	○	○	○	○			○	○ Olfactory rosette ○ Spleen	Quantitative real-time PCR	Shaya et al. 2014
	♂	○	○	○	●	○	○	○		○		○ Olfactory rosette Spleen	Quantitative real-time PCR	Shaya et al. 2014
CYP3C4	♀	○	○	○	○	○	○	○			○	○ Olfactory rosette Spleen	Quantitative real-time PCR	Shaya et al. 2014
	♂	●	●	○	●	○	○	○			○	○ Olfactory rosette Spleen	Quantitative real-time PCR	Shaya et al. 2014

Abbreviations:

n.s. not studied
× studied with negative result

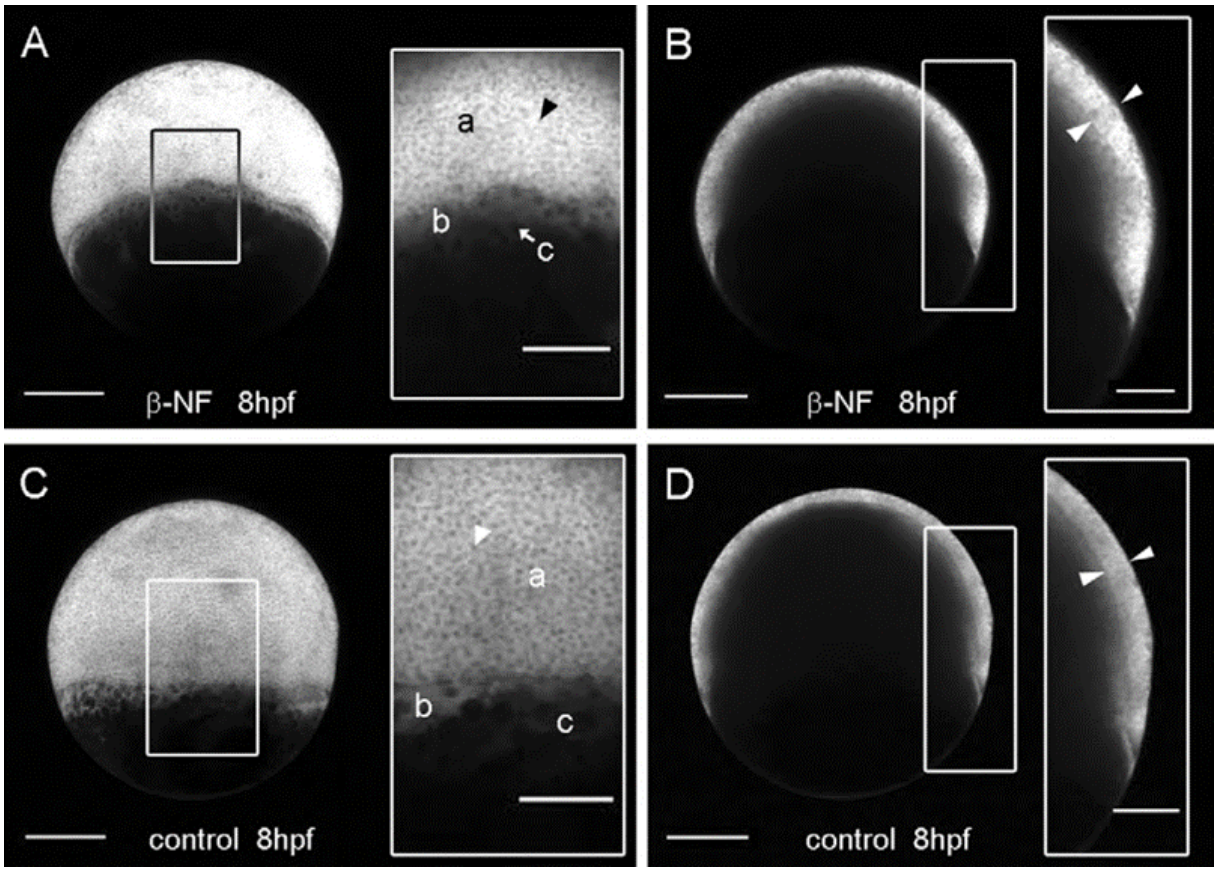
Comparison within the given CYP450 isoform:
○ weak expression
○ moderate expression
● strong expression

Spatial expression patterns of CYP 450 in embryonic and juvenile zebrafish (*Danio rerio*). From Lörracher & Braunbeck (2019)

2.8 CYP-dependent activities

For the understanding of metabolic capacity, it is important to measure CYP-dependent activity. Various preparations have been used to investigate CYP-dependent activities of zebrafish, including whole body, liver microsomes, S9 samples, heterologously expressed CYPs, as well as primary hepatocytes. Most studies have determined CYP-dependent activities using fluorescent-based catalytic activity assays (Braunig et al., 2015; Chng, 2013; Creusot et al., 2015; Jones et al., 2010; Jonsson et al., 2009; Otte et al., 2017; Otte et al., 2010; Pauka et al., 2011; Saad et al., 2016b; Scornaienchi et al., 2010; Verbueken et al., 2017; Wu et al., 2014). The problem with fluorescent-based assays is that their interpretation is complicated due to overlapping and still unknown substrate specificities of CYP isoforms in zebrafish (Scornaienchi et al., 2010). CYP1s have been most extensively studied of all the CYPs. By heterologous expression of CYP1A, CYP1B1, CYP1C1, CYP1C2, and CYP1D1 (e.g. over-expression of zebrafish CYP genes in E. coli or yeast), catalytic activities and specificities towards 11 fluorometric substrates (e.g. 7-ethoxyresorufin, 7-methoxyresorufin, 7-benzyloxyresorufin) have been demonstrated in zebrafish (Scornaienchi et al., 2010).

Figure 31: *In vivo* localization of EROD activity in 8 h old zebrafish (*Danio rerio*) embryos



In vivo localization of EROD activity at 8 hpf zebrafish (*Danio rerio*) embryos exposed to β-naphthoflavone (A, B) or artificial water only (C, D) imaged by CLSM at 8 hpf. Lateral views on the embryos (A, C) showed EROD activity in the cytoplasm of the envelope layer and of the layers below (a). EROD activity also appears in the yolk syncytial layer (b). Nuclei of the envelope layer are marked with arrowheads (A, C) and yolk granules are indicated (c). Sections at the median plane (B, D) indicated EROD activity in the developing germ layers (marked by arrowheads). Scale bar: 200µm (insets: 100µm); animal pole top. From Otte et al. (2010)

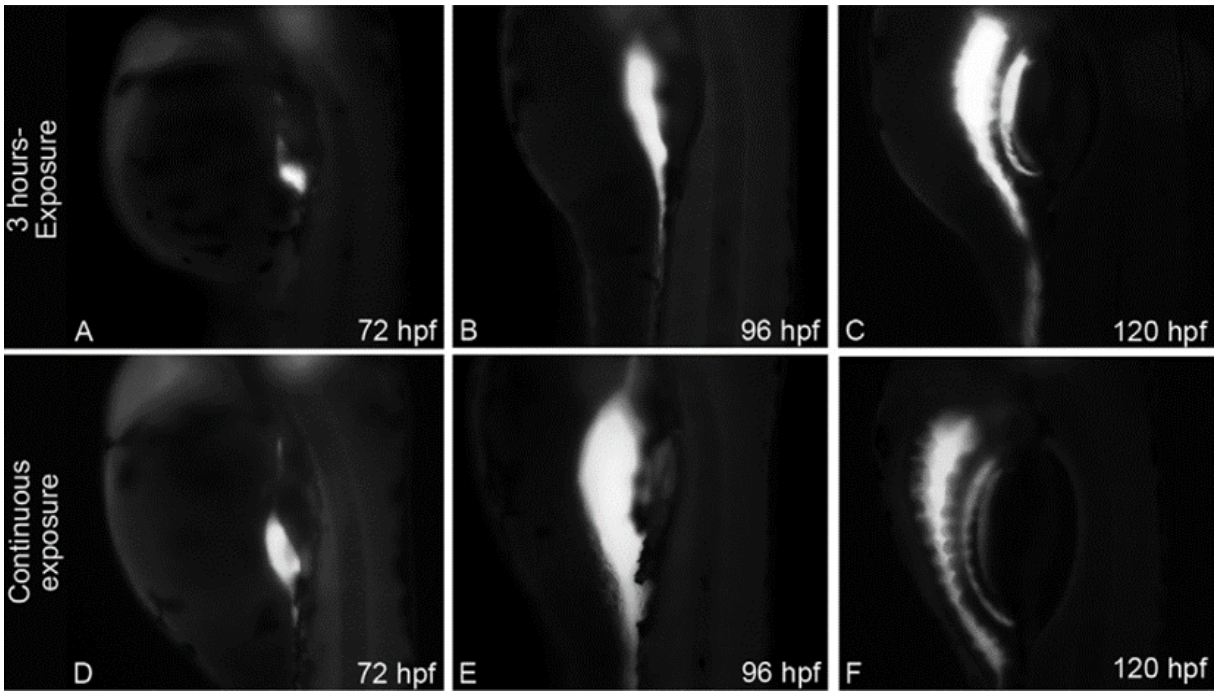
Commonly, ethoxyresorufin-O-deethylase (EROD) activity is used as marker for CYP1 activity. All CYP1s, especially CYP1A, metabolize 7-ethoxyresorufin into the highly fluorescent metabolite 7-hydroxyresorufin (Scornaienchi et al., 2010; Stegeman et al., 2015). In zebrafish, basal CYP1A activity has been detected from 2.5 hpf onwards (Otte et al., 2017). Because of the zebrafish embryo's transparency, CYP1A activity can be measured in vivo. By using confocal laser scanning microscopy, Otte et al. (2010) could demonstrate CYP1 activity as early as 8 hpf (Fig. 31).

Only recently, Kais et al. (2017, 2018) developed the technique by Otte et al. (2010) further and optimized it to a rapid screening test (Fig. 32). Kais et al. (2017, 2018) documented the applicability of in vivo EROD-imaging for a selection of model compounds (Fig. 33 - 37). For more details, see Kais et al. (2017).

Deethylation of 7-ethoxycoumarin (ECOD) is an established marker for CYP2 activities in mammals. Activity towards 7-ethoxycoumarin was detected at 96 hpf (Jones et al., 2010). Earlier life-stages of zebrafish have not been investigated, yet.

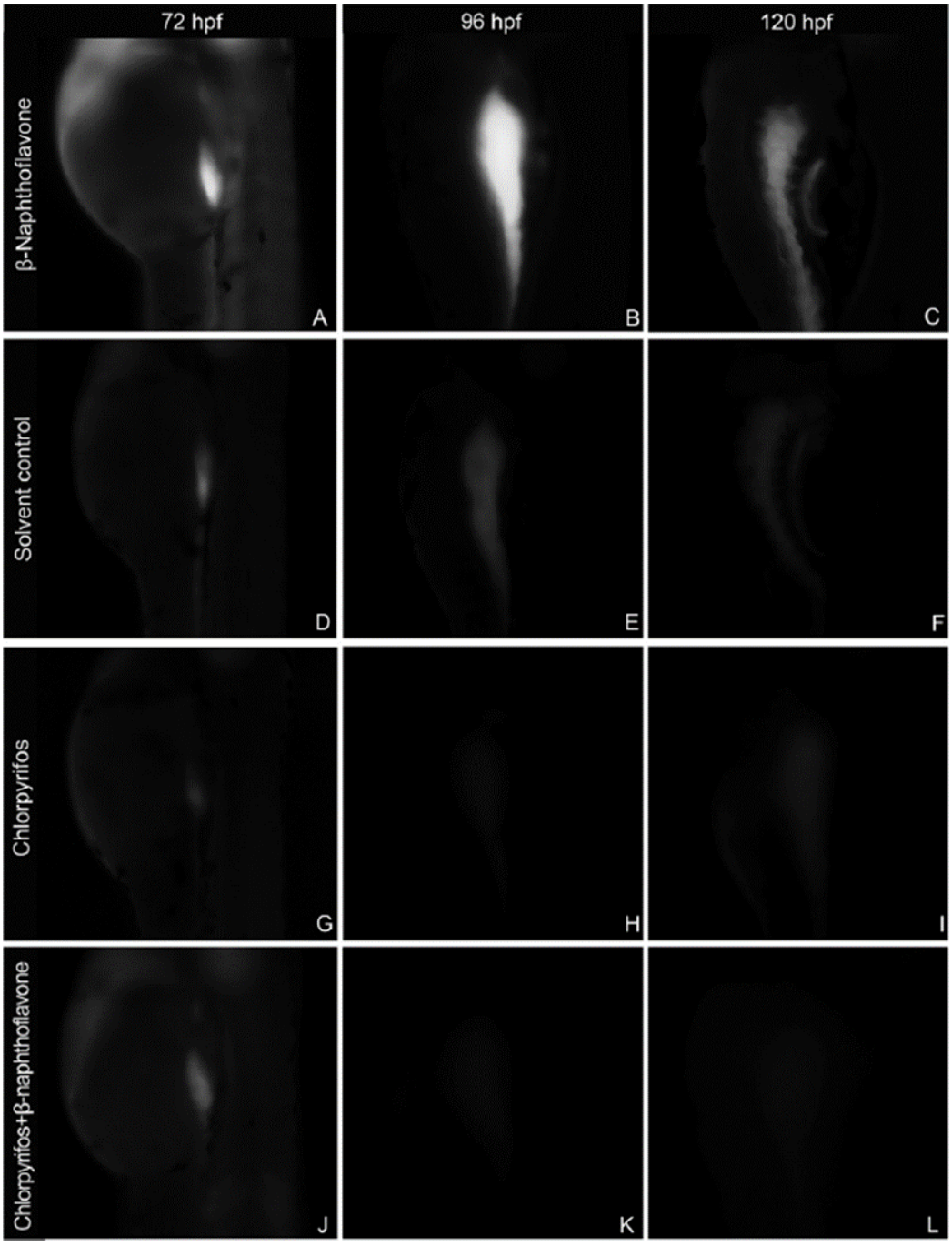
For human CYP3-dependent activities, several substrates are available (e.g. luciferin-derivatives, 7-benzyloxy-4-trifluoromethylcoumarin, and benzyloxy-methylresorufin). CYP3-dependent activities in zebrafish have been detected from 48 hpf onwards, but in most cases starting at 72 hpf (Chng, 2013; Creusot et al., 2015; Jones et al., 2010; Li et al., 2011b; Oziolor et al., 2017; Renwick et al., 2008; Verbueken et al., 2017).

Figure 32: EROD induction patterns in zebrafish (*Danio rerio*) embryos after 3 h pulse and continuous long-term exposure



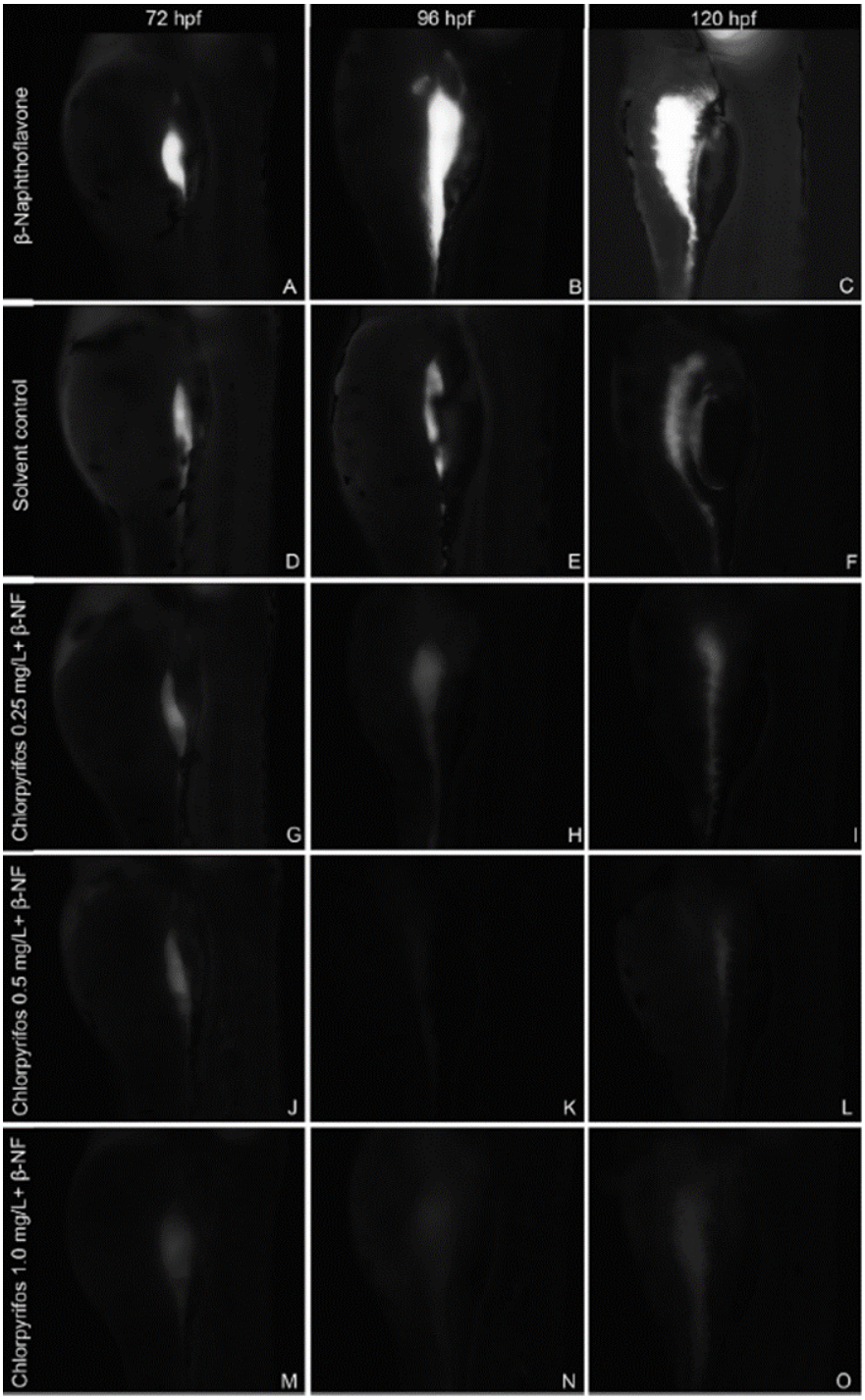
EROD induction patterns in zebrafish (*Danio rerio*) embryos after 72 h (A, D), 96 (B, E) and 120 h (C, F) of exposure to 10 µg/l β-naphthoflavone (maximum EROD induction) following 3 h (A - C) and continuous (D - F) exposure. There is no difference in signal intensity between 3 h and continuous exposure, except for the 120 h exposure, which gives a slightly lower signal following continuous exposure (F). The areas of fluorescence in the embryos exposed for 3 h appear smaller than those seen after continuous exposure. Number of experiments: n = 12 with 4 - 6 embryos each. Epifluorescence microscopy (exposure time: 40 ms). From Kais et al. (2017)

Figure 33: *In vivo* localization of EROD activities in zebrafish (*Danio rerio*) embryos after exposure to chlorpyrifos in various scenarios I



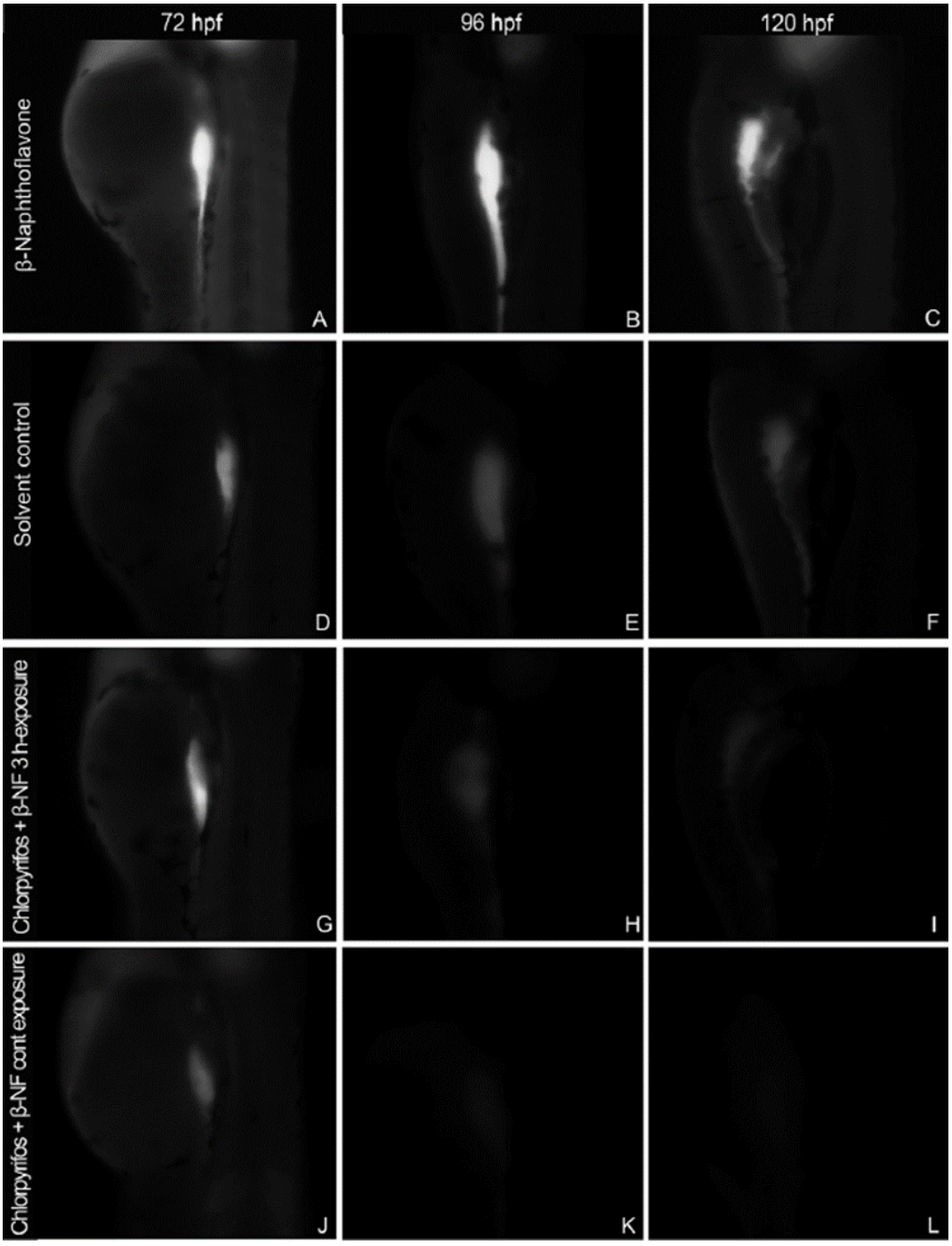
In vivo localization of EROD activities in zebrafish (*Danio rerio*) embryos after 72 (A, D, G, J), 96 (B, E, H, K) and 120 h (C, F, I, L) exposure to 10 µg/L β-naphthoflavone (A - C), solvent (0.1 % DMSO; D-F), 0.6 mg/L (EC_{10}) chlorpyrifos (G - I) and 0.6 mg/L chlorpyrifos + 10 µg/L β-naphthoflavone (J - L). In all developmental stages, embryos exposed to β-naphthoflavone show a bright signal in the liver, whereas embryos of the solvent control show a weaker signal reflecting constitutive activity. The signal intensities after single chlorpyrifos exposure as well as after co-exposure to chlorpyrifos and β-naphthoflavone show lower signal intensities than in the solvent control after both 96 and 120 h. The signal in the co-exposure approach after 72 h is as strong as that of the solvent control. Epifluorescence microscopy, exposure times: 60 ms (72 h), 30 ms (96 h), 40 ms (120 h). From Kais et al. (2018)

Figure 34: *In vivo* localization of EROD activities in zebrafish (*Danio rerio*) embryos after exposure to chlorpyrifos in various scenarios II



In vivo localization of EROD activities in zebrafish (*Danio rerio*) embryos after 72 (A, D, G, J,M), 96 (B, E, H, K, N) and 120 h (C, F, I, L, O) exposure to 10 µg/L β-naphthoflavone (A - C), solvent (0.1 % DMSO; D - F), 0.25 (G - I), 0.5 (J - L) and 1.0 mg/L chlorpyrifos (M - O)+ 10 µg/L β-naphthoflavone each. Embryos exposed to β-naphthoflavone show a bright signal in the liver, whereas the embryos of the solvent control show a weaker signal reflecting constitutive activity. Particularly after 96 and 120 h, signal intensities after co-exposure to 0.5 and 1.0mg/L chlorpyrifos and β-naphthoflavone were lower than in the solvent, whereas 0.25mg/L chlorpyrifos did not inhibit EROD activity completely (G - I). Epifluorescence microscopy, exposure times: 40 ms (72 h), 100 ms (96 h), 100 ms (120 h). From Kais et al. (2018).

Figure 35: In vivo localization of EROD activities in zebrafish (*Danio rerio*) embryos after exposure to chlorpyrifos in various scenarios III



In vivo localization of EROD activities in zebrafish (*Danio rerio*) embryos after 72 (A, D, G, J), 96 (B, E, H, K) and 120 h (C, F, I, L) exposure to 10 µg/L β-naphthoflavone (A - C), solvent (0.1 % DMSO; D - F) and 0.6 mg/L chlorpyrifos following 3 h (G - I) and continuous (cont.) exposure (J - L). In all developmental stages, embryos of short-term exposure to β-naphthoflavone show bright signals in the liver (A - C), whereas embryos of the solvent control show weaker signals (D - F) and demonstrate no differences to continuous exposure. Signals after short-term exposure to chlorpyrifos also show no differences, if compared to continuous exposure, except for the short-term exposure of 72 h embryos (G), which show stronger fluorescence than after continuous exposure (J) and are similar to the signal of the solvent control. Epifluorescence microscopy, exposure times: 60 ms (72 h), 30 ms (96 h), 40 ms (120 h). From: Kais et al. (2018)

2.9 Inducibility of cytochrome P450s in zebrafish

Various CYPs such as CYP1A, CYP1B1, CYP1C1/C2 and CYP3A65 can be induced or inhibited by xenobiotics (Braunig et al., 2015; Cunha et al., 2016; Glisic et al., 2016; Kubota et al., 2013; Tseng et al., 2005). In many cases, the induced CYP is at the same time capable of metabolizing the inducing agent (or other xenobiotics) (Zhu, 2010). Induction of CYP activities, especially in the liver, can therefore lead to accelerated xenobiotic metabolism (Celander, 2011).

In zebrafish, xenobiotic-mediated induction of CYP1s is well documented as, e.g., expression, protein abundance, and 7-ethoxyresorufin-*O*-deethylase activity. In zebrafish, CYP1A, CYP1B1, CYP1C1, and CYP1C2 are induced by aryl hydrocarbon receptor (AhR) agonists such as polycyclic aromatic hydrocarbons (Braunig et al., 2015; Jonsson et al., 2007b). In contrast, CYP1D1 was found not to be inducible by AhR agonists such as PCB126 and TCDD (Goldstone et al., 2009). In contrast, exposure to caffeine, ibuprofen, or carbamazepine (0.05 - 5 µM) leads to a significant down-regulation of CYP1A (Aguirre-Martinez et al., 2017). For the CYP families 2, 3, and 4, mechanism of down- and upregulation, inductors, and inhibitors, are less well understood.

Starting from 8 hpf onwards, induction of CYP1s has been shown to occur in all developmental stages of zebrafish embryos. However, tissue distribution and maximum inducibility vary substantially between developmental stages (Aguirre-Martinez et al., 2017; Cunha et al., 2016; Glisic et al., 2016; Jones et al., 2010; Kubota et al., 2015; Liu et al., 2016; Otte et al., 2010; Zhang et al., 2015).

When assessing metabolization capacities based on CYP expression and activity, respectively, potentially adverse effects by the xenobiotic itself should be considered whenever possible.

2.10 Bioactivation capacity of early-life stages: functional confirmation of CYP activity

It is assumed that only about one quarter of all carcinogenic xenobiotics are tumorigenic in their non-metabolized form; the remaining 75 % xenobiotics require CYP-mediated metabolic activation before elucidating their full carcinogenicity, toxicity, or teratogenicity (Fantel, 1982; Nebert and Dalton, 2006). In fact, a limited biotransformation capacity can lead to an underestimation of protoxicants or proteratogens (Busquet et al., 2008b; Kluver et al., 2014).

So far, the only protoxicant known to be less toxic to zebrafish early-life stages than to the adult and juvenile zebrafish is allyl alcohol (Kluver et al., 2014). In mammals, allyl alcohol is metabolized *via* oxidation into the highly reactive metabolite acrolein. In zebrafish embryos, the reduced toxicity of allyl alcohol is caused by a lack of alcohol dehydrogenase 8a (adh8a), which is responsible for the oxidative formation of acrolein (Kluver et al., 2014).

Concerning proteratogens, there is prominent evidence that zebrafish embryos possess significant biotransformation capacities. Weigt et al. (2011) demonstrated that ten well-known proteratogens were teratogenic in zebrafish embryos exposed for 3 days (Table 6). All ten substances tested (2-acetylaminoflurene, benzo[a]pyrene, aflatoxin B1, carbamazepine, phenytoin trimethadione, cyclophosphamide, ifosfamide, tegafur, and thio-TEPA), are known to undergo CYP-mediated bioactivation in mammals. The result suggests that zebrafish early-life stages (< 72 hpf) are capable of testing proteratogenic substances, even without addition of an exogenous metabolic activation system (e.g. S9 mix, microsomes; Weigt et al., 2011).

Table 6: LC_{50}, EC_{50} and teratogenicity index (TI) of selected proteratogens in 3 d old zebrafish (*Danio rerio*) embryos

Substance	LC_{50}	EC_{50}	TI (LC_{50}/EC_{50})
2-Acetylaminofluorene	6.9 µM	–	< 1
Benzo[a]pyrene	5.1 µM	0.52 µM	9.81
Aflatoxin B1	2.3 µM	2.2 µM	1.05
Carbamazepine	> 500 µM	222 µM	> 1
Phenytoin	> 250 µM	386 µM	> 1
Trimethadione	45.7 mM	23.5 mM	1.95
Cyclophosphamide	8.4 mM	4.7 mM	1.79
Ifosfamide	3.2 mM	3.1 mM	1.03
Tegafur	30.3 mM	3.4 mM	8.91
Thio-TEPA	306 µM	53.2 µM	5.76

LC_{50}, EC_{50} and teratogenicity index (TI) of 2-acetylaminoflurene, benzo[a]pyrene, aflatoxin B1, carbamazepine, phenytoin trimethadione, cyclophosphamide, ifosfamide, tegafur, and thio-TEPA d old zebrafish (*Danio rerio*) embryos. From Weigt et al. (2011)

2.11 Metabolism capacity – chemical analysis

Advances in chemical analytical methods greatly contributed to the understanding of metabolic activities (Guengerich, 2000). Highly sensitive methods (e.g. liquid chromatography tandem-mass spectrometry) allow the detection and measurement of low abundance metabolites in body homogenates and water. Depletion of substances or formation of metabolites allow conclusions to be drawn about metabolic processes and activities of enzymes (Li et al., 2011b).

In zebrafish, studies have been primarily focusing on metabolism of human drugs such as paracetamol, benzocaine, bupropion, verapamil, and phenacetin (Alderton et al., 2010; Brox et al., 2016b; Chng et al., 2012; Jones et al., 2010; Kantae et al., 2016; Saad et al., 2017). So far, several phase-I- and phase-II-related biotransformation products have been described. Based on mass analysis and comparison to standards, the molecular structure of occurring transformation products can be estimated (Alderton et al., 2010).

Studies comparing xenobiotic metabolism of embryonic, juvenile, and adult life-stages are rare. Since there are different analytical approaches using different methods as well as targets (e.g. liver microsomes, primary hepatocytes, whole body homogenates) comparability is not given among the available studies. No investigation yet has systematically studied xenobiotic metabolism within all developmental life-stages of zebrafish. Especially for juvenile zebrafish life-stages, there is hardly any information available (Table 7).

Occurrence of seven transformation products after three hours exposure to clofibric acid, depletion of benzocaine, and increase of its phase II metabolites (4-aminobenzoic acid, 4-acetaminobenzoic acid) indicate early (< 24 hpf) presence of metabolic activity in zebrafish (Brox et al., 2016a; Brox et al., 2016b). In most cases, such as dextromethorphan, diclofenac, and phenacetin, the maximum number and highest level of metabolites appear post hatch (Alderton et al., 2010; Brox et al., 2016b; Chng et al., 2012; Saad et al., 2017). For testosterone, e.g., metabolism capacity of zebrafish early-life stages (< 120 hpf) appears rather limited (Table 8).

Table 7: Phase I and phase II metabolites identified in zebrafish (*Danio rerio*)

Substance	Embryo (< 5 dpf)			Juvenile (> 6 dpf)			Adult (> 3.5 mpf)			References
	Biotransformation	Metabolites identified		Biotransformation	Metabolites identified		Biotransformation	Metabolites identified		
		Phase I	Phase II		Phase I	Phase II		Phase I	Phase II	
2-Acetylaminofluorene	72 hpf Bioactivation of proteratogen: - teratogenic effects	•••		n.s.			n.s.			Weigt et al. 2011
Acetaminophen Paracetamol	24 hpf Quantification of: - Paracetamol-sulfate - Paracetamol-glucuronide Level of metabolites below or close to the limit of quantification 72 hpf Bioactivation of proteratogen: - No teratogenic effects after exposure (2 - 3 hpf) - Co-incubation with metabolic activation system (MAS) leads to significant increase of affected embryos 96 hpf - Acetaminophen-sulfate - Acetaminophen-glutathione			n.s.			Liver microsomes - N-acetyl-p-benzoquinone imine (NAPQI)			Ching et al. 2012, Weigt et al. 2010, Kanbak et al. 2016, Jones 2010
Aflatoxin B_1	72 hpf Bioactivation of proteratogen: - teratogenic effects	•••		n.s.			- Aflatoxicol - Aflatoxicol-glucuronide - Unidentified polar metabolite - Aflatoxin-epoxide	✓	✓	Weigt et al. 2011, Troxel et al. 1997
Albendazole	2- 34 hpf High spontanous transformation to albendazole sulfoxid after 10 h (even in control). It is not shure if embryos contribute to conversation	•••		144 hpf - Albendazole sulfoxide - Albendazole sulfone - Albendazole-2-aminosulfone Albendazole was reduced to a high extend at lower concetrations. At higher concentration the reduce in concentration was not observed or much less evident.		✓	n.s.			Carlsson et al. 2011, Carlsson et al. 2013, Mattsson et al. 2012
Amiodarone	n.s.			n.s.			- Hydroxy-amiodarone - Mono-N-desethylamiodarone - Hydroxy-mono-N-desethylamiodarone	✓		Poon et al. 2017
Benzo[a]pyrene	24-40 hpf Bioactivation of proteratogen: - increased mutant frequency in the rpsL target gene 72 hpf Bioactivation of proteratogen: teratogenic effects	•••		n.s.			Bioactivation of proteratogen: - Formation B[a]P-DNA adducts			Weigt et al. 2011, Hsu et al. 1996 Amanuma et al. 2002,

Substance	Embryo (< 5 dpf)			Juvenile (> 6 dpf)			Adult (> 3.5 mpf)			References
	Biotransformation	Metabolites identified		Biotransformation	Metabolites identified		Biotransformation	Metabolites identified		
		Phase I	Phase II		Phase I	Phase II		Phase I	Phase II	
Benzocain	> 24 hpf -4-aminobenzoic acid 24 -96 hpf hpf -4-aminobenzoic acid -4-acetaminobenzoic acid		✓	n.s.			n.s.			Brox et al. 2016
Benzophenone-2 (BP2)	96 hpf - Two mono-glucuronidated metabolites - Mono-sulfated metabolite - Di-sulfated metabolite - Gluruonidated and sulfated metabolite		✓	n.s.			- Two mono-glucuronidated metabolites - Mono-sulfated metabolite - Di-sulfated metabolite - Gluruonidated + sulfated metabolite - Di-glucuronidated metabolite		✓	Fol et al. 2017
Berberine	n.s.			n.s.			- Demethylated metabolite - Reduced metabolite - Glucuronidated metabolite - Sulfated metabolite	✓	✓	Li et al. 2014
Bisphenol S (BPS)	96 hpf - Mono-glucuronidated metabolite - Mono-sulfated metabolite		✓	n.s.			- Mono-glucuronidated metabolite - Mono-sulfated metabolite		✓	Fol et al. 2017
Bupropion	n.s.			168 hpf - Hydroxybupropion	✓		n.s.			Alderton et al. 2010
Calycosin	72-96 hpf 7 ometabolites could be measured continously in zebrafish larvae. All of them showed steady increase during the experimental period. - Mono-glucuronide metabolite - Mono-glucosylated metabolite - Mono-sulfated metabolite - Di-conjugated: glucosylated and sulfated metabolite - Di-conjugated: gluruonidated and glucossylated metabolite - Di-conjugated: glucuronidated and sulfated metabolite - Mono-hydroxylated metabolite	✓	✓	n.s.			n.s.			Hu et al. 2012
Carbamazepine	72 hpf Bioactivation of proteratogen: - teratogenic effects	---		n.s.			n.s.			Weigt et al. 2011
Cisapride	72 hpf No metabolite detected			168 hpf - Cisapride N-sulfate		✓	n.s.			Alderton et al. 2010
Clofibric acid	7 hpf - 7 Transformation products 18 hpf - 14 Transformation products 52 hpf - 72 hpf - 18 Transformation products	✓	✓	n.s.			n.s.			Brox et al. 2016
Coptisine	n.s.			n.s.			- Demethylated metabolite - Reduced metabolite - Methylated matbolite	✓		Li et al. 2014

Substance	Embryo (< 5 dpf)			Juvenile (> 6 dpf)			Adult (> 3.5 mpf)			References
	Biotransformation	Metabolites identified		Biotransformation	Metabolites identified		Biotransformation	Metabolites identified		
		Phase I	Phase II		Phase I	Phase II		Phase I	Phase II	
Cyclophosphamide	48 hpf Bioactivation of proteratogen: - no teratogenic effects after incubation (2 - 3 hpf) 72 hpf Bioactivation of proteratogen: - teratogenic effects	•••		n.s.			n.s.			Weigt et al. 2011, Busquet et al. 2008
Dextromethorphan	Whole embryo microsomes: 5 hpf- 48 hpf - 3-Methoxymorphinan dextrorphan :below the limit of quantification 72 hpf - Dextrorphinan - 3-Methoxy: below the the lower limit of detection 96 hpf - Dextrorphan: significant higher levels than at 120 hpf - 3-Methoxymorphinan: unquantifiable 120hpf - Dextrorphan - 3-Methoxymorphinan: unquantifiable	✓		168 hpf - O-desmethyl dextromethorphan - N-desmethyl dextromethorphan - On the methoxyphenyl ring hydroxylated metabolite - Dextrophan	✓		Liver microsomes: - 3-Methoxymorphinan - Dextrorphan	✓		Saad et al. 2017, Alderton et al. 2010
Diclofenac	Whole embryo microsomes: 5 hpf - 72 hpf - No metabolite detected 96 hpf - Hydroxylated metabolite (close to the lower limit of detection) - Level of hydroxylated metabolites 10 times lower than in liver microsomes of adults.	✓		168 hpf - Hydroxy-diclofenac			Liver microsomes: - 4 hydroxy-diclofenac - 5-hydroxy-diclofenac (Distinction not possible)	✓		Alderton et al. 2010, Saad et al. 2017
Epiberberine	n.s.			n.s.			- Demethylated metabolite - Methylated matbolite	✓		Li et al. 2014
Febantel	n.s.			144 hpf - Fenbendazole - Oxfendazole	✓		n.s.			Carlsson et al. 2013
Fenbendazole	n.s.			144 hpf - Oxfendazole	✓		n.s.			Carlsson et al. 2013
Ibuprofen	96 hpf - Hydroxyibuprofen	✓			✓		n.s.			Jones et al. 2008, Jones et al. 2012
Ifosfamide	72 hpf Bioactivation of proteratogen: - teratogenic effects	•••		n.s.			n.s.			Weigt et al. 2011
Jatrorrhizine	n.s.			n.s.			- Demethylated metabolite - Methylated matbolite - Hydroxylated metabolite - Glucuronidated metabolite - Sulfated metabolite	✓	✓	Li et al. 2014
Lauric acid	n.s.			168 hpf - More polar, earlier eluting metabolites (HPLC)	•••	•••	n.s.			Alderton et al. 2010,

Substance	Embryo (< 5 dpf)			Juvenile (> 6 dpf)			Adult (> 3.5 mpf)			References
	Biotransformation	Metabolites identified		Biotransformation	Metabolites identified		Biotransformation	Metabolites identified		
		Phase I	Phase II		Phase I	Phase II		Phase I	Phase II	
Midazolam (MDZ)	5-120 hpf (microsomes) - No depletion of midazolam 72 hpf Five metabolites detected: - Mono-oxydated metabolite - Oxydated metabolite - N-demethylated metabolite - Di-oxydated metabolite	✓		168 hpf A total of 5 metabolites - Mono-oxydated metabolite - Oxydated metabolite - N-demethylated metabolite - Dioxydated metabolite	✓		low level of - 1'-Hydroxymidazolam - 4- Hydroxymidazolam - N-Glucuronidation metabolites Liver microsomes - No depletion of midazolam - Low, non-quantifiable concentrations of 1-hydroxy-midazolam	✓	✓	Alderton et al. 2010 Ching et al. 2012, Saad et al. 2017, Poon et al. 2017
Nefazodone	n.s.			n.s.			- Hydroxy-nefazodone	✓		Poon et al. 2017
Palmatine	n.s.			n.s.			- Demethylated metabolites - Hydroxylated metabolites - Glucuronidated metabolite - Sulfated metabolite	✓	✓	Li et al. 2014
Phenacetin	< 24 hpf - Paracetamol 24 -96 hpf - Paracetamol - Paracetamol glucuronide - Paracetamol sulfate	✓	✓	n.s.			n.s.			Brox et al. 2016
Phenytoin	72 hpf Bioactivation of proteratogen: - teratogenic effects	---		n.s.			n.s.			Weigt et al. 2011
Tacrine	n.s.			168 hpf - Hydroxy-tacrine	✓		n.s.			Alderton et al. 2010
Tegafur	72 hpf Bioactivation of proteratogen: - teratogenic effects	---		n.s.			n.s.			Weigt et al. 2011
Testosterone	Whole embryo microsomes: 5 hpf - 120hpf - No substrate consumption 120 hpf In total 2 hydroxytestosterone metabolites - Main metabolite, was unique, and not found in microsomes of adult zebrafish - 6ß-Hydroxytestosterone - testosterone glucuronide	✓		168 hpf - Testosterone glururonide conjugate - One hydroxylated metabolite	✓	✓	Liver microsomes In total 6 hydroxylated metabolites with several isomers - 6ß-Hydroxytestosterone - 16ß-Hydroxytestosterone - 2α-Hydroxytestosterone - Other metabolites not identified.	✓		Alderton et al. 2010, Ching et al. 2012, Saad et al. 2017
Thio-TEPA	72 hpf Bioactivation of proteratogen: - teratogenic effects	---		n.s.			n.s.			Weigt et al. 2011
Trimethadione	72 hpf Bioactivation of proteratogen: - teratogenic effects	---		n.s.			n.s.			Weigt et al. 2011
Triphenyl phosphate (TPHP)	n.s.			n.s.			- Diphenylphosphate - Monohydroxylated diphenylphosphate - Monohydroxylated triphenylphosphate - Dihydroxylated triphenylphosphate - Glucuronic acid conjugated metabolites (after hydroxylation) Study of 10 additional metabolites: non of these metabolites were detectable or below the detection limit.	✓	✓	Wang et al. 2016

Substance	Embryo (< 5 dpf)			Juvenile (> 6 dpf)			Adult (> 3.5 mpf)			References
	Biotransformation	Metabolites identified		Biotransformation	Metabolites identified		Biotransformation	Metabolites identified		
		Phase I	Phase II		Phase I	Phase II		Phase I	Phase II	
Valproic acid	72 hpf Bioactivation of proteratogen: - teratogenic effects	...		n.s.			n.s.			Weigt et al. 2011,
Verapamil	72 hpf In total 12 metabolites - Oxidated metabolites - N-, or O-demethylated metabolites - Glucuronide conjugated metabolites - N-dealkylated metabolites	✓	✓	168 hpf In total 12 metabolites - Oxidated metabolites - N-, or O-demethylated metabolites - Glucuronide conjugated metabolites - N-dealkylated metabolites	✓	✓	n.s.			Alderton et al. 2010,

Abbreviations:

n.s. not studied

✓ studied with positive result

... further clarification is needed

Biotransformation metabolites identified in embryonic, juvenile, and adult stage zebrafish. From Lörracher and Braunbeck (20199

Table 8: Testosterone metabolite profiles zebrafish (*Danio rerio*) at various developmental stages

Metabolites	Embryo (microsomes)	Juvenile	Adult (microsomes)
Phase I	5-120 hpf: no testosterone consumption 120 hpf: two hydroxylated metabolites: (1) main metabolite, unique, and not found in microsomes of adult zebrafish, (2) 6β-hydroxytestosterone	168 hpf: hydroxylated metabolite	Six hydroxylated metabolites with several isomers: 6β-hydroxytestosterone 16β-hydroxytestosterone 2α-hydroxytestosterone Three not identified metabolites
Phase II	Testosterone glucuronide conjugate	Testosterone glucuronide conjugate	Not studied

Testosterone metabolite profiles of embryo, juvenile, and adult zebrafish (*Danio rerio*) reveal differences in the metabolism capacity. Data from Alderton et al. (2010) and Saad et al. (2017), compiled by Lörracher and Braunbeck (2019)

2.12 Conclusions and recommendations for future research into the biotransformation capacities of zebrafish (*Danio rerio*)

From the considerations above, the following conclusions can be drawn:

- So far, zebrafish embryos, juveniles and adults have been studied to a very different extent with respect to their biotransformation capacities. Since especially juveniles have been neglected largely, transfer of biotransformation data from juvenile fish to adult fish does not appear justified. Overall, our knowledge about xenobiotic transformation capacities in zebrafish can – at best – be called fragmentary.
- However, whenever studied in more detail, biotransformation in zebrafish embryos could be documented. So far, only for rare exceptions such as allyl alcohol and albendazol, a lack of biotransformation (allyl alcohol to acrolein) could be demonstrated due to a lack of the enzyme required (alcohol dehydrogenase in the case of allyl alcohol).
- Studies on biotransformation gene expression (induction) is not as conclusive as studies in enzyme biosynthesis (transcriptomics, proteomics) or – even better – biochemical activity of enzymes. Since our knowledge on the spectrum of substances accepted as substrates by various cytochrome P450 isoforms is little, there is no way to extrapolate gene activation to biochemical functionality.
- There is no adequate database to extrapolate observations from mammalian studies to fish and fish embryos. For many P450 isoforms, we do not have conclusive evidence of congruence in cytochrome P450 terminology between mammalian studies and studies in

lower vertebrates. Therefore, transfer of a link between nomenclature and metabolic function from mammals to fish or vice versa ("same nomenclature means same function") is not necessarily justified.

- With respect to biotransformation phase II reactions, our knowledge is even more fragmentary than for phase I metabolization.
- In case there is suspicion from studies with mammalian models that metabolic activation or inactivation by biotransformation might play a role for the toxicity profile of a given substance, additional experiments with external biotransformation via, e.g., mammalian S9 preparations may help to compensate for a potential lack of biotransformation in zebrafish.
- As a gold standard for studies into biotransformation capacities in (zebra)fish embryos, juveniles and adults, gene expression studies need to be linked to transcriptome / proteome analysis data as well as chemical analytical analyses into the metabolites formed by cytochrome P450 enzymatic activity. Such studies, however, are most challenging in terms of expertise, resources, time, number of animals used and funding.
- Given the limitations of resources and the multitude of potential substrates for cytochrome P450 conversion, studies providing indirect evidence of the existence of active biotransformation seem more appropriate for screening purposes. Such indirect evidence may come from
  - studies designed to visualize the formation of fluorescent or colored metabolites (at best, in living embryos: in situ live imaging); unfortunately, such studies so far suffer from an apparent lack of appropriate fluorescent metabolites;
  - studies based on chemical analyses of emerging metabolites; however, in many instances, protocols for the chemical identification of metabolites still need to be developed;
  - studies based on the detection of biological effects by substances activated by cytochrome P450 action.

3 Analysis of the relevance and adequateness of the Fish Acute Toxicity Test (AFT) according to OECD TG 203 to fulfil the information requirements and addressing concerns under REACH

3.1 Summary

The aim of this study was a re-evaluation of historical AFT data produced according to OECD TG 203. The selection of compounds used for the present study was identical to that used by (Scholz et al., 2016) for the analysis of the relevance and adequateness of using the Fish Embryo Acute Toxicity Test (FET; OECD TG 236) to fulfill the information requirements and to address concerns under REACH. The data were retrieved from the ECHA database in July 2017 and were analyzed for the relevance and adequateness of using the AFT to fulfill the information requirements and addressing concerns under REACH. The selection criteria used in the present study to obtain the AFT database are identical to those that were used for the ECHA FET study (Scholz et al., 2016; Sobanska et al., 2018).

Starting with a dataset of 2936 studies of a total of 1842 substances, in a first step (I), all studies with LC_{50} entries not precisely defined (e.g. LC_{50} data given as range only) were removed. In the second step (II), duplicate studies were eliminated. These two initial steps reduced the number of studies to 56.4 % of all studies and 54.3 % of the test substances originally contained in the dataset. Further filtering steps included: (III) studies with LC_{50} values above half the water solubility; (IV) studies into substances with log P_{OW} > 4, when test concentrations were not verified by chemical analytics; and (V) inorganic substances. Application of filtering steps 3 to 5 resulted in a remainder of 38 % of studies and of 38 % of the substances of the original dataset.

An intra- and interspecies comparison of the remaining data revealed robust values for AFT data: The analysis illustrated that almost all studies of intra- and interspecies comparison (93.45 % and 96.22 %) lie within a deviation range between 1 and 100, which is currently accepted within the REACH process. A comparison of AFT data from the present study to the data from the corresponding FET study (Scholz et al., 2016; Sobanska et al., 2018) showed some comparability, although not all filtering steps were identical in both studies. Namely, the filtering steps “Keep studies with predicted pH at saturation >5 or <9” and “Remove studies with no toxicity and maximum test concentration < 10fold above baseline toxicity” were not performed in the analysis of AFT studies due to the tools being unavailable. In the end, when only zebrafish (*Danio rerio*) studies are considered (as was done in the ECHA FET study), the potential for elimination of data from the database was similar for both FET and AFT data. However, the rationale for only using zebrafish data is debatable, because data from other fish species is used for regulatory purposes and also correlates well with FET data (Belanger et al., 2013). Since these findings indicate that the robustness of FET and AFT data are similar, the suitability of the FET for REACH purposes should be reconsidered, and political action(s) should be taken to make FET data acceptable for application in REACH.

With regard to the use of OECD 203 for regulatory purposes, more attention should be paid to the physicochemical properties of the test substances and to the correct preparation and use of test substance concentrations. Additionally, chemical verification of test concentrations should be mandatory for substances with log P_{OW} > 4 or tested above water solubility for a better reliability of the data.

3.2 Introduction

The ECHA database represents a valuable tool to quickly obtain information on chemical components. Apart from substance description and physical and chemical properties, it provides information on the environmental fate and pathways as well as ecotoxicological and toxicological information. Given the rapid pace of scientific developments and the significant increase of information within the REACH process, the ECHA database undergoes constant modification of data and is updated frequently.

The aim of the present study was a retrospective re-evaluation of a dataset for the short-term Acute Fish Toxicity Test (AFT) according to OECD TG 203, which had been retrieved from the ECHA database (https://www.echemportal.org/echemportal/propertysearch/) in July 2017, with respect to its suitability for the information requirements and addressing concerns under REACH. Furthermore, the study has been designed as an analysis in parallel to the already existing analysis of FET data conducted by Scholz et al. (2016).

3.3 Treatment of datasets

The dataset was extracted in July 2017 and has been worked with since then, being aware that some data especially on significant ecotoxicological studies in the REACH process may have been changing in the meantime. Therefore, this analysis should be regarded as a snapshot of the AFT data available in the ECHA database from July 2017.

In collaboration with Dr. Stefan Scholz and Dr. Nils Klüver (UfZ Leipzig), the ECHA database was screened for historical AFT data. All studies performed with the major species used for ecotoxicological testing, rainbow trout (*Onchorhynchus mykiss*), carp (*Cyprino carpio*), fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*), Japanese medaka (*Oryzias latipes*), bluegill sunfish (*Lepomis macrochirus*) and guppy (*Poecilia reticulata*) were incorporated.

Table 9: Overview of the search criteria in the ECHA database for historical AFT data

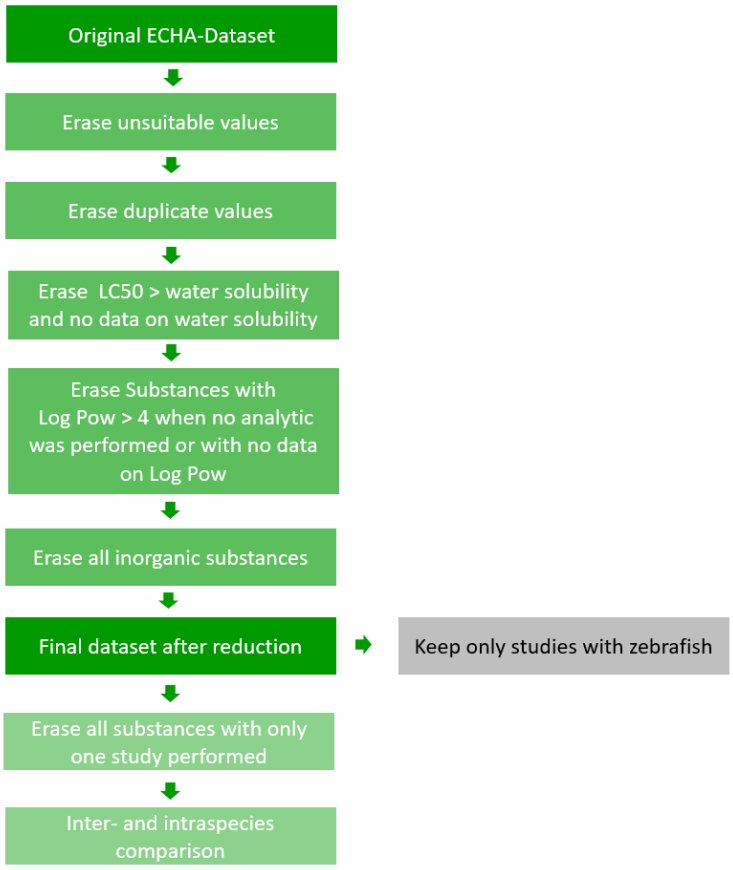
Search criterion	Keyword
Study result type	Experimental result
Reliability (Klimisch score)	1, 2
Test guideline	OECD Guideline 203
Test guideline, qualifier	According to, equivalent or similar to
GLP compliance	yes
Test type	Static, semi-static, flow-through
Test type; Water media type	all
Total exposure	96 h
Effect concentrations, Endpoint	LC_{50}
Effect concentrations, Effect conc.	overlapping 0 – 10,000 g/L
Effect concentrations, Basis for effect	all

Overview of the search criteria in the ECHA database for historical Acute Fish Toxicity data

In order to include preferably all AFT data, studies containing old species names such as *Salmo gairdneri* for rainbow trout and *Brachydanio rerio* for zebrafish were also included. Subsequently, the search criteria listed in Tab. 9 were used.

Primary search results were organized in an Excel spreadsheet. Subsequent analyses were performed on the Excel sheet with the acute toxicity data in fish, as obtained from the ECHA database and modified by means of the KNIME software (www.knime.org) by Dr. Stefan Scholz. The actual analyses were conducted using modified versions of the Excel data sheet as well as a KNIME workflow and appropriate pivot tables and filters. The final search dataset after re-formatting with KNIME consisted of a collection of 2936 studies covering 1842 substances. The single consecutive steps of the analysis are summarized in Fig. 36.

Figure 36: Overview on the filtering steps in the ECHA dataset



Flow-chart representation of the filtering steps in the ECHA dataset

Step I: Filtering of unsuitable LC_{50} values

In order to obtain suitable LC_{50} concentrations, the dataset was first filtered by removing any data from plates with unsuitable entries (e.g. "LC_{50} = x to y mg/L"; "LC_{50} >..." or "LC_{50} <..." or acute tests without concentration units (e.g. g/ha or ml/ha).

Step II: Filtering of duplicate studies

By revising the dataset, it became obvious that numerous studies on one substance showed the same (sequence of) LC_{50} values as those of other substances. Thus, it was assumed that data for either single studies or structurally related substances were duplicated in the dataset. These studies were removed, if either no studies for analogues of the corresponding substances could be found in the ecotoxicological information in the ECHA database or in some cases it became

obvious that mixtures of different components were used that were enlisted separately in the dataset (e.g."A mixture composed primarily of resin acids and modified resin acids such as dimers and decarboxylated resin acids" was used to cover all substances with overall 10 studies).

Steps III – V: Identification of physicochemical properties

The ECHA database was checked for the most important physicochemical properties of the substances including water solubility and log P_{OW}.

In the third step, all studies tested with an LC_{50} above the water solubility were excluded. In the fourth step, the criteria were exacerbated by eliminating all studies from the dataset, if the LC_{50} was higher than half the level of water solubility. Substances without any specifications on water solubility were also excluded.

In the fourth step, a similar procedure was used for substances with a log Pow > 4 with the exception, that substances with log Pow > 4 were kept, if the studies were performed with a chemical analysis.

In the fifth step, all remaining studies performed with inorganic substances were removed.

Studies with zebrafish (*Danio rerio*)

Since the purpose of this study was also to compare the AFT dataset to the FET dataset from a recent study, in a final step the remaining dataset was filtered for Acute Fish Toxicity Tests, performed with the zebrafish (*Danio rerio*).

Intra- and interspecies comparison

For a better appreciation of the reliability and consistency of LC_{50} data, inter- and intraspecies comparisons were performed. For this purpose, all substances with only one single entry per substance in the database were excluded, since for these neither intra- nor interspecies comparisons could be made.

For the interspecies comparison, the dataset was filtered to include all substances, where studies for more than one fish species were available. For the intraspecies comparison, all substances analyzed in more than one study and fish species were used.

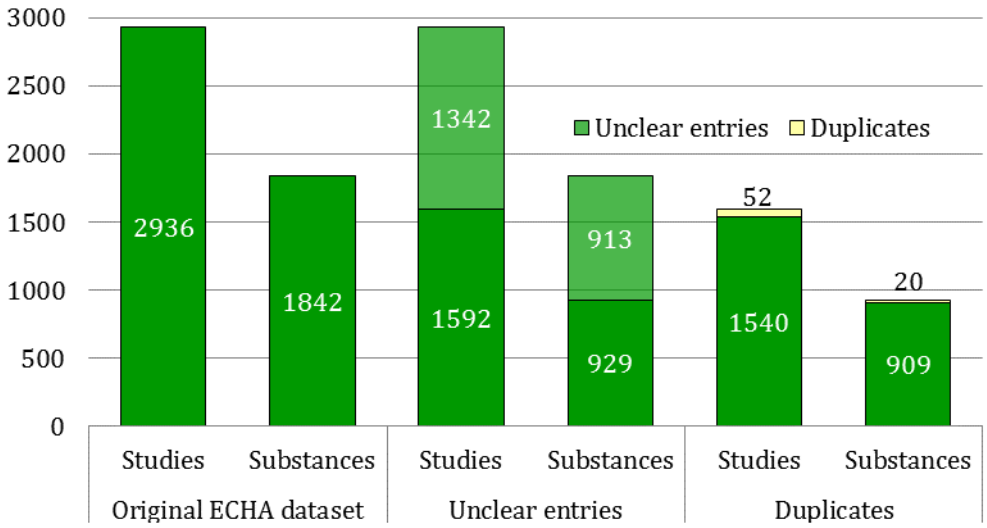
3.4 Results of the data filtering procedure

The study was carried out with a dataset generated in July 2017, containing 2936 studies for AFT tests retrieved from the ECHA database representing a total of 1842 substances (for an overview of absolute numbers and ratios of the different steps, cf. Fig. 42 and Table 10).

3.4.1 Filtering steps I and II: Unsuitable LC_{50} values and duplicate studies

After identification and deletion of all database entries containing no clearly defined values (LC_{50} value ranges only given: "x to y", "≥", "≤", "ca."), 1594 studies out of original 2936 studies, representing 929 out of 1842 substances remained within the database (Fig. 37). The first filtering step ("unclear entries"; 1342 studies, 9113 substances) thus resulted in a reduction to 54.3 % and 50.5 % remaining studies and substances for further evaluation, respectively. In filtering step II, 52 studies (1.77 % of the original dataset) dealing with 20 substances (1.09 % of the original dataset) could clearly be identified as either duplicate studies of the same substance or substances tested with the same mixture of components; these were therefore removed from the dataset. Filtering steps I and II thus resulted in an elimination of 47.5 % of all studies (n = 1394) and of 50.7 % of all substances (n = 933) of the original dataset.

Figure 37: Elimination of unclear entries and duplicate studies in the ECHA dataset

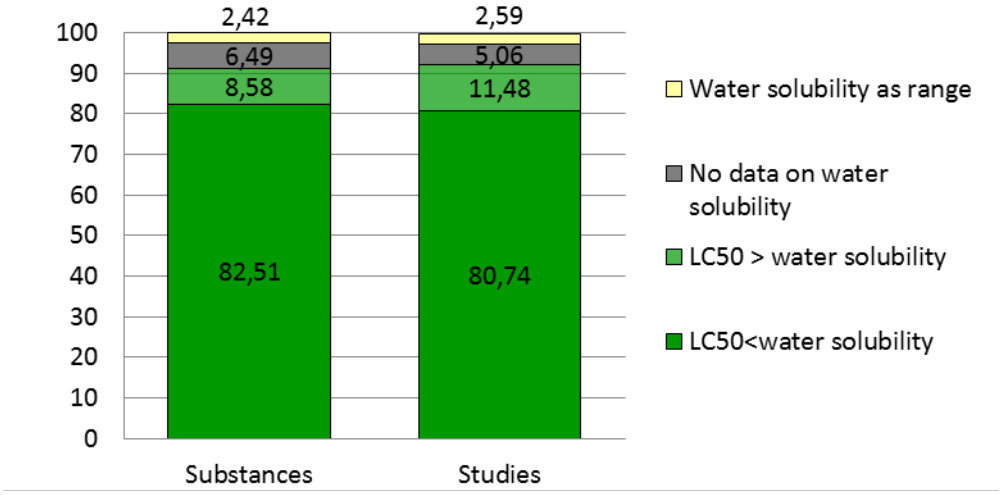


Absolute numbers of studies and substances in the database during the elimination of unclear entries and duplicate studies. The elimination of unclear entries and duplicates reduced the number of studies and substances in the dataset by around 50 %.

3.4.2 Filtering steps III and IV: LC_{50} and water solubility

When comparing the LC_{50} values with the water solubility of the substances given in the ECHA database, it became obvious that the LC_{50} of 177 studies out of the 1542 studies after filtering step II (11.49 % of studies after filtering step II) lies above water solubility. This correlates to 78 (8.58 % of studies after filtering step II) from overall 909 remaining substance (Fig. 38).

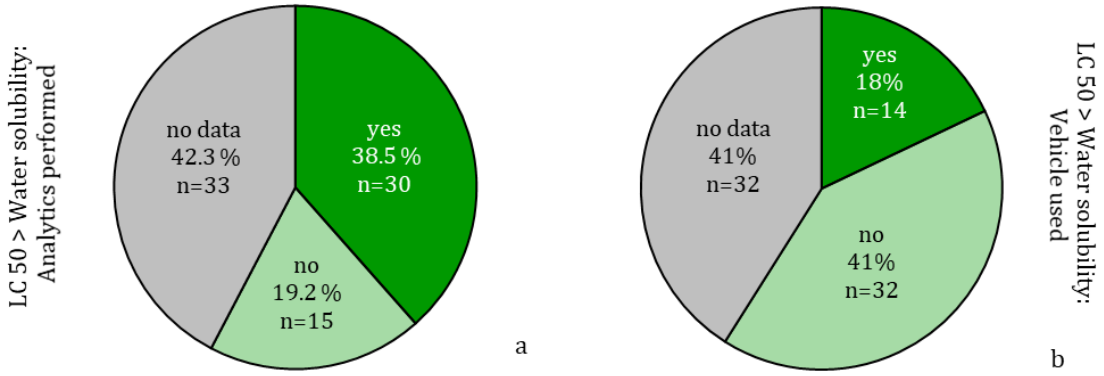
Figure 38: Entries in the ECHA database with LC_{50} > water solubility, no data on water solubility and water solubility given as range with the LC_{50} lying within this range



Overview on the ratio of studies and substances with LC_{50} > water solubility, no data on water solubility and water solubility stated as range with the LC50 lying within this range. The data refers to the remaining 909 substances and 1542 studies after step I and II. Studies and substances with LC50 above water solubility (light green) or without any information on water solubility (grey) were excluded from the dataset.

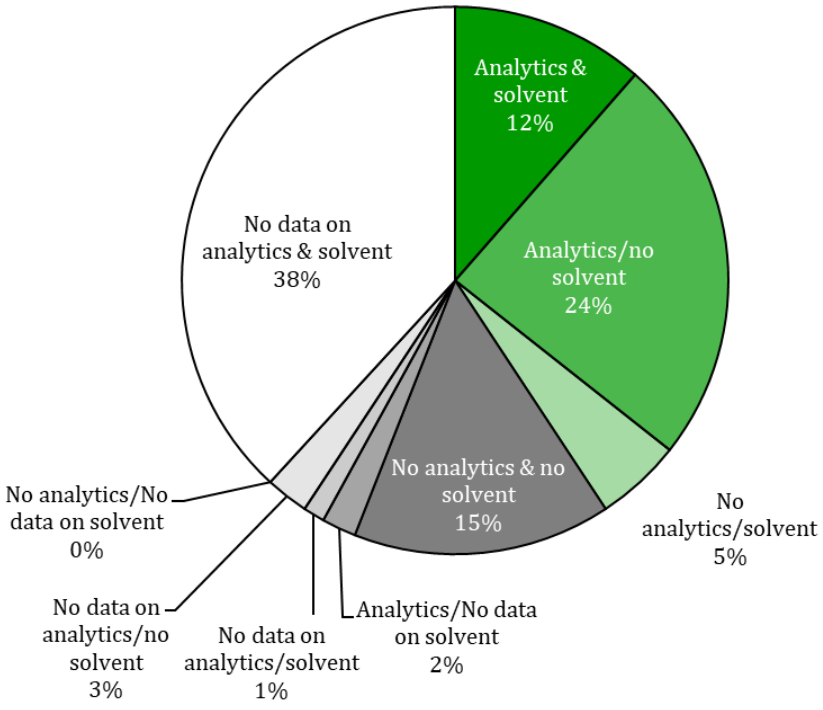
For 78 studies (5.06 % of the remaining dataset after step I and II) and 59 substances (6.49 % of the remaining dataset after step I and II), no data on water solubility was available in the ECHA database. Both, studies with LC_{50} > water solubility (177 studies with 78 substances) as well as substances, where no data on water solubility exists, were therefore excluded from the dataset. Additionally, for 40 studies (2.60 %, referring to 1594 studies after step I and II) and 22 substances (2.42 %, referring to 909 substances after step I and II) , the water solubility was given as range with the LC_{50} lying within that range. These studies and substances remained in the datasheet for the next step, resulting in an overall of 1287 studies and 772 substances.

Figure 39: Ratio of the studies in the ECHA database with use of analytics and vehicle in studies with LC_{50} tested above water solubility



Ratio of the use of analytics and vehicle in studies with LC_{50} tested above water solubility. In almost the same ratio of studies, no data are available on analytics or vehicle use

Figure 40: Solvent use and analytical verification in substances from the ECHA database tested with LC_{50} over water solubility



Overview on solvent use and analytical verification in substances tested with LC_{50} over water solubility

In only 38.5 % of the studies tested with an LC_{50} over water solubility, a chemical analysis was performed (Fig. 39a). In 42.3 % of the cases, no information on analytics was available, and 19.2 % of the studies were conducted without any analysis. Although the selected concentrations of the test substances were above water solubility, surprisingly 41 % of the studies were not performed with a solvent. For the same ratio (41 %), no information on the use of a vehicle was available. Only in 18 % of the studies, a vehicle was used (Fig. 39b).

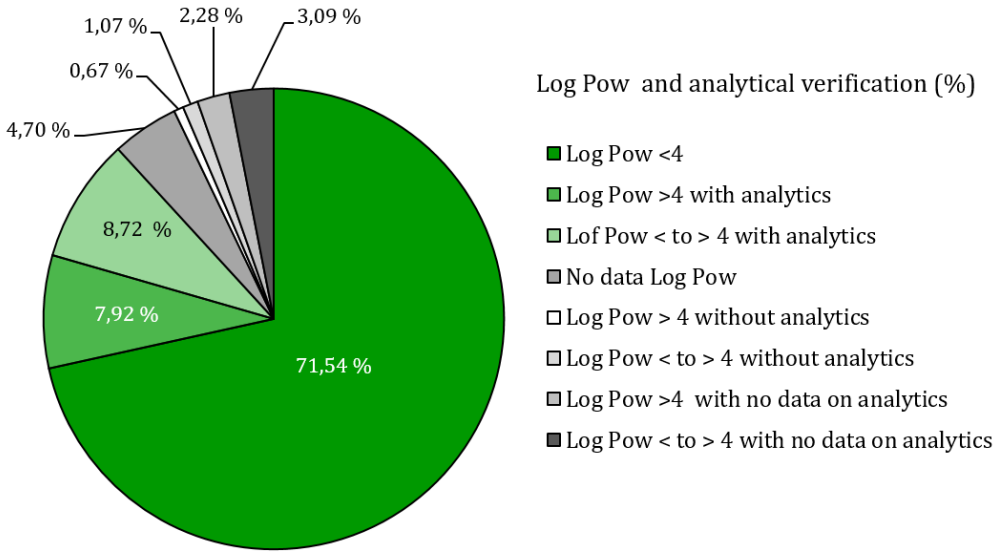
A detailed analysis revealed a differentiated picture (Fig. 40): For the majority (38 %) of studies, information on neither solvent use nor analytical verification was available. In 24 % of the cases, analytics were performed although the LC_{50} lies over the value of water solubility. In these studies, the measured concentrations often were available; however, the nominal concentration was used for the LC_{50}. 15 % of the studies were conducted without solvent or analytical verification. Only in 12 % of studies both, a solvent was used and analytics were performed.

The elimination of all studies with an LC_{50} higher than half the level of water solubility resulted in a further elimination of 40 studies (1.36 % of the original dataset) and 27 substances (1.47 % of the original dataset). Thus, 1247 studies corresponding to 745 substances remained for the next step, meaning 42.47 % of the studies and 40.45 % of the substances from the original dataset, respectively.

3.4.3 Elimination step V: Analysis of log Pow data

Since log Pow values refer to a specific substance, the elimination took place on the substance level, eliminating all studies from one substance, when it was not meeting the criteria. On the other hand, since the ecotoxicological information in the ECHA database does not normally include all study results, the information on analytical results was adopted for the substance. Criteria for elimination were as follows: log Pow > 4, when the studies were not supported by analytics and no data available for log Pow within the ECHA database. Additionally, another cohort was formed, if the log Pow was specified as a range from < 4 to > 4, with the LC_{50} lying within this range. Analogously to the data on water solubility, these data remained in the dataset for further calculation. Results from the analysis of log Pow values are summarized in Fig. 41.

Figure 41: Analytical verification of the test concentrations of substances tested within the ECHA database, when log Pow > 4



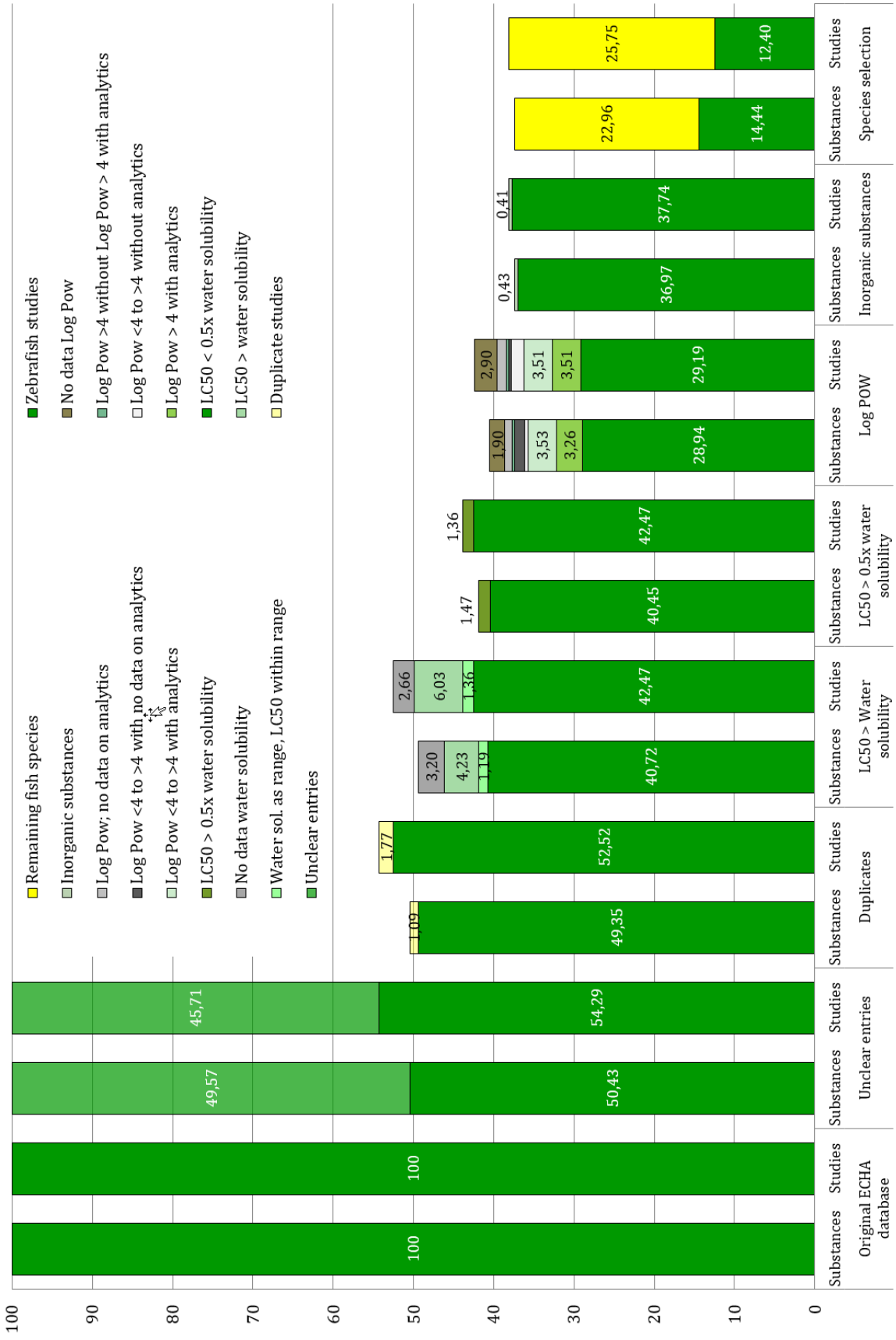
Analysis of log Pow values of substances and analytical verification of the test results when log Pow > 4

Table 10: Absolute number of studies/substances removed or considered for final analysis

Reduction steps	Original ECHA database		Step I		Step II		Step III		Step IV		Step V		Step VI		Step VII	
	Subst.	Studies	Unclear entries		Duplicates		LC_{50} > Water solubility		LC_{50} > 0.5 x Water solubility		Log P_{OW} > 4		Inorganic substances		Species selection	
			Subst.	Studies	Subst.	Studies	Subst.	Studies	Subst.	Studies	Subst.	Studies	Subst.	Studies	Subst.	Studies
Total dataset	1842	2936														
Without unclear entries			929	1594												
Erased unclear entries			913	1342												
Without duplicate studies					909	1542										
Erased duplicate studies					20	52										
LC_{50} < water solubility (ws)							750	1247								
LC_{50} > water solubility (ws)							78	177								
No data (n.d.) on water solubility							59	78								
Water solubility stated as range							22	40								
Dataset without LC_{50}>ws & n.d.							772	1287								
LC_{50} < 0.5x water solubility									745	1247						
LC_{50} > 0.5x water solubility									27	40						
Log Pow < 4											533	857				
Log Pow > 4											81	145				
No data Log Pow											35	85				
Log Pow < 4, with analytics											59	104				
Log Pow < 4, without analytics											5	10				
Log Pow < 4, n. d. on analytics											17	32				
Log Pow <4 to >4 total											96	160				
Log Pow <4 to >4 with analytics											65	103				
Log Pow <4 to >4 without analytics											8	46				
Log Pow <4 to >4 with n. d. on analytics											23	10				
Remaining Studies/substances after removal of Log Pows											689	1120				
Inorganic substances													8	12		
Organic substances													681	1108		
All fish species except zebrafish															416	744
Zebrafish (*Danio rerio*) studies															265	364

Processing of the ECHA dataset for AFT data: number of studies/substances removed or considered for the final dataset. Green fields: values removed from the dataset

Figure 42: Summary of study elimination per filtering step relative to original data (%)



Overview on studies/substances eliminated per filtering step relative to original data (%)

Based on the remaining number of substances (745), the majority (71.54 %) of substances (n = 533) tested had a log Pow below 4. Overall, 10.9 % of the substances (n=81) had log Pow values > 4; yet, the majority (8.1 %) of these substances were verified by analytics and, thus, kept in the dataset. For 96 of the substances (12.9 %), log Pow was given as range. Referring to the original dataset, only substances with log Pow < 4 were kept as well as log Pow stated as range and Log Pow > 4 when chemical analysis were performed. Thus, 37 % of substances and 38% of studies from the original dataset remained after the elimination of log Pow data, representing 689 substances and 1120 studies (Table 10; Fig. 42).

3.4.4 Further filtering: Inorganic substances

The ECHA dataset also contained a number of inorganic substances for which the log Pow is not significant. Most of them had already been deleted in previous steps. The remaining dataset still contained 8 inorganic substances with altogether 12 studies (Table 10). Following this step, 36.97 % of substances and 37.74 % of the studies relative to the original dataset remained.

3.4.5 Further filtering: AFT studies with zebrafish (*Danio rerio*) only

For comparison with the results from the FET study, in this step only studies with zebrafish (*Danio rerio*) were included (Table 10). 364 studies and 266 substances of the whole dataset were performed with zebrafish (*Danio rerio*), representing 12.40 % of studies and 14.44 % of substances of the total dataset.

3.4.6 Further filtering: Interspecies and intraspecies comparisons

In a further step and as preparation for subsequent inter- and intraspecies comparisons, all studies for substances with only one single entry in the database were excluded (i.e. no comparison possible). When compared to the original number of studies and substances in the ECHA database (100 %), this step resulted in a reduction of the number of substances from 682 to 453 (remaining 12.4 % of original number of substances) and a reduction of the number of studies from 1108 to 655 (remaining 22.3 % of original number of studies).

For intraspecies comparisons, 493 of the remaining studies (72.3 %) covering 181 substances (16.34 %) were available. For interspecies comparison, only 178 studies (26.0 %) representing 53 substances (4.8 %) were used.

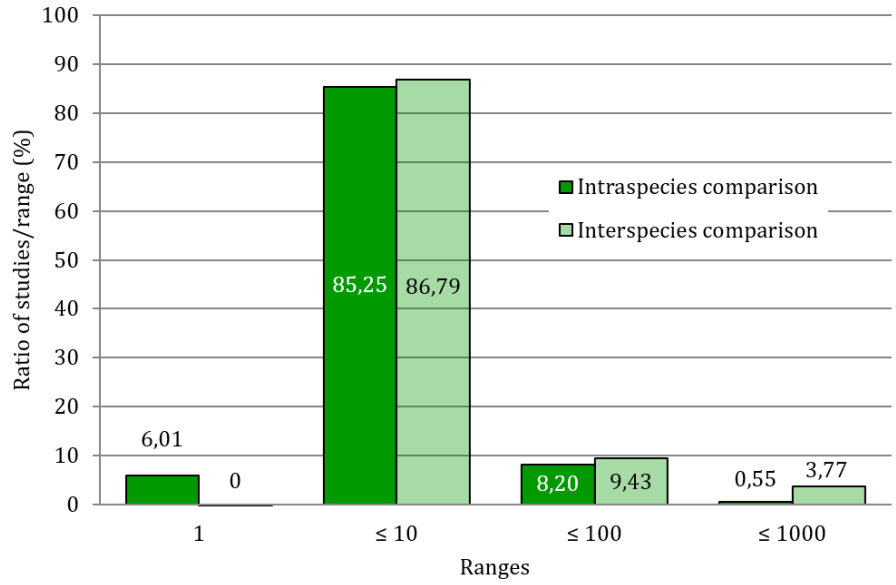
The minimal and maximal LC_{50} values of a substance were identified, and the maximum deviation (maximum deviation = maximum value/minimum value) for each substance was calculated. For a better overview, deviations were categorized as follows (Fig. 43):

= 1	identical value
≤ 10	deviation by a factor between 1 and 10
≤ 100	deviation by a factor between 10 and 100
≤ 1000	deviation by a factor between 100 and 1000

Since in the intraspecies comparison two substances were tested with several studies in two species, two substances for the calculation of the percentile of ranges were added. The analysis illustrated that almost all studies of intra- and interspecies comparison (93.45 % and 96.22 %) are within the deviation ranges from 1-100, which is currently accepted within the REACH process.

The majority of studies per substance (85.3 % intraspecies and 86.8 % interspecies, respectively) varied by a factor ≤ 10. In the next category with a factor ≤ 100, interspecies comparison showed with 9.4 % of the substances nearly the same value as the intraspecies comparison with 8.2 %. Only few studies showed a deviation factor ≤ 1000 in intra- as well as in interspecies comparison. The value of the interspecies comparison (3.8 %) was about a sevenfold higher when compared to the intraspecies value (0.6 %). In the intraspecies comparison in 6.0 % of the substances, two or more studies per substance were found with exactly the same LC_{50} values, resulting in a deviation factor of 1. On the other hand, no substance with a deviation factor of 1 could be detected in the interspecies comparison.

Figure 43: Range of deviation factors in intra- and interspecies comparisons within the ECHA database



Range of deviation factors in intra- and interspecies comparisons: values were categorized into 4 ranges: 1 for identical same values; ≤ 10 for deviations by a factor between 1 and 10; ≤ 100 for deviation by a factor between 10 and 100; ≤ 1000 deviation by a factor between 100 and 1,000

There is a difference to the distribution in the first inter- and intraspecies comparison in the AFT status report presented to the UBA in November 2017 prior to most of the processing steps. In the November 2017 status report, 22.2 % of interspecies studies met the deviation factor ≤ 100. In this dataset, nearly twice as many studies in the interspecies (5.6 %) and 4 times as many studies in the intraspecies comparison (1.9 %) showed a deviation factor ≤ 1000, indicating that the processing of the initial dataset, especially the exclusion of studies with LC_{50} > water solubility erased most of the outlier studies.

3.5 Conclusions drawn from the analysis of AFT data from the ECHA database and recommendations for future AFT studies

3.5.1 Comparison of data handling in the present AFT study and the FET by (Scholz et al., 2016)

One purpose of the present study was to give an overview on the reliability and possible elimination capacities of AFT data by setting similar criteria as in the ECHA FET study by (Scholz et al., 2016) and (Sobanska et al., 2018). However, the procedure and order of the processing steps varied between the present study and the study by (Scholz et al., 2016), and not all steps were equally performed. In case of the ECHA FET study, unclear values were not explicitly removed as were duplicate studies; in the present AFT study, studies with "predicted pH at saturation >5 or <9" and studies with "no toxicity and the maximum test concentration <10 fold above baseline toxicity" were not taken into account due to the tools not being available.

Nevertheless, parts of the findings allow a direct comparison (Table 11). The processing of studies with an LC_{50} above 0.5 times of water solubility in the ECHA FET study resulted in elimination of about 22 % for both tests and substances. In the present AFT study, this step (separated in two steps: LC_{50} > water solubility and LC_{50} > 0.5 times water solubility) reduced the number of tests by only 10.1 and % and the number substances by only 5.6 %. This indicates that – in this respect – the AFT data are obviously more robust than the FET data. On the other hand, the elimination potential of the analysis of log Pow data is almost identical in both the FET and the present AFT study.

Although the last step of the present study, the reduction of studies to those performed with the zebrafish (*Danio rerio*), is not necessarily a valid step, since historically for the AFT more fish species are used. However, it could be shown that by keeping only zebrafish data, the percentage of remaining studies is 7.6 % for the FET and 12.5 % for the AFT, i.e. very similar for both test systems. Thus, the selection of only zebrafish data does not lead to a massive loss of data for neither the FET nor the AFT analysis.

A closer look at the ECHA FET study reveals the biggest potential for elimination of data in the two steps: "Keep studies with predicted pH at saturation >5 or <9" and "Remove studies with no toxicity and maximum test concentration <10 fold above baseline toxicity", which were not performed in the analysis of AFT studies due to the tools being unavailable. This indicates that these two steps might be important for a more significant result and should be analyzed in additional studies.

Table 11: FET and AFT sets and numbers of substances/studies considered in final dataset

	Studies (n)		Substances (n)		Studies (%)		Substances (%)	
	FET	AFT	FET	AFT	FET	AFT	FET	AFT
Initial Fet and AFT dataset	2065	2936	1415	1842	2065	2936	1415	1842
Remove studies with unclear LC_{50}- values		1594		929		54.3		50.4
Remove duplicate studies		1542		909		52.5		49.4
Remove studies when LC50 > water solubility		1287		772		43.81		41.91
Keep only studies conducted with zebrafish	2036	*	1415	*	98.60	*	100	*
Keep only studies with organic substances	1996	*	1391	*	96.66	*	98.30	*
Keep tests with exposure duration of 96 to 120	1610	**	1191	**	77.97	**	84.17	**
Keep studies with LC50 or maximum test concentration below 0.5 times of water solubility	1155	1247	882	745	55.93	42.40	62.33	40.45
Keep studies with predicted pH at saturation >5 or <9	1131	n.p.	873	n.p.	54.77	n.p.	61.70	n.p.
Remove studies with no toxicity at maximum test concentration < 10 fold above baseline toxicity	411	n.p.	343	n.p.	19.90	n.p.	24.24	n.p.
Keep compounds with corresponding AFT data for fathead minnow, rainbow trout, bluegill and zebrafish	238	n.r.	185	n.r.	11.53	n.r.	13.07	n.r.
Keep only substances with Kow < 4 and/or Log Kaw <4, unless the test concentrations were confirmed by analytical chemistry	156	1120	123	689	7.55	38.15	8.69	37.40
Keep only studies with organic substances		1108		681		37.74		36.97
Keep only studies conducted with zebrafish		364		266		12.40		14.44

*conducted in AFT analysis at a later timepoint; **already selected within the filtering procedure of original dataset ; n.p. - not performed; n.r. - not relevant

Overview of processing the FET and AFT datasets and absolute numbers of substances and studies considered for the final dataset. Green fields represent the last filtering step for both, FET and AFT analysis, the elimination of all studies except those with zebrafish.

3.5.2 Conclusions and recommendations for future AFT testing

Analyzing the AFT data from the ECHA database clearly showed that the number of studies removed from the database after applying the filtering criteria is nearly as the same as in the ECHA FET study. The largest filtering of the AFT data took place when the entries with unclear LC_{50} values were excluded. Another very critical filtering step was the analysis of LC_{50} tested above water solubility, indicating that such studies might not be fully reliable.

With regard to conducting future AFT studies for regulatory purposes, the following recommendations can be given:

- Only studies with clearly defined toxicity values should be accepted. Existing studies with readings like e.g. "LC_{50} = x to y mg/L"; "LC_{50} >..., %w/t" or "LC_{50} <..."should be revised or rejected.
- The recently revised OECD Guidance Document 23 should be consulted when testing difficult-to-test test substances (OECD, 2018).
- In the process of test design and the selection of test concentrations, the limits of water solubility as well as other physicochemical properties of a substance must be considered.
- For tests above water solubility, analytical validation of test concentrations should be mandatory.
- If the test concentration needs to be above water solubility, the thorough use of a vehicle should be considered.
- Measured concentrations should be given preference for ecotoxicological results. Given the importance of e.g. LC_{50} values for risk assessment, nominal concentrations should only be accepted, if there is evidence that degradation or other losses from the system (e.g. metabolism, volatilization, adsorption to the surface of the tank, etc.) do not occur.
- Within the REACH process, elevated attention should also be paid to the preparation of test solutions. Procedures such as "ultrasonic treatment followed by filtration" or "test media with visible unsolved matter" should be discarded. In any case, as mentioned above, such procedures need to be accompanied by thorough sampling and analytical verification.
- In particular, studies with a log Pow > 4 should generally be accompanied by analytical verification.
- Given that approx. 60 % of the existing AFT data would not fulfil the requirements set in the ECHA FET study (Scholz et al., 2016; Sobanska et al., 2018), massive re-testing of acute fish toxicity would be required.
- Study results for substances tested at concentrations close or above their water solubility, but without analytical verification should be interpreted with care or considered for revision.

4 Contributions to OECD project no. 2.54: „Guidance Document on an Integrated Approach on Testing and Assessment for Fish Acute Toxicity Testing“ – Integration of the Fish Embryo Test into the Threshold Approach (OECD Guidance Document 126)

In 2010, the threshold approach was adopted by the OECD (OECD, 2010) based on the observation that fish are not always the most sensitive species (Hutchinson et al., 2003; Weyers et al., 2000). In this approach, an initial Acute Fish toxicity Test (AFT) according to OECD TG 203 (OECD, 1992) is conducted at one concentration, i.e. the lowest EC_{50}/LC_{50} derived from test responses in Daphnia and algae, and continued testing is triggered only if death is observed at this threshold concentration. Since the threshold approach was developed, the Fish Embryo Toxicity Test (OECD TG 236) has been validated and accepted by the OECD (OECD, 2013a). It seemed, therefore, appropriate to update the threshold approach to incorporate OECD TG 236, because this test offers a significant 3Rs (refinement) benefit compared to the AFT as embryos are used instead of juvenile or adult fish. As a consequence, a project proposal to update the threshold approach to incorporate the Fish Embryo Acute Toxicity Test (in particular OECD TG 236) was submitted to the OECD by Austria as the lead country and the International Council on Animal Protection in OECD Programs (ICAPO) in 2015 (OECD project no. 2.54: „Guidance Document on an Integrated Approach on Testing and Assessment for Fish Acute Toxicity Testing“).

Incorporation of OECD TG 236 into the threshold approach was deemed appropriate, because LC_{50} values from the FET correlate very well with LC_{50} values from OECD TG 203 (Belanger et al., 2013; Klüver et al., 2015; Knobel et al., 2012; Lammer et al., 2009; Ratte and Hammers-Wirtz, 2003). However, some exceptions to this good correlation have been described and a review was performed by the European Chemicals Agency (ECHA) suggesting some limitations of TG 236 for regulatory use that require further consideration (Scholz et al., 2016).

Within the present UBA project, the authors of this report made continuous contributions during telephone conferences and face-to-face meetings as well as in written especially to the background document, which is designed to accompany the central updated guidance document. This background document provides relevant background information and discussions considered when updating OECD GD 126 with the aim of integrating OECD TG 236. It focuses on the characterization of the performance of the OECD TG 236 in terms of its reliability within the threshold approach and the applicability domain of OECD TG 236.

In this context, chapter 3 of this report (“Analysis of the relevance and adequateness of the Fish Acute Toxicity Test (AFT) according to OECD TG 203 to fulfil the information requirements and addressing concerns under REACH”) will attempt to provide a counterpart to the corresponding ECHA FET study (Scholz et al., 2016). Likewise, the literature review in chapter 2 of the present report (“Biotransformation and bioactivation capacities in early life stages of zebrafish (*Danio rerio*)” will provide valuable input to the discussion of the applicability of the Fish Embryo Test. Since the background document has not been finalized yet, further input will be given by the authors of this report in the near future.

5 References

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