Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2020:69
Faculty of Veterinary Medicine and Animal Science

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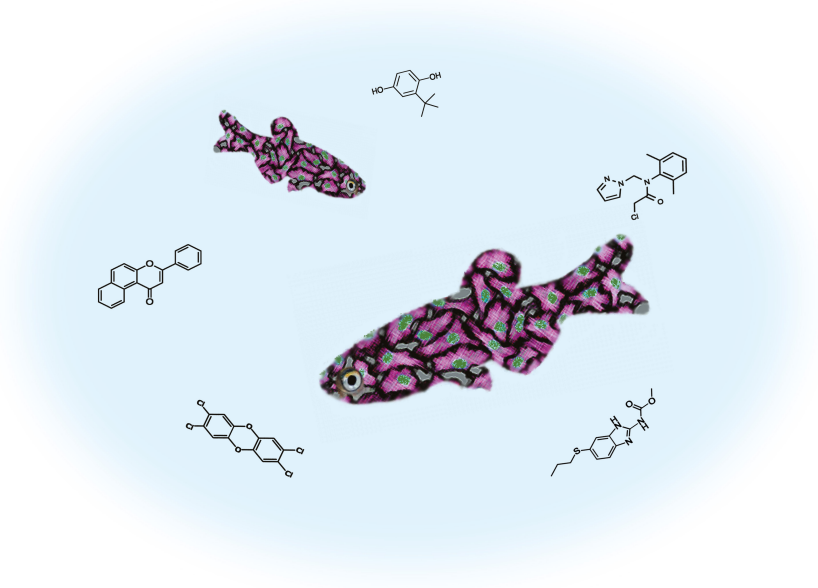

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Sebastian Lungu-Mitea
Faculty of Veterinary Medicine and Animal Science
Department of Biomedical Sciences and Veterinary Public Health
Uppsala

SLU
SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

DOCTORAL THESIS
Uppsala 2020

Acta Universitatis agriculturae Sueciae
2020:69

Cover: illustration designed by the author

ISSN 1652-6880
ISBN (print version) 978-91-7760-656-7
ISBN (electronic version) 978-91-7760- 657-4

Uppsala
Print: SLU Service/Repro, Uppsala 2020

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Abstract

Standard toxicological *in vivo* testing has been challenged as the procedures are time-consuming, expensive, and require a large number of animals; given the number of problematic chemicals. Novel toxicological frameworks, such as "toxicity testing in the 21st century", proposed the use of "new approach methods" (*in vitro* and *in silico* techniques), that can be applied in high-throughput setups and would allow for the testing of a large number of compounds. However, such new approach methods need to be designed and evaluated first. Especially within ecotoxicology, the coverage of species-specific bioanalytical tools, e.g. for fish, is rather scarce. Currently, mainly *in vitro* assays of mammalian and bacterial origin are used. This thesis outlines how to design and scrutinise fish transient reporter gene assays. We have established transient reporter gene assays in permanent zebrafish fibroblasts and hepatocytes of the oxidative stress response and the xenobiotic metabolism toxicity pathways. We identified non-specific effects caused by transient transfection itself and suggested preventive strategies. Further, we identified toxicity pathways' cross-talk as a significant driver of uncertainty in regards to the assessment of receptor-mediated toxicity. Additionally, we evaluated the correlation between cytotoxicity in cultured zebrafish cells and the acute toxicity observed in zebrafish embryos. When using chemical distribution models to derive bioavailable concentrations, we observed a good positive correlation between the two test systems. The results advocate an intensified use of fish *in vitro* assays in integrated testing strategies. Conclusively, new approach methods, as developed and applied in this thesis, show great potential in future toxicity testing and environmental monitoring.

Keywords: toxicity pathways, Tox21, 3Rs, reporter gene assays, cytotoxicity assays, mass-balance modelling, cross-talk, AOP

Author’s address: Sebastian Lungu-Mitea, SLU, Department of Biomedical Sciences and Veterinary Public Health, PO Box 7028, 75007 Uppsala, Sweden

Dedication

To family, friends, and everyone who accompanied me on this journey.

"All models are wrong, some are useful."

George E. P. Box

"Noli turbare circulos meos!"

Archimedes

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Lungu-Mitea, S*.**, Oskarsson, A., Lundqvist, J. (2018). Development of an oxidative stress *in vitro* assay in zebrafish (*Danio rerio*) cell lines. Scientific Reports, 8 (1; 12380).

II. **Lungu-Mitea, S.***, Lundqvist, J. (2020). Potentials and pitfalls of transient *in vitro* reporter bioassays: interference by vector geometry and cytotoxicity in recombinant zebrafish cell lines. Archives of Toxicology, 94, pp. 2769–2784

III. **Lungu-Mitea, S*.**, Vogs, C., Carlsson, G., Montag, M., Frieberg, K., Oskarsson, A., Lundqvist, J. (2020). Modelling bioavailable concentrations in zebrafish cell lines and embryos increases the correlation of toxicity potencies across test systems. (submitted)

IV. **Lungu-Mitea, S*.**, Han, Y., Lundqvist, J. (2020). Modulation of transient reporter gene vectors of the xenobiotic metabolism pathway in permanent zebrafish hepatocytes. (manuscript)

*Corresponding author

The contribution of Sebastian Lungu-Mitea (SLM) to the papers included in this thesis was as follows:

I. Main responsibility in preparing and conducting the study. SLM compiled the literature and wrote the manuscript with support from co-authors. Journal correspondence was managed by SLM.

II. Main responsibility in design and execution of the study. SLM compiled the literature and wrote the manuscript with support from co-authors. Journal correspondence was managed by SLM.

III. Main responsibility in design and execution of the study. SLM compiled the literature and wrote the manuscript with support from co-authors. Journal correspondence was managed by SLM.

IV. Main responsibility in design and execution of the study. SLM compiled the literature and wrote the manuscript with support from co-authors.

Other publications

The following publication was prepared during the time of thesis and is discussed in this manuscript as well, but not part of the thesis evaluation.

I. Lundqvist, J.*, Mandava, G., **Lungu-Mitea, S.**, Yin Lai, F., Ahrens, L., (2019). *In vitro* bioanalytical evaluation of removal efficiency for bioactive chemicals in Swedish wastewater treatment plants. Scientific Reports, 9 (7166).

*Corresponding author

Abbreviations

AFT	acute fish toxicity test
AhR	aryl hydrocarbon receptor
AO	adverse outcome (within the AOP concept)
AOP	adverse outcome pathway
ARE	anti-oxidative stress response element
ARNT	aryl hydrocarbon receptor nuclear translocator
BEQ	bioequivalent value (in molarity or concentration)
DLR	dual-luciferase reporter system
FET	fish embryo acute toxicity test
GOI	gene of interest
KE	key event (within the AOP concept)
Keap1	kelch-like ECH-associated protein 1
KER	key event relationship (within the AOP concept)
MB	mass-balance model (chemical distribution model)
MIE	molecular initiating event (within the AOP concept)
MOA	mechanism of action
MoA	mode of action
NAM	new approach method

Nrf2	nuclear factor erythroid 2-related factor 2 (transcription factor)
PBTK/TD	physiology-based toxicokinetics/dynamics
qHTS	quantitative high-throughput screening
QSAR	(quantitative) structure-activity relationship (model)
REACH	Registration, Evaluation, Authorisation and restriction of Chemicals
REF	relative enrichment factor
ROS	reactive oxygen species
Tox21	toxicity in the 21st century (conceptual framework and screening program)
ToxCast	toxicity forecaster (screening program)
TP	toxicity pathway
TSCA	toxic substances control act
WFD	water framework directive
XRE	xenobiotic response element (a.k.a. DRE – dioxin response element)

1. Background & introduction

1.1 The dawn of the Anthropocene: humanity in a chemical environment

Undoubtedly, modern chemistry is changing the world in an unprecedented fashion. The standard of living recently experienced by nearly all of humanity is also based on revolutionising inventions and developments in the field of chemical engineering (Fischman 2013). However, chemical science and industry failed to live up to former high hopes (R. 1939). Instead, anthropogenically-derived pollution is detectable in geological terms, and the dawn of the "Anthropocene" epoch has been proclaimed (Corcoran et al. 2014; Waters et al. 2016). This multifarious pollution adds to civilisational and environmental threats, such as climate change and the Holocene mass extinction event (Dirzo et al. 2014; Ceballos and Ehrlich 2018). Thus, it is legitimate to say that most of humanity currently dwells in a chemical environment.

The plethora of chemicals surrounding us has evoked the "exposome" scenario (Miller 2014; DeBord et al. 2016; Vermeulen et al. 2020); whose adverse effects on the individual, the population, and the overall environment are hardly assessable. In detail, the exposome is defined as a cumulative measure of all environmental exposure influences and associated biological responses throughout one individual's lifespan (Miller 2014). "Exposomics" are an integrative term that interlaces chemical exposure with multiple levels of biological complexity (genome, transcriptome, proteome, metabolome, and epidemiology) (Vineis et al. 2017). Most lifestyle diseases, such as cardiovascular diseases and cancer, and therefore most deaths within the

industrialised nations, can be associated with the exposome scenario (Gakidou et al. 2017). On the one side, specific classes of compounds are designed to have certain effects on biological targets (e.g., pesticides and pharmaceuticals), and their potential risk is calculable. On the other side, a multitude of industrial chemicals proved to have unintended adverse effects on biota, and their intrinsic hazard has to be assessed appropriately. Given the sheer numbers of environmental pollutants, elusively appearing and synergistically acting mixture toxicity may be one of the most significant challenges today and in the near future (Altenburger et al. 2013). Thus, it is of everyone's vital interest to regulate the chemical environment.

1.2 "Toxicity testing in the 21st century": a new era of chemical regulation

During the last two decennia, legislation on regulatory risk assessment has been drafted and ratified internationally (The European Parliament and the Council 2006; US EPA 2016), in an attempt to solve above-stated issues. Within the European Union, regulations and directives were adopted on a transnational level. Several consumer and environmental protection bills were ratified, completing or overruling national laws (The European Parliament and the Council 2003, 2006, 2009). On the one hand, these novel bills standardise and guide toxicity testing, while on the other, these novel regulations drastically increased the demand in animal testing (Goldberg 2010; Hartung 2010, 2011). The latter is a contradictive development to the common attempts within the scientific community to minimalise animal testing in the context of the "3Rs" (refine, reduce, replace; Russell and Burch 1959) and beyond ("6Rs": relevance, reliability, regulatory acceptance; Lillicrap et al. 2016). Noteworthy, most directives and regulations encourage the use of non-animal or alternative test methods; however, they are mostly non-mandatory. Hence, scientists are facing the dilemma of chemical regulation versus ethical and economic concerns.

In consideration of the dilemma, public authorities established scientific frameworks to identify potential solutions. The report on the "21st-century toxicology" (Tox21) by the US National Research Council (NRC) and the Environmental Protection Agency (EPA) (NRC 2007) is considered a paradigm shift in toxicology testing. The general intent was to utilise *in vitro*

toxicity tests for quantitative high-throughput screenings (qHTS) accompanied by *in silico* methods and, thereby, transition toxicology into a predictive and mechanism-based science (Collins et al. 2008; Tice et al. 2013); in contradiction to classic toxicology, which records apical endpoints of toxicity *in vivo*. The report launched an unprecedented screening program that is applying automated robotic platforms and big data approaches (Inglese et al. 2006; Shukla et al. 2010), commonly known as "Tox21". The US National Toxicology Program, an interagency body, endorses the Tox21 project. Subsequently, similar but smaller qHTS screening projects started in the EU with the SEURAT (2008-2016, Gocht and Schwarz 2016) and the following EU-ToxRisk (2016-ongoing, Krebs et al. 2020) programs (see also tab. 1). Additionally, governmental evaluation laboratories and institutes (see tab. 2, reviewed in Balls et al. 2018) became important stakeholders throughout this process by promoting, designing, establishing, and evaluating "new approach methods" (NAMs) to be used in qHTS (Halder et al. 2014; Worth et al. 2014).

The Tox21 report proposed NAMs, comprised of *in vitro* and *in silico* techniques, as vital alternatives to *in vivo* methods. Such alternate applications are supposed to be utilised for the assessment of "toxicity pathways" (TPs), given their rather simplistic nature in comparison to manifold *in vivo* systems. A TP is defined as a sequence of intracellular events, which maintain cellular homeostasis under physiological conditions; although, once perturbed by a xenobiotic may lead to adverse effects on the cellular, and beyond, on the organismal level of biological complexity (Collins et al. 2008; Whelan and Andersen 2013; Kleensang 2014). A TP can be assessed by measuring the perturbation of a specific intracellular event caused by the xenobiotic. Cellular *in vitro* systems are ideal sentinels for such endpoints. However, for a correct assessment and prediction of the integrated effect (e.g., the exposome), all potential TPs need to be identified. The ToxCast screening, within Tox21, is trying to narrow down specific TPs by initially testing a defined set of compounds (approximately 10,000) in multiple *in vitro* bioassays (approximately 500) for diverse endpoints of cellular toxicity. Huang et al. deciphered the ToxCast dataset and recommended the assessment of 1,658 TPs via 362 bioassays (Huang et al. 2016, 2019).

To make TPs accessible for environmental risk assessments, they were complemented by the "adverse outcome pathway" (AOP) concept (Ankley et al. 2010; Villeneuve et al. 2014a, b) (see fig. 1 for details). AOPs project the TP framework onto the individual, population, and community scale, but also add the element of network plasticity via interconnections. They are defined as conceptual constructs portraying available knowledge of the linkage between a molecular initiating event (MIE) and an adverse outcome (AO) at a higher level of biological complexity. The MIE and AO are linked via key events (KE), such as cellular and organ toxicity endpoints, and their interactions are defined as key event relationships (KER). These nodes, though, can be activated by multiple inputs. Thus, in contrary to TPs, AOPs are not linear but plastic (Knapen et al. 2018). Further, AOPs integrate former concepts of toxic effect categorisation, such as mode of action (MoA) and mechanism of action (MOA). MoA and MOA definitions are sometimes arbitrarily handled within the scientific literature and often used interchangeably. Per se, MoA is defined as a common set of measured responses that characterise an adverse biological response, whereas MOA is described as the detailed mechanism of a sequence of events that culminate in a toxic outcome (Borgert et al. 2004). Thus, the older framework definitions are rather head or tail-heavy - from whatever perspective we are backing this conceptual horse – and AOPs are trying to unify and reconcile the latter. In order to promote the concept, the scientific community is invited to develop novel AOPs and share them among their peers via the AOP-Wiki tool (https://aopwiki.org/) in a regulated, "best practice" manner (AOP-knowledgebase: https://aopkb.oecd.org) (Villeneuve et al. 2014a, b; OECD 2017). Comprising all AOPs into one database empowers their intrinsic network plasticity via KEs and KERs and makes them accessible for *in silico* evaluations.

Table 1: Synopsis on major quantitative high-throughput screening projects and conceptually associated projects.

Project	**Explanation/abbreviation**	**Period**	**Selected references**
Tox21	21st-century toxicology	2008-ongoing	(NRC 2007; Collins et al. 2008; Tice et al. 2013)

ToxCast	Toxicity Forecaster: First major screening program within Tox21 – CompTox database	2008-ongoing	
SEURAT	Safety evaluation ultimately replacing animal testing	2008-2016	(Gocht and Schwarz 2016)
EU-ToxRisk	Integrated European flagship program driving mechanism-based toxicity testing and risk assessment for the 21st century	2016-ongoing	(Krebs et al. 2020)
SOLUTIONS	Pollution management for land and water resources	2013-2018	(Brack 2019)

Table 2: Governmental stakeholders and institutions promoting and evaluating quantitative high-throughput screening. An overview is given in (Balls et al. 2018).

Institution	**Explanation/abbreviation**	**Governmental body/agency**
EURL ECVAM	EU reference laboratory, European Centre for Validation of Alternative Methods	European Commission
CAAT	Center for Alternatives to Animal testing	Johns Hopkins University
ICCVAM	Interagency Coordination Committee on the Validation of Alternative Methods	US NIEHS + FDA + EPA
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods	US NIEHS + FDA + EPA
NTP	National Toxicology Program	US NIEHS + FDA + EPA

1.3 *In vitro* bioassays in qHTS

For the AOP to work, pathway nodes (MIE, KE) and connections (KER) need to be populated with data. Adverse outcomes (AO) can mostly be populated with data from historic acute *in vivo* toxicity testing. However, *in vivo* data is often not available. Alternatively, algae tests (OECD 2011), aquatic invertebrate tests (OECD 2004), and the fish embryo test (FET) (OECD 2013) can be utilised. From a legislative point of view, these tests are considered *in vitro* and can also be conducted on multi-well microtiter plates, thus suitable for qHTS. Upper KE levels, such as developmental or organ toxicity, are more challenging to populate if historic histopathology data is not available. 3D cell culture models could be an appropriate

surrogate in this regard, but the technology is still in its infancy. As well, cellular *in vitro* test systems are optimal sentinels to uncover effects on the MIE and lower KE levels. KERs mostly describe a mathematical model, such as a dose or concentration-response relation derived from a regression analysis. In the case of higher complexity KERs, biology-based response-response relationships can be derived in theory (Lau et al. 2000). Here, we will concentrate on cellular *in vitro* assays that cover the assessment of MIEs and lower complexity KEs. Once appropriately established, these could be utilised to predict effects on higher levels of biological complexity.

In comparison to *in vivo* tests, cellular *in vitro* assays have the advantage of the small setup (Bols et al. 2005; Hartung and Daston 2009). This can be utilised for miniaturisation and automation processes, as applied in qHTS. Thus, it is easily achievable to conduct high numbers of replicates and minimise the response variability. Additionally, cellular *in vitro* assays are more cost-effective and adaptable to novel imaging and *omics* technologies. Further, they facilitate the disclosure of molecular mechanisms. On the other hand, cellular *in vitro* assays face several disadvantages (Segner 2004; Bols et al. 2005; Gülden et al. 2005; Gülden and Seibert 2005). The lack of biological complexity precludes the assessment of effects beyond cell-cell interactions. In particular, permanent mammalian cell lines are often derived from cancerous lines, thus, extensively differing in genotypic and karyotypic terms from their tissue of origin. Further, biotransformation is often reduced or non-existent. Finally, cellular *in vitro* assays are cultured in complex nutrition media. For instance, the serum, as necessary for cell culturing, is a significant sink for hydrophobic compounds within spiked exposure media. Thus, the actual bioavailable concentration of the applied compound is drastically lower than the nominal concentration. Also, due to differences in toxicokinetics, *in vitro* results cannot be equally compared to *in vivo* test from aqueous environments. Accordingly, *in vitro* to *in vivo* extrapolations (IVIVE) require either chemical analysis or toxicokinetic and toxicodynamic modelling of the actual bioavailable and target concentrations (Kramer et al. 2012).

Cellular *in vitro* assays can assess specific and non-specific toxic MoAs to populate MIEs and KEs. Here, we discuss three types of toxic MoAs: baseline toxicity (narcosis; non-specific), receptor and transcription factor-

mediated toxicity (specific), and reactive toxicity (specific) (fig. 2). Narcosis describes the intercalation and disruption of cellular membranes (cells or organelles) at high concentrations of neutral organic compounds, leading to apoptosis or necrosis due to membrane disintegration. Receptor and transcription factor-mediated toxicity occurs via the activation or inhibition of particular TPs by non-endogenous ligands binding to intrinsic receptors. Reactive toxicity describes a form of energy transfer initiated by physical agents (radiation) or reactive oxidative species (ROS, electrophiles), leading to alternating covalent bindings within a biomolecule (amongst others). These novel covalent bindings hinder the biomolecule's original function, impede downstream processes, and may lead to toxicity. Narcosis is recorded via so-called viability or cytotoxicity assays (Kepp et al. 2011). Reporter gene assays are optimal for the assessment of receptor-mediated toxicity (Wood 1998). Given its multifarious nature, reactive toxicity is not assessed by one specific type of assay. However, numerous applications are covering diverse endpoints, such as the Comet-assay (Singh et al. 1991) or the micronucleus test (Heddle et al. 1983) for the assessment of genotoxicity. Noteworthy, other types of specific-toxicity can also be recorded via cellular *in vitro* assays, e.g. enzyme inhibition, but are not considered in this categorisation.

In this thesis, we will mainly focus on cytotoxicity and reporter gene assays, covering non-specific (narcosis) and specific (receptor-mediated) toxicity. The main emphasis is on receptor-mediated toxicity because assays measuring narcosis are mostly employed to safeguard from cytotoxic exposure concentrations. Further, receptor and transcription factor (TF)-mediated toxicity can be subclassified into three groups: TPs of the xenobiotic metabolism, the adaptive stress response, and the hormone response (for a selected synopsis see also tab. 3). Specifically, TPs of the xenobiotic metabolism (AhR) and the adaptive stress response (Nrf2) were examined in this thesis.

Table 3: Synopsis on receptor and transcription factor-mediated toxicity pathways (incomplete listing).		
Class	**Pathway/transcription factor**	**Selected reference**
Xenobiotic metabolism	**Aryl hydrocarbon receptor (AhR)**	Reviewed in (Perdew et al. 2018)
	Constitutive androstane receptor (CAR)	
	Peroxisome proliferator-activated receptors (PPARα/δ/γ)	
	Pregnane X receptor (PXR)	
Adaptive stress response	**Oxidative stress (Nrf2)**	Reviewed in (Simmons et al. 2009)
	Heat shock response (HSF1)	
	DNA damage response (p53)	
	Hypoxia (HIF1)	
	Metal stress (MTF1)	
	Inflammation (NFkB)	
Hormone response	Estrogen receptor (ER)	Reviewed in (Rüegg et al. 2009)
	Androgen receptor (AR)	
	Thyroid receptor (TR)	
	Glucocorticoid receptor (GR)	
	Progesterone receptor (PR)	

1.4 Reporter gene assays in ecotoxicology and the Water Framework Directive-reevaluation

The Tox21 strategy publication (NRC 2007) proposed reporter gene assays as an ideal tool for identifying and measuring TPs, given their intrinsic ability to define MIEs and MOAs. Reporter gene assays are pro- and eukaryotic cellular systems bearing stably or transiently introduced reporter gene cassettes (see M&M section, fig. 3). Upon activation of the TP-specific response element, the utilised reporter is synthesised in a parallel fashion to the TP-specific target genes and enzymes. Luciferases and fluorescent proteins are employed as reporters, by placing their specific coding sequence adjacent to the TP-specific response element and the promoter, within the reporter gene cassette (fig. 3B+C). The reporter signal is quantifiable, thus disclosing the turnover of the TP-specific target genes to the investigator.

Within ecotoxicology, the coverage of specific TPs by existing reporter gene assays is relatively scarce in comparison to human toxicology. The utilisation

of biology-based assays (bioassays) within environmental screening is termed as "bioanalytics". So far, bioanalytical studies mainly rely on reporter gene assays of bacterial and mammalian origin, given their general technological establishment (Leusch and Snyder 2015). Although stipulated ahead (Ankley et al. 1998), the development of relevant bioassays for the risk assessment of aquatic habitats has been somewhat neglected, and reminders are reappearing in more recent literature (Halder et al. 2014; Lillicrap et al. 2016; Villeneuve et al. 2019). Fish-specific assays are thereby of higher priority, given that plants and invertebrates can also be tested in an HTS-manner per se, and the ethical standards are differing.

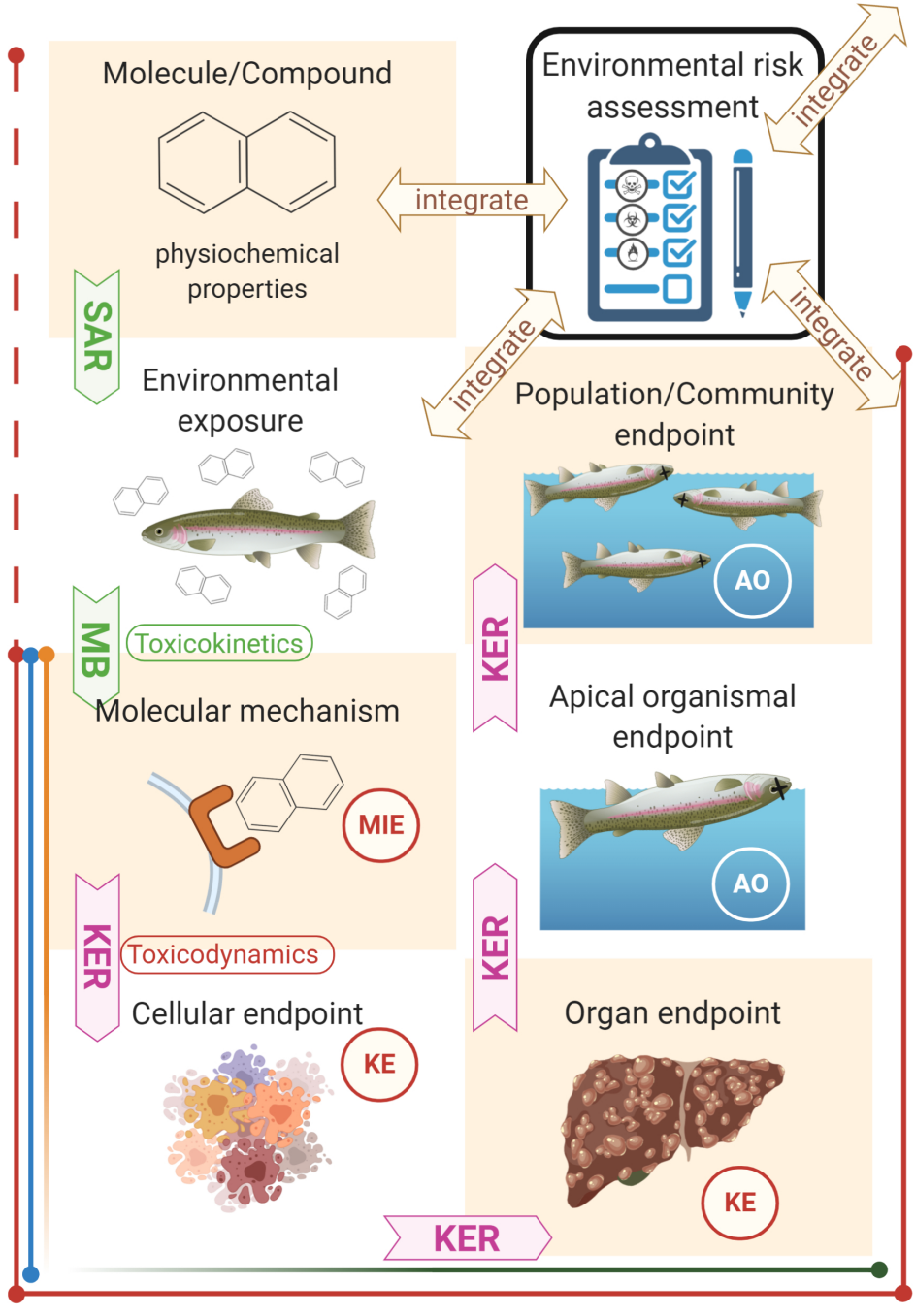
The potential advantages of fish-derived bioassays in bioanalytics have been discussed previously (Ankley et al. 1998; Castaño et al. 2003; Bols et al. 2005) and their application for environmental risk assessment has been considered a while ago (Schirmer 2006). Fish cells have two significant advantages in comparison to mammalian cell lines. First, they can be cultured at lower temperatures, proliferate slower, and are therefore less demanding in handling and maintenance. Second, permanent, immortal cell lines are not cancerous, but they proliferate from primary tissue explants and remain vivid in culture, likely due to increased telomerase activity in fish tissue. Thus, permanent fish cell lines reflect the original tissue properties better than most mammalian cell lines. When developing novel fish-based cell lines and bioassays, the emphasis should be on reporter gene assays for the assessment of receptor-mediated TPs; given that in terms of non-specific toxicity (e.g., narcosis), eukaryotic cell lines should generally be interchangeable due to Ekwall's principle of basal cytotoxicity (Ekwall 1983). Further, receptor-mediated TPs need to be investigated in terms of evolutionary conservation among vertebrates. A better understanding is needed of the difference in TP sensitivity, inducibility, and architecture among species, such that, a parallel assessment is worthwhile or could alternatively be retrieved from human data (Villeneuve et al. 2019). The discussion remains inconclusive as to what amount established mammalian assays should be incorporated, or if assays derived from aquatic organisms are more representative (Lillicrap et al. 2016; Neale et al. 2020).

The lack of available assays is further problematic in the context of the upcoming European Water Framework Directive (WFD) (European

Commission 2009, 2016) reevaluation. The WFD aims to achieve a "good biological and chemical status" of all European surface waters. Currently, 45 priority substances are monitored via chemical analyses, and a few complementary bioassays are listed. However, these bioassays are not mandatory. Problematically, a multitude of unknown anthropogenic substances and mixture effects cannot be assessed by chemical analysis. The EU-wide SOLUTIONS program (tab. 2) was concluded in 2018 and compiled diverse strategies to address legacy, current, and future pollutants that display a threat to water resources concerning human and ecosystem health status (Brack 2019). These strategies were recommended for incorporation into the upcoming WFD-reevaluation. In order to bridge the gap between chemical analytics and biomonitoring and also account for the unknown, the application of a "triad" approach (Altenburger et al. 2015) consisting of advanced chemical analytics (e.g. non-target screening), effect-based tools (bioanalytics/bioassays), and effect-directed analysis has been recommended (Brack et al. 2017, 2018, 2019). However, most of the proposed effect-based tools (bioassays) are of either mammalian or bacterial origin (Wernersson et al. 2015), as they have standardly been utilised in bioanalytics (Leusch and Snyder 2015). As mentioned above, this emphasises the necessity of fish-derived or at least aquatic organism-related *in vitro* bioassays, especially, reporter gene assays.

We postulate the use of transient reporter bioassays to be a quick, easy, and economical solution to fill the current gap in fish-derived *in vitro* reporter gene assays. In comparison to stably transfected constructs, transient reporter gene assays have the advantage of being more flexible in terms of logistics, maintenance, and genetic alteration. One primary permanent cell line can be used and transiently transfected with different constructs, thus covering the assessment of various TPs. The presented thesis aimed to develop transient reporter gene assays of specific xenobiotic metabolism and cellular stress response TPs in relevant aquatic organisms (fish), assess their reliability and robustness, investigate their potential to predict effects *in vivo*, and interpret their fit to the AOP concept. Permanent cell lines derived from the zebrafish (*Danio rerio*) of different tissue origins were utilised as model systems, given its overall establishment in toxicity testing and feasibility to biotechnological tools (Garcia et al. 2016).

Figure 1: The adverse outcome pathway (AOP) concept. Organisms are exposed to potential pollutants. Once the compound has permeated into the organism and becomes bioavailable (**toxicokinetics**; PBTK modelling) at a specific target molecular site (e.g., a receptor), it triggers the molecular initiating event (MIE) of a specific AOP. During the ensuing cellular processes (**toxicodynamics**; PBTD modelling), downstream toxic effects are triggered, so-called key events (KE). Specific key events are linked via key event relationships (KER). Finally, on the individual and population scales, the pollutant might cause apical adverse effects (AO). The sequence of the AOP is marked with a red line. The dashed red line identifies optional validation layers, such as structure-activity-relationship (SAR) and mass-balance (MB) modelling (alternatively: PBTK) that can be additionally evaluated to retrieve a quantitative AOP (qAOP). The range of a toxicity pathway (TP) is marked in blue. Mechanism of action (MOA) and mode of action (MoA) are marked in orange and green, respectively. AOP information can be integrated into environmental risk assessment decision making. The illustration was created in the licensed BioRender application.


Molecule/Compound
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Toxicokinetics
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Figure 1

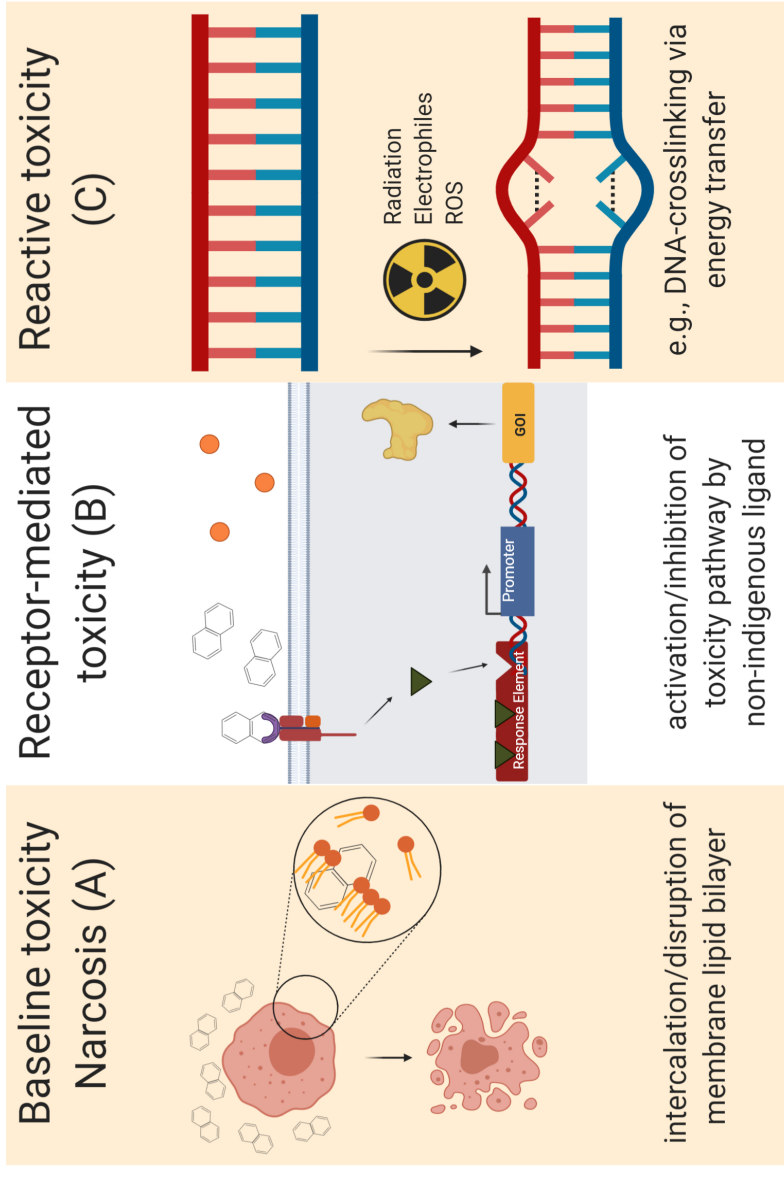


Figure 2: Several modes of action that can be recorded via cellular *in vitro* bioassays: (A) baseline toxicity/narcosis (non-specific) via cytotoxicity/viability assays, (B) receptor/transcription factor-mediated toxicity (specific) via reporter gene assays (GOI = "gene of interest"), and (C) reactive toxicity (specific) via various cellular biomarkers. The illustration was created in the licensed BioRender application.

2. Aims & objectives

The overarching aim of the thesis was to develop transient reporter gene assays in zebrafish cell lines, as fish-derived bioassays are considered essential for an appropriate organism-based environmental risk assessment. Such assays can be employed in future test batteries, for the assessment of multiple TPs, and in the AOP context. In brief, it was proposed to establish and validate transient reporter gene assays for an adaptive stress response pathway (oxidative stress – Nrf2) and a xenobiotic metabolism pathway (aryl hydrocarbon receptor – AhR). The former has been conducted in papers I and II, the latter in paper IV. Further, the *in vitro*-measured effects should be extrapolated to a low-tier *in vivo* fish model. A correlation of acute toxicity data from zebrafish cell lines and embryos has been conducted in paper III. Finally, it was the desired plan to compare established assays with data generated in standardly used mammalian bioassays, as presented in the additionally depicted data (tab. 4). In the course of this thesis project, additional minor objectives were considered, such as how to handle spurious and artefact effects of the test system induced by transgenesis per se, and, additionally, how to handle cross-talk between analysed TPs. In summary, the objectives of the study were to:

- Develop transient reporter gene assay in zebrafish cell lines, covering TPs of interest (Nrf2, AhR)
- Validate developed assays
- Correlate *in vitro* and low-tier *in vivo* data
- Evaluate the need for species-specific assays in ecotoxicology
- Reason strategies on how to handle cross-talk and artefact effects within test systems

3. Commentary on materials and methods

This section will provide a summary of the experimental design and methods used. A detailed description of each method, including technical details, is available in each specific paper.

3.1 Chemicals

Specific positive controls were used that are known inducers of the investigated TPs. Positive controls were mainly used for assay validation and standard curves. Designed assays were assessed for applicability via exposure to environmental pollutants. Pesticides and pharmaceuticals were the major groups investigated since they comprise primary categories of legacy, current, and potential future pollutants. Please consult the attached publications for more details on the specific test chemicals.

3.2 Test organism

The zebrafish (*Danio rerio*) cell lines were selected as a test system, given the zebrafish's establishment as a toxicological test platform, especially *in vivo* (Garcia et al. 2016; Shao et al. 2019). Further, the fish embryo test (FET) (OECD 2013) was one of the first *in vitro* assays to gain partial regulatory acceptance and is popularly conducted with zebrafish embryos. Therefore, the FET can be considered as a vital alternative for the acute fish toxicity test (AFT) (Lammer et al. 2009; Knöbel et al. 2012; Belanger et al. 2013; OECD 2019). The TPs investigated in this thesis are well studied in zebrafish. Respective receptors, translocators/transducers, co-factors, response elements, transcriptomic and proteomic responses are characterised for both the Nrf2/Keap1/ARE (Carvan et al. 2000, 2001; Kobayashi et al. 2002, 2009;

Timme-Laragy et al. 2012; Hahn et al. 2015; Fuse and Kobayashi 2017; Sant et al. 2017) and the AhR/ARNT/XRE (Tanguay et al. 1999, 2000; Andreasen et al. 2002; Zeruth and Pollenz 2005, 2007; Hahn et al. 2017) TPs. Thus, enabling the investigator to draw precise mechanistic conclusions.

A handful of permanent zebrafish cell lines has been established over the years and is available via cell banks or lab-to-lab propagation. In this thesis, two fibroblasts lines were used, PAC2 (RRID:CVCL_5853) (Culp 1994; He et al. 2006; Senghaas and Köster 2009) and ZF4 (RRID:CVCL_3275) (Driever and Rangini 1993; He et al. 2006); and an adult hepatocytes line, ZFL (RRID:CVCL_3276) (Ghosh and Collodi 1994; Ghosh et al. 1994; Eide et al. 2014). Permanent zebrafish cell lines were incubated at 28°C and subcultured weekly. Further information is given in each paper regarding specific culturing conditions and culture media formulations.

Zebrafish FET data were not derived from experiments conducted during the timeframe of this thesis but from a former study (Carlsson et al. 2013), which was conducted within our facilities. The historical data was re-analysed to fit the requirements of paper III. In the Carlsson et al. study, the authors modified the standard FET test by adding sublethal endpoints to the apical endpoint evaluation. See also the supplementary information of paper III for further details.

3.3 Assessing non-specific toxicity: bioassays for cytotoxicity/viability

Non-specific toxicity (e.g., narcosis) has been either assessed as the primary toxicity endpoint (paper III) when investigating acute toxicity, or as a safeguarding mechanism to ensure that the specific/reactive toxicity is investigated under conditions that do not cause non-specific toxicity (papers I, II, IV, and additional data). A plethora of cytotoxicity/viability assays has been developed and is commercially available. In general, cytotoxicity assays score an adverse effect, such as LDH or NR release (see explanations below). Thus, the higher the damage inflicted to the cells, the higher the recorded score of the endpoint. Membrane integrity assays can be considered as cytotoxicity assays. Viability assays, on the other hand, measure vital cellular metabolism function. Thus, the healthier the cells are, the stronger

the recorded signal is. Energy metabolism assays can be considered as viability assays. Nevertheless, since it is common to normalise recorded exposures to healthy controls and display the data as a percentage or induction ratio, cytotoxicity/viability assays are interchangeable after data evaluation. There are different approaches to categorise cytotoxicity/viability assays, such as by MoA or by the recorded output (dye-exclusion, colourimetric, fluorometric, and luminometric). All utilised assay types are shortly presented in the following section, in an MoA-like manner. Extensive reviews on commonly used cytotoxicity/viability assays can be found in the scientific literature (e.g., Kepp et al. 2011; Riss et al. 2015; Aslantürk 2018).

3.3.1 Assays of energy metabolism: MTS, ATP, AB

Endogenous cellular NADPH reduces tetrazolium salts to insoluble formazan, which can be recorded colourimetrically. Thus, mitochondrial activity is reflected by formazan turnover (Mosmann 1983; Berridge et al. 2005). MTS (5-(3-carboxymethoxyphenyl)-2-(4,5-dimethyl-thiazoly)-3-(4-sulfophenyl) tetrazolium) is a water-soluble tetrazolium derivative that can be directly applied to the cells and is available as a commercial kit ("CellTiter 96® AQueous One Solution"; Promega, Madison, USA). The reaction is recorded "alive" and does not require fixing, staining, or cell lysis.

Cellular ATP catalyses the oxidation of luciferin to oxyluciferin. Luciferin substrate is applied to cells, and oxyluciferin turnover can be measured luminometrically after incubation (Fan and Wood 2007; Auld et al. 2009). However, an assessment of luminescence requires cell lysis.

The water-soluble dye resazurin, also known as Alamar Blue (AB), is reduced by cellular NADPH to resorufin, which can be detected fluorometrically (De Jong and Woodlief 1977; Winartasaputra et al. 1980). Thus, the mechanism is identical to the MTS assay.

3.3.2 Assays of membrane integrity: LDH, NR, CFDA-AM

Lactate dehydrogenase (LDH) is a cytosolic enzyme that is released into the surrounding nutrition medium once the cell membrane loses stability. The LDH-assay substrate contains additional lactate, NADPH, and resazurin. After exposure, LDH-containing medium is extracted and separately

incubated with the substrate. In an NADPH-coupled redox reaction, LDH is reducing resazurin to resorufin, which can be recorded as described for the AB assay (De Jong and Woodlief 1977; Winartasaputra et al. 1980).

The cationic dye neutral red (NR) has no charge at physiological pH and permeates into the cells. Once it reaches the lysosomes, it protonates due to the lower organelle pH and gets trapped (ion-trap effect). Apoptotic cells cannot maintain NR due to lysosome instability. The cells are fixed, and the extracted dye is measured colourimetrically (Borenfreund and Puerner 1985; Borenfreund et al. 1988).

Membrane-bound esterases mainly convert the dye CFDA-AM (5-carboxyfluorescein diacetate, acetoxymethyl ester) into CF (carboxyfluorescein), which is assessed fluorometrically (Cavarec et al. 1990). Thus, CFDA-AM conversion by esterases reflects membrane stability since their functionality is only given in intact cells.

3.3.3 Assays of cell proliferation (BCA, EdU)

BCA (bicinchoninic acid) forms a strong, insoluble complex with supplemented Cu^{2+} and cysteine, cystine, tryptophan, and tyrosine residues of present proteins (Smith et al. 1985; Olson 2007), which can be recorded colourimetrically. Cellular proteins are extracted via lysis buffer. Thus, the overall amount of protein reflects the cell culture's vivacity and proliferation.

The synthetic thymidine derivate EdU (5-ethynyl-2′-deoxyuridine) can be added to the nutrition medium during incubation and exposure and is incorporated into the culture's genome, as the cell culture proliferates in a relative manner (Salic and Mitchison 2008). Thus, the amount of incorporated EdU represents the culture's overall vivacity and ongoing proliferation. The EdU is quenched via bioconjugation and fluorometrically detected.

3.3.4 Multiplexing cytotoxicity/viability assays

Relying on only one endpoint of cytotoxicity/viability can be cumbersome, especially given that specific compound classes can act via various MoA/MOA and impact endpoints differently. Secondly, compounds can also cause false positives by reacting with the assay substrate. Therefore, it is

recommended to use multiple endpoints (Schirmer 2006; Stepanenko and Dmitrenko 2015; Lungu-Mitea and Lundqvist 2020). By multiplexing cytotoxicity/viability assays, diverse endpoints can be assessed from the same microtiter plate, thus increasing throughput and reliability. Within this thesis, the AB/CFDA/NR (Schirmer et al. 1997, 2004; Dayeh et al. 2005, 2013; Fischer et al. 2019)) and ATP/LDH (Farfan et al. 2005) multiplex assays were applied. Further, we developed the MTS/BCA multiplex assay (paper III and IV).

3.4 Assessing receptor-mediated toxicity: DLR reporter gene assays

As mentioned before, reporter gene assays are an ideal tool for identifying and measuring TPs. They are vital in the AOP concept, given their intrinsic ability to identify and investigate MIEs and MOAs. Reporter gene assays are pro- or eukaryotic cellular systems bearing stably or transiently introduced reporter gene cassettes. In this thesis, we focused on the establishment of transient reporter gene assays, given the scarcity of species-specific assays in ecotoxicology. Transient assays are developed and distributed faster; therefore, they were prioritised over stable reporter assays.

In contrast to stable transfection, the reporter gene cassette is not incorporated into the host's genome during transient transgenesis. Instead, the plasmid DNA construct is guided into the nucleus, where it transiently persists as an episomal target gene until degradation (fig. 3A). Therefore, one host cell line can be utilised for multiple reporter gene constructs. However, transgenesis must be conducted separately for every experiment. Several types of transfection methods exist (Kim and Eberwine 2010; Kaestner et al. 2015): physical (electroporation), biological (virus-mediated), and physio-chemical (transfection reagents). From a toxicological perspective, transfection reagents are favoured since they are the least stress-inducing in comparison to the other methods. Physio-chemical reagents exploit lipofection or hijack cellular phagocytosis and endocytosis mechanisms. Many reagents employ multiple mechanisms. However, the transfection reagents are mostly proprietary. Thus, their specific mechanisms are unknown to the investigator, which can be disadvantageous. As follows, the investigator needs to test several commercial transfection reagents in order

to identify the best fit for the cell line of interest (Sandbichler et al. 2013). Such a transfection reagent cohort test has been conducted here in the paper I (supplementary information).

The principle of the reporter gene assay is to replicate the genomic recognition site (response element) of a specific TP and recruit the cellular transcription/translation machinery in a parallel manner (fig. 3B) (Manley et al. 1980; Nordeen 1988). The reporter gene cassette bears the same response element as certain genes-of-interest (GOIs) of the investigated TP. Nevertheless, instead of the GOI, a gene coding sequence of a specific reporter enzyme (e.g., luciferase) is located adjacent to the response element and promoter. If a specific stressor activates the TP, episomal reporter vectors will recruit specific transcription factors, leading to the transcription and translation of the luciferase reporter enzyme, which can be quantified. An example of the Nrf2-responsive pGL4.37 reporter vector is given in fig. 3C.

In principle, transient and stable reporter plasmid vectors are identical except for an antibiotic resistance gene. Classic stable transgenesis is conducted via random genome integration of the reporter construct using the same methodologies as mentioned above. At random, a few cells will recombine and integrate the reporter construct. If the construct is bearing an antibiotic resistance gene, clones can be screened and selected via antibiotic incubation. However, the procedure is very lengthy and faulty, given that even positive clones are often epigenetically silenced after an extended period in culture (Stepanenko and Heng 2017). More sophisticated transgenesis methods have been developed *in vivo*, such as RNAi and Tol2-mediated transgenesis, to increase efficacy (Kawakami 2007; Clark et al. 2011; Lee et al. 2014; Long 2014). The CRISPR/Cas technology has the potential to revolutionise transgenesis in *in vitro* systems (Lo et al. 2017; Li et al. 2018, 2019).

Given that transient transgenesis is a stochastic process and only a certain percentage of the cells within a culture dish get transfected, the investigator has to account for transfection efficiency. The Dual-Luciferase® reporter system (DLR) corrects for this (Sherf et al. 1996; Wood 1998). In principle, two reporter gene constructs are administered in parallel. The first construct

bears the primary reporter, which is responsive to the TP under investigation (e.g., fig. 3C). The secondary construct bears an alternating reporter whose expression is coupled to a constitutive viral promoter. Thus, every transiently transfected cell is expressing the secondary reporter on a background level. Subsequently, the primary reporter signal can be normalised to the secondary reporter. Hence, the signal is corrected for transfection efficiency. Fig. 3D abstractly illustrates the DLR system. Fig. 4 comprehensively depicts the process of culturing, transfecting, exposing, and measuring TP-induction in zebrafish cell lines, as conducted in the papers I and II. Unfortunately, DLR systems have their pitfalls as well (Shifera and Hardin 2010; Stepanenko and Heng 2017). Within this thesis, we investigated artefact effects induced by transient transgenesis and developed strategies to tackle the issue.

3.5 Plasmid vectors

In this thesis, primary reporter vectors were all utilising Firefly luciferase (Fluc; *Photinus pyralis*). Response elements within the reporter gene cassettes were either generic or genomic. Generic response elements are synthetically engineered from the response elements' consensus sequence; thus, generic reporters retain conserved response across species and within different tissue types and cell cultures. Alternatively, genomic response elements are directly derived from endogenous genes of test species. They can be cloned into plasmid vectors and have the advantage of specificity. Secondary reporter vectors were all utilising Renilla luciferase (Rluc; *Renilla reniformis*). Rluc normalisation vectors were purchased with differing plasmid backbones and constitutive promoters, mainly to test their impact on the overall reporter signal. The following, commonly used constitutive promoters were applied: TK (herpes simplex virus thymidine kinase promoter), SV40 (simian virus 40 promoter), CMV (cytomegalovirus promoter), and minP (truncated minimal promoter). For details and plasmid geometries, see also supplementary information in papers II and IV.

3.6 Statistics & data evaluation

Study design and statistical evaluation of data were conducted according to recommendations and guidelines (Lazic 2010; Lazic et al. 2017; Green et al. 2018; Musset 2018), where feasible. The following mathematical models

were applied throughout the thesis: variance and multivariate analysis (n-way ANOVA), regression analysis (linear type I and II, nonlinear four-parameter log-logistic (4PL) and probit), and toxicokinetic mass-balance modelling (Fischer et al. 2017; Bittner et al. 2019). Further details are given in the different papers.

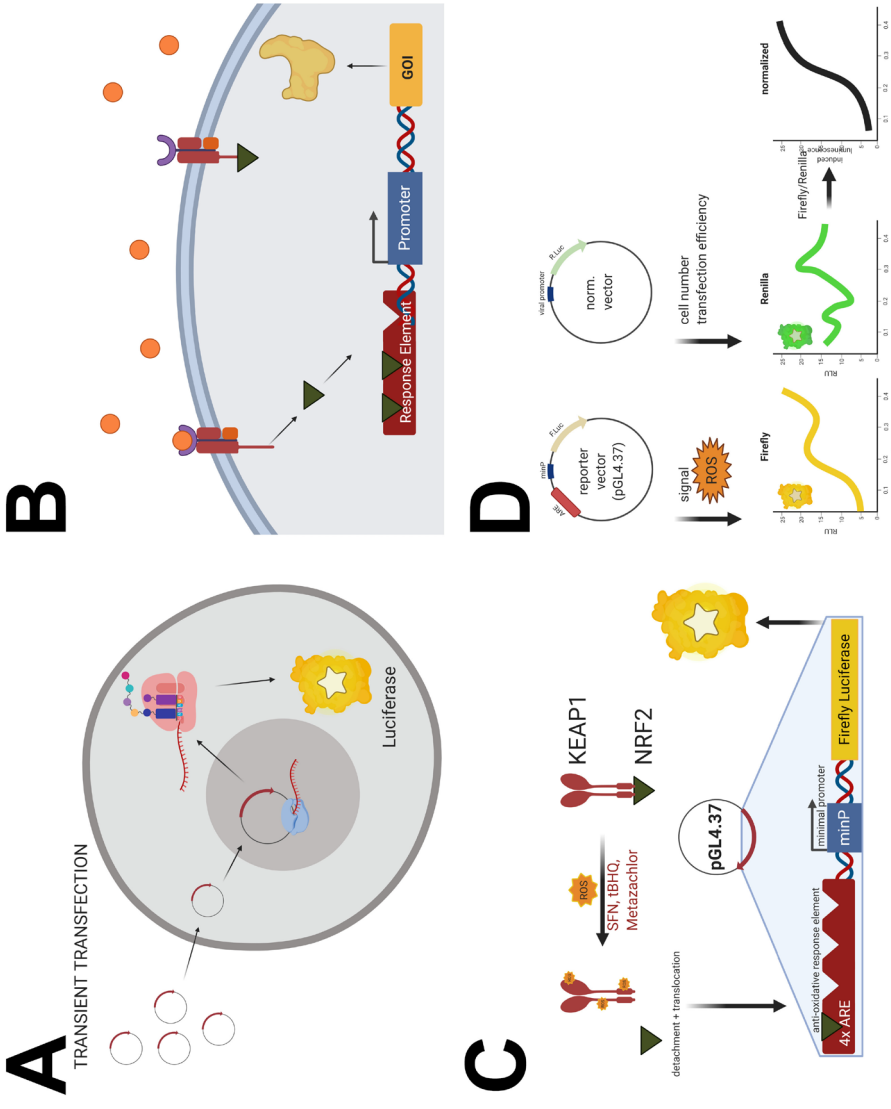


Figure 3: (A) Transient transgenesis of a reporter gene plasmid vector. (B) Reporter gene assays are utilising TP's transactivation domains (GOI = gene of interest). (C) Topography, functionality, and activation of the Nrf2-responsive reporter plasmid pGL4.37 (SFN = sulforaphane; tBHQ = tert-butylhydroquinone). (D) The DLR system (FLuc = firefly luciferase; RLuc = renilla luciferase). The illustration was created in the licensed BioRender application.

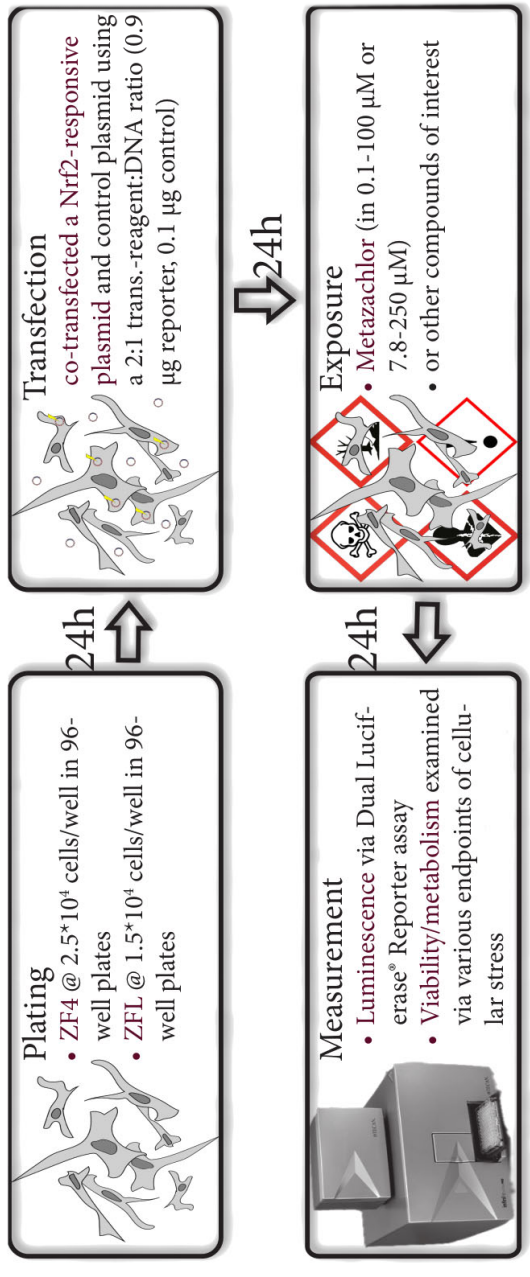


Figure 4: Synopsis of a DLR experiment to measure the induction of specific TPs in zebrafish cell lines. In this case, cells were transfected with an Nrf2-responsive primary reporter vector (fig. 3C) of the oxidative stress TP.

4. Results & discussion

In the results and discussion section, all publications and manuscripts are shortly presented with an emphasis on the principal results and findings. The results and findings of each objective comprising this thesis study are presented in their respective papers (I to IV). Here, the papers are not discussed chronologically, but by topics. Subsequently, the results are followed by a general discussion focusing on the overall implications and the future perspectives that evolve from the conducted work.

4.1 Transient reporter gene assays of the Nrf2 adaptive stress response pathway (papers I+II)

In "**Development of an oxidative stress *in vitro* assay in zebrafish cell lines**" (paper I) we screened a group of commercially available transfection reagents for transfection efficiency in three permanent zebrafish cell lines: hepatocytes (ZFL), embryonic fibroblasts (ZF4), and adult fibroblasts (Pac2). The most efficient reagent for each cell line was selected for further experiments: FuGene HD ("FHD"; Promega) for the ZF4 and Pac2 cell lines and Xtreme-Gene HP ("XHP"; Roche) for ZFL (see supplementary information of paper I for specific illustrations). Chosen reagents were utilised in the transient transfection of an Nrf2-responsive Firefly luciferase plasmid vector ("pGL4.37", see fig. 3C) and a Renilla luciferase normalisation vector ("pRL-TK"). The transcription factor "nuclear erythroid 2-related factor 2" (Nrf2) is a key regulator of the cellular defence against oxidative stress and, thus, a primary TP of the cellular stress response. Known inducers of oxidative stress were initially tested in the designed assays (fig. 5A). Upon positive outcomes, bioassays were employed in testing a group of pesticides that were associated with oxidative

stress in fish (Slaninova et al. 2009). We found the ZFL and ZF4 cell lines responsive to an Nrf2-regulated stimulus when transiently transfected with specific transfection reagents and constructs and exposed to known inducers (e.g., fig. 5A). Further, to the best of our knowledge, our results are the first to identify the compound metazachlor as a potent activator of the Nrf2-modulated oxidative stress TP (fig. 5B).

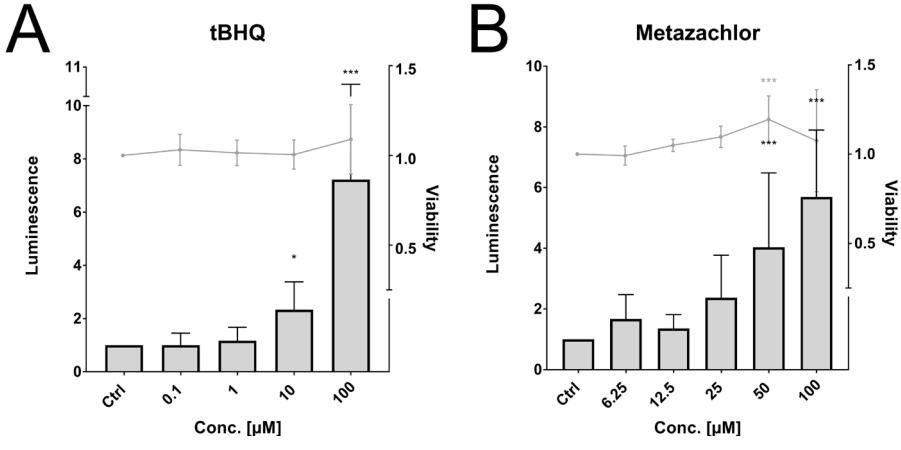


Figure 5: Relative luminescence induction (bars) and cellular viability (lines) in ZF4 cells after exposure to the positive control tertbutylhydroquinone (A, "tBHQ") and the pesticide metazachlor (B). Luminescence corresponds to quantitative Nrf2 activation measured via the DLR assay. Viability corresponds to measured absorbance of formazan production via the MTS-assay. Each bar and point represent the mean (experimental units n = 3–4; observational units N = 10–16) including SD. Asterisks indicate significance tested in a two-way ANOVA mixed model with Dunett's post-hoc test (*P < 0.05, **P < 0.01, ***P < 0.001; grey = viability; black = luminescence). Images modified from Lungu-Mitea et al. 2018.

In "**Potentials and pitfalls of transient *in vitro* reporter bioassays: interference by vector geometry and cytotoxicity in recombinant zebrafish cell lines**" (paper II), we refined the previously established oxidative stress TP reporter assay. Noteworthy, transient transfection itself might interfere with cellular homeostasis and impact the system beyond the function of the manipulated gene, thus leading to non-specific results. In this publication, we described how varying vector geometry and different regulatory gene elements on vector plasmids used for transient transfection in ZFL and ZF4 cell lines led to an almost ten-fold difference in assay efficacy (fig. 6A+B) when exposed to a specific stressor. Additionally, we

uncovered how transient transgenesis increases stress to the cellular test system per se (fig. 6C) and in a construct/size-dependent manner (see paper II). We concluded that a thorough bioassay design is needed to ensure reliability and regulatory acceptance of newly designed reporter gene assays.

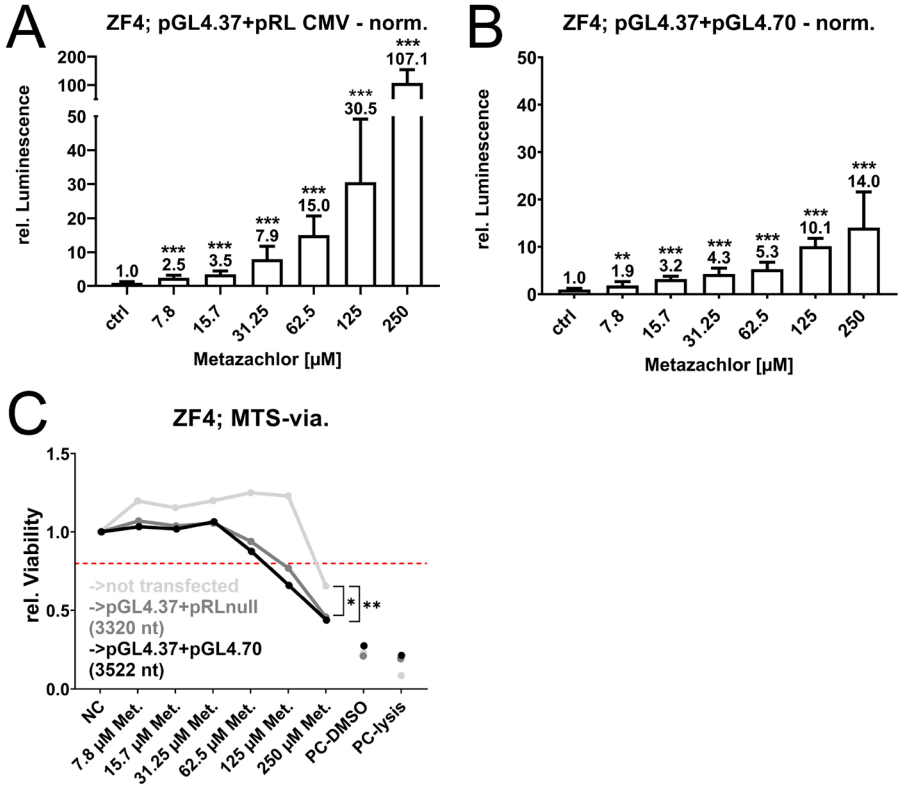


Figure 6: Effects on luminescence (white bars) measured in the ZF4 cell line exposed to metazachlor. Normalised relative luminescence induction corresponds to quantitative Nrf2 activation measured via the DLR assay in cells co-transfected with pGL4.37 and the normalisation vectors pRL-CMV (A) and pGL4.70 (B). Effects on the MTS viability endpoint (cellular metabolism; dots connected by lines) measured in the ZF4 cell line after exposure to metazachlor. Cells were transiently transfected with constructs of increasing size, as depicted (nt = nucleotides) (C). Each bar and point represent the mean (experimental units n = 3–4; observational units N = 9–16) including SD. Numerical means are depicted on top of bars. Asterisks indicate significance tested in a two-way ANOVA mixed model with Dunnett's/Holm-Sidak's post hoc test (*P < 0.05, **P < 0.01, ***P < 0.001). Images modified from Lungu-Mitea et al. 2020.

4.2 Transient reporter gene assays of the AhR xenobiotic metabolism pathway (paper IV)

In "**Modulation of transient reporter gene vectors of the xenobiotic metabolism pathway in permanent zebrafish hepatocytes**" (paper IV) we developed an AhR-responsive transient reporter assay in the ZFL cell line by applying previously developed technologies and strategies (paper I + II). We reported the viral constitutive promoter-induced squelching of the primary reporter signal in transient reporter assays (fig. 7B), as depicted by low overall inducibility in comparison to non-squelched reporter signals (fig. 7A). Squelching is defined as the epigenetic competition of gene-regulatory units for the recruitment of limited transcription (co-)factors and, thereby, the overall transcription/translation machinery (Natesan et al. 1997; Simon et al. 2015). We designed a novel normalisation vector bearing an endogenous zebrafish-derived genomic promoter ("zfEF1aPro") instead of a generic, synthetic promoter. The new construct rescued the squelching-delimited system. This finding provided new insights into the modulation of transient reporter systems under stress (fig. 7A) and depicted the overall higher efficacy in concentration-response relationships (fig. 8). As well, our results aligned with data of the xenobiotic metabolism TP in adult zebrafish, as reported in other literature. Zebrafish-derived systems are considered to be intrinsically low responders to dioxin-like compounds, as the pattern can be derived from potencies and effect concentrations in fig. 8B (mammalian-derived assays are 2-3 log scales more sensitive (Eichbaum 2014)). Hence, better evaluation of the conditions under which zebrafish assays of the AhR-responsive TP can be utilised in environmental screenings is needed. Finally, we discussed how the ubiquitously used ligand beta-naphthoflavone (BNF) promiscuously activates multiple TPs of the xenobiotic metabolism and cellular stress response in an orchestral manner, leading to a concentration-related inhibition of some TPs and non-monotonous concentrations response curves (fig. 8A). We named such a multi-level inhibitory mechanism that might mask effects as "maisonette squelching".

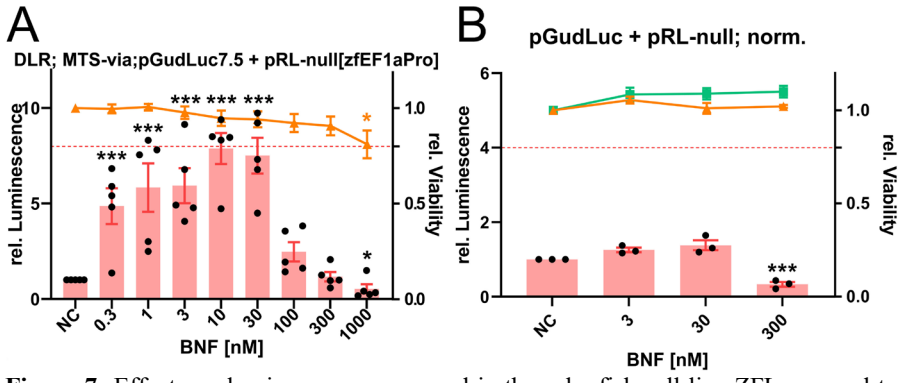


Figure 7: Effects on luminescence measured in the zebrafish cell line ZFL exposed to beta-naphthoflavone (BNF). Luminescence corresponds to quantitative AhR transcription factor activation measured via the DLR assay in cells co-transfected with the pGudluc7.5 reporter and depicted normalisation vectors: (A) pRL-null[zfEF1aPro] (zebrafish genomic promoter); (B) pRL-null (minimal, synthetic promoter). Mean normalised luminescence induction is illustrated as red bars, black dots represent means of single experiments, red whiskers represent the SEM (experimental units n = 3–5; observational units N = 9–15). Cellular viability corresponds to endpoints measured via the MTS/BCA-multiplex assay. Each point (MTS orange, BCA green) represents the mean including SEM (experimental units n = 3–4; observational units N = 9–12). A threshold value of 0.8 was considered as biologically significant (dotted red line). Asterisks indicate significance tested in a one-way ANOVA with Dunnett's post hoc test (*P < 0.05, **P < 0.01, ***P < 0.001).

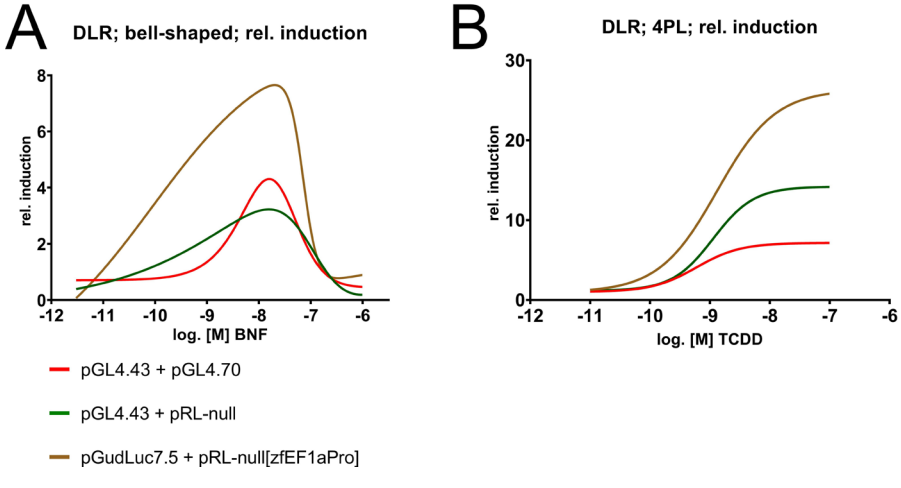


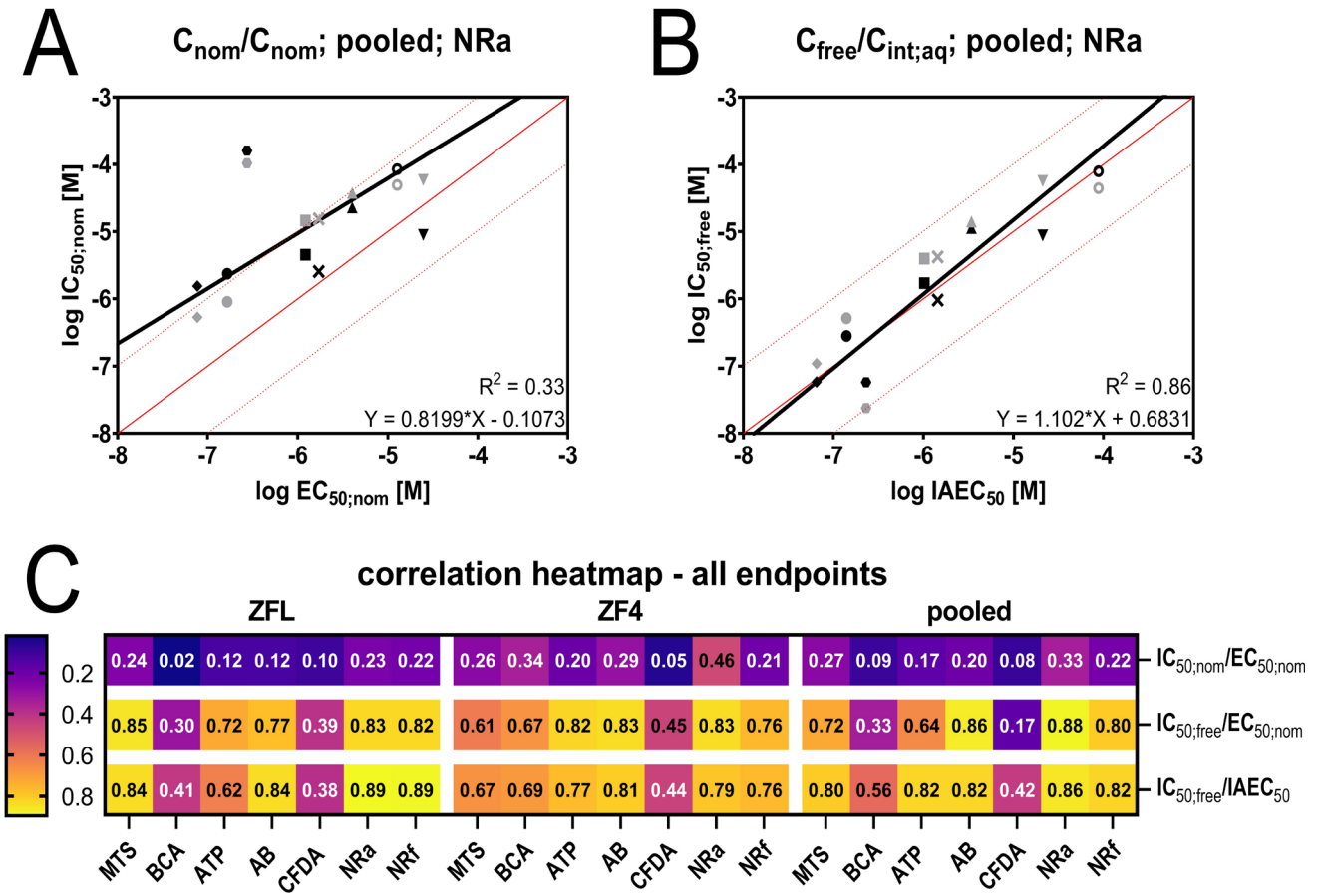
Figure 8: Concentration-response curves of depicted co-transfection setups after beta-naphthoflavone (BNF) (A) and tetrachlorodibenzo-p-dioxin (TCDD) (B) exposure. Results of the DLR assays were fitted as relative induction to either a bell-shaped (A) or 4PL (B) nonlinear regression.

4.3 Correlating *in vitro* to low-tier *in vivo* data

In "**Modelling bioavailable concentrations in zebrafish cell lines and embryos increases the correlation of toxicity potencies across test systems**" (paper III) we recorded endpoints of cytotoxicity in zebrafish cell lines (ZFL and ZF4) and apical endpoints in zebrafish embryos after 48h and 144h of exposure. Cells and embryos were exposed to veterinary pharmaceuticals, previously shown to have effects *in vivo* and *in vitro* (Carlsson et al. 2013), in zebrafish. Derived nominal effect concentrations were utilised to compare sensitivity, potency, and predictability between the cell- ($IC_{50;nom}$) and embryo-derived ($EC_{50;nom}$) data. State-of-the-science mass-balance-models (Fischer et al. 2017; Bittner et al. 2019) were applied to compute the actual bioavailable concentrations to the cells ($IC_{50;free}$) and embryos ($IAEC_{50}$), and structure-related internal concentrations (see paper III). Modelled bioavailable concentrations strongly increased correlations (up to $R^2 = 0.98$ for several endpoints and combinations tested; see paper III) compared to nominal concentrations and placed regression lines close to the line-of-unity and axis-origin (fig. 9B). Additionally, we modelled the bioavailable concentrations of literature-derived pesticide data and obtained similar results, thus demonstrating the general applicability of our study concept. Conclusively, appropriate cytotoxicity assays accompanied by mass-balance-modelling showed a high correlation to embryotoxicity and therefore, great potential in bridging *in vitro* to *in vivo* toxicity testing when utilising the FET as a linking platform.

Figure 9: Deming regression of depicted IC_x (cells) and EC_x (fish embryo) values for pooled cell lines (ZF4 + ZFL) and the NRa (neutral red, absorbance) endpoint of cellular toxicity. (A) Nominal concentration in cells vs. nominal concentrations in zebrafish embryos ($logIC_{50;nom}$ vs. $logEC_{50;nom}$). (B) Modelled bioavailable concentrations in cells vs. modelled bioavailable concentrations in zebrafish embryos ($logIC_{50;free}$ vs. $logIAEC_{50}$). The regression line is plotted in solid black, the line of unity in solid red, and one order of magnitude deviations from the line of unity are plotted as dotted red lines. Regression equations and adjusted R^2 values of Pearson correlations are given for every setup of comparison. (C) A heatmap of adjusted R^2 values derived from Pearson correlation of the various IC_x (cells) vs. EC_x (fish embryos) comparisons per endpoint of cellular toxicity. Correlations of nominal and bioavailable median effect concentrations are depicted for ZFL, ZF4, and pooled cell lines (column stacks).

Figure 9


A
C_{nom}/C_{nom}; pooled; NRa
log $IC_{50;nom}$ [M]
log $EC_{50;nom}$ [M]
R^2 = 0.33
Y = 0.8199*X - 0.1073
B
$C_{free}/C_{int;aq}$; pooled; NRa
log $IC_{50;free}$ [M]
log $IAEC_{50}$ [M]
R^2 = 0.86
Y = 1.102*X + 0.6831
C
correlation heatmap - all endpoints
ZFL
ZF4
pooled
0.24 0.02 0.12 0.12 0.10 0.23 0.22 0.26 0.34 0.20 0.29 0.05 0.46 0.21 0.27 0.09 0.17 0.20 0.08 0.33 0.22
0.85 0.30 0.72 0.77 0.39 0.83 0.82 0.61 0.67 0.82 0.83 0.45 0.83 0.76 0.72 0.33 0.64 0.86 0.17 0.88 0.80
0.84 0.41 0.62 0.84 0.38 0.89 0.89 0.67 0.69 0.77 0.81 0.44 0.79 0.76 0.80 0.56 0.82 0.82 0.42 0.86 0.82
$IC_{50;nom}/EC_{50;nom}$
$IC_{50;free}/EC_{50;nom}$
$IC_{50;free}/IAEC_{50}$
0.2
0.4
0.6
0.8
MTS BCA ATP AB CFDA NRa NRf

4.4 Transient zebrafish reporter gene assays as bioanalytical tools

Additionally, comparable to "***In vitro* bioanalytical evaluation of removal efficiency for bioactive chemicals in Swedish wastewater treatment plants**" (Lundqvist et al. 2019), we used the above-established transient zebrafish reporter bioassays to perform a bioanalytical evaluation of the presence and removal efficiency of bioactive compounds in Swedish wastewater treatment plants. Wastewater samples were selected that have previously been identified as positive in standard mammalian bioanalytical assays (stably transfected HepG2 cells; unpublished data). Preliminary results (unpublished, manuscript to be prepared) detected removal efficiency-related effects on the induction of the cellular stress response TP (Nrf2) in the zebrafish assay, similar to the mammalian bioassays (tab. 4). Moreover, bioequivalent values (BEQ) and relative enrichment factors inducing the 1.5-fold threshold concentrations (EC IR1.5) values were comparable between the mammalian (stably transfected HepG2 cells) and transient zebrafish assays. Noteworthy, the zebrafish assays were slightly more sensitive, based on the responses from the positive controls and wastewater samples. This highlights the suitability of the zebrafish assay as a bioanalytical tool in water quality assessments. In contrast, the AhR-related TP bioassay in the ZFL cell line demonstrated reduced applicability due to its lower responsiveness and unexpected synergistically acting matrix/solvent effects, leading to increased cytotoxicity of the probes (data not shown). Therefore, results seemed non-specific but related to the "cytotoxicity burst" (Escher et al. 2013; Judson et al. 2016) and could not appropriately be evaluated for BEQ values.

Table 4: A bioanalytical evaluation of chemical removal efficiency in three selected Swedish wastewater treatment plants in the context of the oxidative stress response TP (Nrf2), conducted via mammalian (Lundqvist et al. 2019) and fish (paper I + II) effect-based tools.

			EC IR1.5 [REF]	**tBHQ-BEQ [M]**	**tBHQ-BEQ [µM]**	**Removal [%]**
Plant A	mammal	in	0.96	2.23E-06	2.23	
	fish	in	1.39	4.45E-06	4.45	
	mammal	out	4.90	4.35E-07	0.43	**80.72**
	fish	out	11.25	5.49E-07	0.55	**87.64**
Plant B	mammal	in	1.44	1.48E-06	1.48	
	fish	in	1.43	4.34E-06	4.34	
	mammal	out	6.27	3.40E-07	0.34	**77.03**
	fish	out	13.82	4.47E-07	0.45	**89.63**
Plant C	mammal	in	1.50	1.42E-06	1.42	
	fish	in	1.23	5.03E-06	5.03	
	mammal	out	13.00	1.64E-07	0.16	**88.73**
	fish	out	34.35	1.80E-07	0.18	**96.42**
			EC IR1.5 [M]			
Control	mammal	tBHQ-PC	2.13E-06			
	fish	tBHQ-PC	6.18E-06			

EC IR1.5: effect concentration causing a 1.5-fold induction of the specifically recorded response

tBHQ: tertbutylhydroquinone

BEQ: bioequivalent value; tBHQ-BEQ: the bioequivalent concentration of tBHQ causing the same effect in the bioassay

5. General discussion

5.1 Potentials and pitfalls of transient *in vitro* fish reporter gene assays

Initially, we hypothesised transient fish *in vitro* reporter assays to be a quick, easy, and economical solution to the recent scarcity of specific-toxicity centred bioanalytical tools in ecotoxicology. As presented above, we successfully designed and applied transient reporter bioassays of the xenobiotic metabolism (AhR) and cellular stress response (Nrf2) TPs in zebrafish cell lines. Additionally, we proved the applicability of diverse cytotoxicity/viability assays in the zebrafish cell lines test systems; and beyond, the potential to multiplex these assays. Indeed, once established, the transient zebrafish reporter assays appropriately assessed a toxic burden inflicted by known inducers, proprietary substances, and, partly, environmental samples. This thesis at hand is thus a proof-of-concept of the original hypothesis, and the established assays could be an ideal bridging technology until more sophisticated methods are available.

However, we encountered drawbacks and pitfalls of the applied technology that might hinder a simple adaptation to other TPs and its suitability for qHTS. We highlighted how transient transgenesis perturbs cellular homeostasis and might lead to non-specific effects, especially within cellular systems under xenobiotic stress. Stepanenko & Heng reviewed how transgenesis might affect the biochemical processes that are specifically under investigation (Stepanenko and Heng 2017), in a biomedical and molecular biology context. They identified the potential induction of systemic cellular stress as the primary driver. In our study, we demonstrated

that this is especially problematic in a toxicological context (paper II), given that "stress" or toxicity are the focus of the investigation. Thus, an adequate assessment (see above) of the transfection method is necessary to neglect the introduction of additional stress via the applied methodology. We also encountered squelching effects when utilising various vector combinations in transient co-transfection. Precisely, we encountered squelching on multiple levels, and we termed the phenomenon "maisonette squelching" (fig. 10). Apparently, TPs exhibit cross-talk and can regulate each other in an orchestral manner (Safe et al. 1998; Ohtake et al. 2003; Miao et al. 2005; Tian 2009; Simmons et al. 2009). Consequently, a cellular stress response TP might squelch the activation of a hormone or xenobiotic metabolism TP, given that it activates all of the cell's resources in a final stand against the stressor. Therefore, when applying a transient reporter assay in toxicity screenings, a lack in signal may be caused by maisonnette squelching and not reflect the actual cellular response. First, the primary reporter signal might be squelched in co-transfection by the normalisation signal. Second, TP cross-talk and downregulation might squelch the primary reporter signal in favour of more imminent transcription/translation processes.

We found the final DLR assay signal output to be integrated and influenced by various parameters specific to the utilised cell lines, transfection reagents, vector plasmids combinations in co-transfection, vector sizes, vector backbones, and gene regulatory units. Unfortunately, the vice-versa interference of these factors is not universal, but cell line and TP-specific, as we have seen in our studies. Therefore, it is necessary to develop and optimise strategies to deal with spurious regulation within the DLR system, such as: (A) test the suitability of specific transfection reagents in specific cell lines; (B) test diverse cell lines for a specific TP, given that its components might not be preserved; (C) test if co-transfection is interfering with cellular viability; (D) test if gene regulatory units of the reporter and normalisation vectors are affecting each other. We have conducted such strategies in papers II and IV.

Equally noteworthy, such a test array binds resources and diminishes the throughput and feasibility for the development of transient reporter assays in a toxicological context. However, a thorough scrutinising of reporter-biased artefacts is mandatory, given that false negatives can significantly hamper

qHTS (Schuck et al. 2017). On the one hand, transient reporter assays could be a prerequisite for stable reporter assays. A reporter construct that proved functional in transient transgenesis will most likely be functional, once stably integrated into the host's genome. On the other hand, since we have seen significant interference by the normalisation vectors, it might be legitimate to undertake stable transgenesis directly. Most likely, it is required to conduct a case-by-case assessment on what transgenesis method to use.

The rise of new technologies has the potential to circumvent above-described issues. Organ-on-a-chip and 3D cell culture systems are rapidly evolving (Godoy et al. 2013; Jensen and Teng 2020). The investigator can utilise these technologies to co-culture manifold cell types, as they co-exist naturally within the specific tissues and organs, thus mimicking their integrated response to a stressor. Such a system per se is closer to *in vivo* than traditional 2D monolayer cell cultures. Recent advancements in the field also facilitate the transition of 3D cell culture applications from biomedicine and pharmacology to toxicology (Lelièvre et al. 2017). The establishment of the "clustered regularly interspaced short palindromic repeat" CRISPR/Cas system in genome engineering allows for the guided introduction of exogenous genetic material into specific host loci of interest. The potential pitfalls of stable transgenesis were mentioned in the materials & methods section, e.g., position effects, cell population heterogeneity, continuous antibiotic selection pressure, and exogenous (mostly viral) constitutive promoters (Lo et al. 2017). The CRISPR/Cas technology enables the investigator to introduce reporter genes exactly adjacent to endogenous gene-regulatory units (Lo et al. 2017; Li et al. 2018, 2019). Thus, it is possible to monitor the direct modulation of a TP. This heavily facilitates the design of TP-responsive stable reporter gene assays, shortens clone selection processes, and diminishes the risk of post-transgenesis epigenetic silencing. Conclusively, the CRISPR/Cas technology will accelerate the establishment of novel recombinant cell lines.

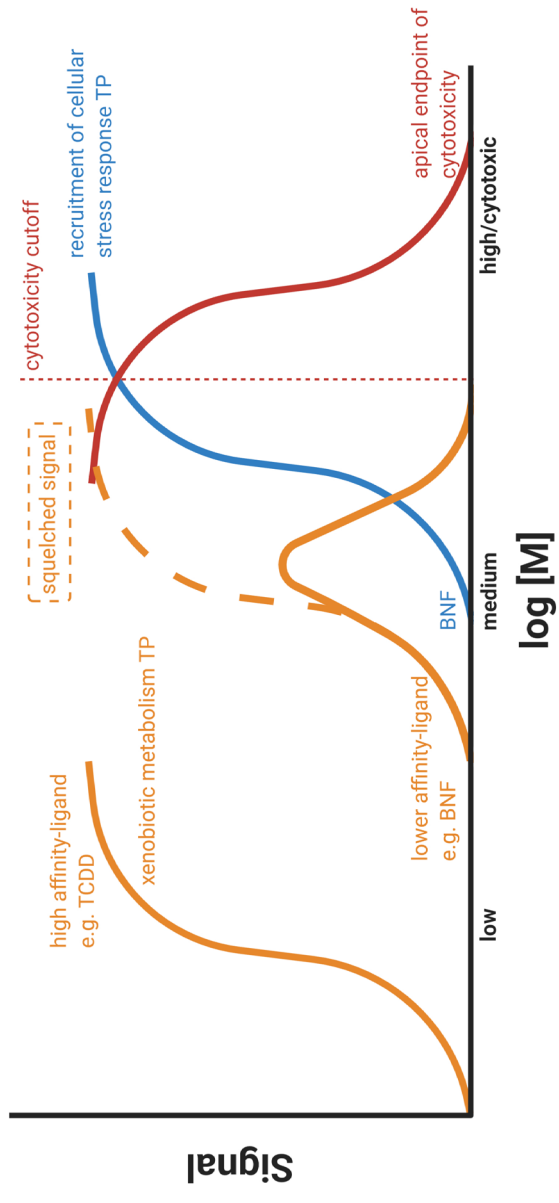


Figure 10: Schematic representation of the squelching of the AhR TP-related luciferase signal (orange) due to activation of alternate cellular stress response pathways (blue) in a system under growing toxic stress up to cytotoxicity (red) (beta-naphthoflavone (BNF); tetrachlorodibenzo-p-dioxin (TCDD)). The illustration was generated in the licensed BioRender application.

5.2 Species-specific bioanalytical tools

Recently, Villeneuve et al. renewed the request for species-specific bioanalytical tools (Villeneuve et al. 2019), at least for non-conserved pathways. However, not all TPs have been investigated in regards to the evolutionary conservation of their pathway architecture. Thus, there are no defined criteria by which to prioritise TPs in terms of bioassay development. More recently, Neale et al. assessed species-specific differences of receptor-activation via bioassays (Neale et al. 2020). They focused on TPs of the hormone response (amongst others), namely the estrogen receptor (ER), androgen receptor (AR), glucocorticoid receptor (GR), progesterone receptor (PR), mineralocorticoid receptor (MR), pregnane X receptor (PXR), and peroxisome proliferator-activated receptor gamma (PPARγ) pathways. Overall, species-specific differences in activity and potency were negligible between human and zebrafish reporter gene assays. Environmental samples were more active in zebrafish PXR compared to human PXR, whereas the opposite was the case for PPARγ. Interestingly, these results are partly contradictive to results published previously (Grimaldi et al. 2015), showing overall lower activity in fish-specific receptors of the ER and PPARγ TPs. Noteworthy, these experiments were conducted in chimeric *in vitro* systems (mammalian cell line, zebrafish-specific reporter gene cassette), and artefacts may have been introduced by pathway architecture interference.

In parallel, we conducted a similar approach for our transient zebrafish Nrf2 and AhR-activated reporter assays. Concerning the Nrf2-related pathway, the zebrafish assays showed higher activity and potency, leading to an increased calculated removal efficiency in regards to the tBHQ-BEQ values. It should be mentioned that the differences were apparent but not substantial. The zebrafish assay of the AhR-related pathway worked sufficiently with controls and spiked compounds. However, it showed higher cytotoxicity when exposed to environmental samples than the cells in the mammalian assay, arguably due to synergistic effects of the solvent and sample matrix. It can be speculated that the test system was already near the limit of systemic stress due to transgenesis and the additional solvent/matrix synergistic effects imploded the test system. Thus, bioanalytical assays should always be evaluated with environmental samples and various solvent carriers. Further, it can be speculated that AhR-related reporter bioassays in fish cell lines could be less sensitive by default, given the reduced sensitivity for fish

in comparison to birds or mammals (Eichbaum et al. 2014), in the AhR activation context. Our data and the few studies published up to date on the utility of species-specific bioanalytical tools are inconclusive. Thus, more investigations of all major TPs of interest are necessary to draw a final judgement. The current knowledge supports a continuous use of mammalian bioanalytical tools for the environmental monitoring of the aquatic habitat, such as recommended for the WFD-reevaluation (Wernersson et al. 2015), as known differences are apparent but not substantial.

5.3 Closing the *in vitro* to *in vivo* gap via integrated testing strategies

In vitro to *in vivo* extrapolation (IVIVE) has been termed "the philosopher's stone" in toxicology (Kisitu 2019). IVIVE describes the qualitative or quantitative transposition of effects recorded *in vitro* to predict toxic exposure levels *in vivo* (Blaauboer 2010; OECD 2018). As such, it belongs to a new test strategy to replace animal testing in toxicology, and further, be included in a battery of methods to estimate toxic exposure levels using non-animal tests. A quantitative IVIVE (QIVIVE) is a holistic approach on *in vivo* exposure level prediction, combining *in vitro* and *in silico* techniques (NAMs); including quantitative structure-activity-relationship modelling (QSAR), physiologically-based toxicokinetic/dynamic modelling (PBTK/TD), and additional input on the compound's absorption, distribution, metabolism, and excretion (ADME) (Yoon et al. 2012; Wilk-Zasadna et al. 2015). Among the latter models, some require reliable *in vitro* biotransformation, distribution, and absorption-defining test systems, to be developed and evaluated. Such test systems are scarce in ecotoxicology.

Until such sophisticated test systems are available, other strategies can be employed to progress the field. Tanneberger et al. and Natsch et al. undertook first promising steps by measuring the actual bioavailable concentrations in RTL-W1 (rainbow trout, *Oncorhynchus mykiss*) cytotoxicity assays and correlated their results to *in vivo* data (Tanneberger et al. 2013; Natsch et al. 2018). Brinkmann et al. utilised reversed-dosimetry PBTK modelling to relate internal concentrations in fish to nominal concentrations in primary fish cell lines when looking at the induction of vitellogenin and CYP450 biomarkers (Brinkmann et al. 2014). Further, they designed a multispecies

PBTK model for the cross-species evaluation of neutral organic compounds and their respective bioaccumulation potential (Brinkmann et al. 2016). A PBTK model was also used to correlate fish cytotoxicity data with acute fish data and even derive a bioaccumulation based IVIVE model (Stadnicka-Michalak et al. 2014; Stadnicka-Michalak and Schirmer 2019). However, the latter studies employed the ISO21115:2019 RTL-W1 cytotoxicity assay (ISO 2019; Fischer et al. 2019), which contains no serum in the culture and exposure medium. On the one hand, this has the advantage that the medium constitutes less as a sink to hydrophobic and lipophilic compounds (see also paper III for details). On the other hand, binding to culture plastics may be increased, and not all permanent cell cultures are vivid in serum-less nutrition medium.

In paper III, we instead opted to use classic, serum-containing medium and employ "serum-mediated passive dosing" (SPME) (Fischer et al. 2018a, b, 2019a). SPME secures the culture's vivacity and prevents a decline of the exposure concentration within the tested timeframes. In order to account for the decreased bioavailability when using the serum, we employed chemical distribution modelling (mass-balance modelling) to compute the "free concentrations" (bioavailable concentrations to the cells within the cytotoxicity assay) (Fischer et al. 2017; Bittner et al. 2019). Thus, in comparison to Brinkmann's and Stadnicka-Michalak's approaches, we utilised forward dosimetry of empirically derived mass-balance models. In paper III, we showed correlations close to unity when comparing cytotoxicity *in vitro* data to FET data of our dataset, but also partly AFT literature data. Hypothetically, a combination of both forward and reverse-dosimetry approaches, presented here, could be a powerful approach and pave the way for QIVIVE in ecotoxicology.

Alternatively, we proposed the use of our approach in paper III in integrated test strategies (ITS). Previously, several publications reported a high correlation between FET and AFT data up to the 96 hpf exposure time point (Lammer et al. 2009; Knöbel et al. 2012; Belanger et al. 2013). However, this is not universally true, given that some compound classes, such as neurotoxins, relate differently (Scholz et al. 2016). The AFT itself suffers from diverse limitations (low replication, no mechanistic information, broad range of test species, etc.) and would probably not be validated in its current

form (Braunbeck et al. 2020), regarding current standards. In this context, the FET is gaining momentum regarding its utilisation in the threshold approach (Rawlings et al. 2019) or ITS (Paparella 2020). We showed a good overall correlation in acute short-term toxicity between zebrafish cytotoxicity assays and the FET when considering actual bioavailable concentrations. Therefore, the FET can be exploited within the AOP concept to bridge the gap between MIE and low-tier KE, as recorded *in vitro*, to AO on the individual level, as recorded with the AFT (see also discussion and illustrations in paper III). Further, we are advocating the increased use of fish cytotoxicity assays in ITS, given the data at hand. Beyond, we postulate that the utilised zebrafish cell lines and designed transient reporter gene assays are feasible for a specific-toxicity centred IVIVE if bioavailable concentrations are considered in the study design.

5.4 Toxicity pathways: from linearity to networks

As mentioned in the introduction, Huang et al. identified 6,800+ TPs within the ToxCast data, an amount that cannot be realistically assessed for a plethora of chemicals (Huang et al. 2016). There have been attempts to unify and map the TP-landscape, such as the XTalkDB (http://www.xtalkdb.org/home) (Sam et al. 2017) and the BioPlanet (https://tripod.nih.gov/bioplanet/#) (Huang et al. 2019) databases. However, both approaches are lacking data, publicity, and a community in order to be effective tools. Hence, TP linearity is confined to hazard assessment and cannot define risk. Instead, TPs should be contemplated in a multidimensional fashion, as we have observed and discussed related to strong interdependencies and cross-talk (paper IV).

Such a TP-network approach has been formulated with the AOP concept that also regards for network plasticity (Ankley et al. 2010; Villeneuve et al. 2014a, b). In principle, AOPs are TPs that have been prolonged to the AO event on the individual and population scale, if they are considered as single AOPs. Single AOPs are unique, non-branching pathways (Villeneuve et al. 2014a). However, single AOPs are only an intellectual game used for AOP-design. In reality, toxic exposure operates in a systemic context, given that cross-talk and pathway interaction (paper IV) are the norm. A *de novo* conceptualisation of a holistic AOP network would, however, be a vain

endeavour. Given that multiple AOPs can be branched via shared MIEs and KEs, previously designed single AOPs can be used as modular building blocks to devise novel AOPs and entire AOP networks. Building the AOP network is an ongoing process by tying historic AOPs within the AOP-Wiki to new pieces until a complete network is available in the future. Therefore, AOP networks are conceptualised as a living document (Villeneuve et al. 2014b). One might say that single AOPs/TPs are the veins of the AOP body (network).

AOP networks are viewed as the most likely units of prediction. But how should we relate from an MIE to an AO which can be assessed in regulatory terms? Especially, given the unfathomable amount of data, once a holistic AOP network is established, akin to biological complexity itself. Knapen et al. and Villeneuve et al. reviewed *in silico* big data approaches, such as graph theory and network science that can be utilised to interpret a holistic AOP network (Knapen et al. 2018; Villeneuve et al. 2018). In principle, mathematical models and filters can be used to superimpose specific information of interest as layers on top of the AOP network entity. These layers can be viewed alike data layers employed in geographic information systems (GIS). A future investigator or regulator might then navigate the AOP topography via to-be-devised software and identify most-likelihood "paths" of exposure and toxicity. Noteworthy, these paths are not AOPs but a meta-abstraction of the latter. The paths are overlaid Bayesian network probability functions that collate all potential cross-talk (Perkins et al. 2019). Further, such *in silico* tools are supposed to transpose the recent qualitative nature of AOPs into quantitative units of assessment (qAOPs). A qAOP is defined as a biologically-based mathematical model that describes and predicts all relationship between nodes (KER: concentration/dose-response; time-dose; response-response relationships) (Conolly et al. 2017; Perkins et al. 2019). Given that the AOP is supposed to be a simplification of biological complexity, the qAOP is a parsimonious simulation of the latter.

However, these conceptual frameworks are in their infancy and mostly theory-based. In practice, the scientific community is decades away from their actual manifestation. For instance, the Aromatase-inhibition AOP (https://aopwiki.org/aops/25) took approximately 15 years to develop in a laborious joint-effort of multiple groups. Even if it were possible to narrow

down 6,800+ TPs to several hundred AOPs, the pace must increase drastically. In parallel to other scientific developments, once appropriate methods are established, and knowledge has been propagated, the turnover would increase exponentially. Nevertheless, incentives need to be created first, as AOP-development is still very laborious, time-consuming, and, so far, mostly meritless (from an academic perspective; personal communication, AOP-workshop, ECCVAM). Moreover, we should bear in mind that such new approach methods are replacing biological complexity with systemic complexity. On the one hand, there is the advantage that the scientific community possess the experience and knowledge to assess systemic complexity mathematically. On the other hand, there is, of course, the possibility that these mathematical models are immature or even wrong.

In the long term, the AOP concept stimulates necessary advancements in toxicology. Just as physics and chemistry, biology is a multidimensional discipline. The frameworks and models that we are building in our minds to conceptualise highly complex toxicological interrelations, such as the exposome with its myriad of influencing parameters, are mostly linear, rarely three-dimensional; given that we are unable to fathom multidimensionality. The biology of TPs' cross-talk and modulation might even be compared to quantum mechanics, in a slightly exaggerated manner. Scientific research can measure and define individual nodes within the AOP network but will not be able to encompass its totality, at least in the near future; just as a particle's duality is not recordable simultaneously. The unknown needs to be theorised and modelled in appropriate ways.

The described concepts are the first step in the right direction. Nevertheless, considerable research is still necessary to develop and populate these models. The Tox21 platform endorses the AOP concept, as they complement one another. Only qHTS platforms, such as Tox21, have the potential to populate AOP networks with enough data to test the robustness of the devised *in silico* models. The AOP concept and the Tox21 platform go hand in hand, and will progressively co-evolve in the future to pave the way for a new form of toxicity testing and environmental risk assessment. So far, these frameworks represent the cutting-edge state-of-the-science in toxicology and are the most appropriate tools conceptualised by the scientific community to tackle the

challenges of the Anthropocene epoch, such as the chemical environment and the exposome scenario.

6. Conclusion

This thesis outlines how to design and scrutinise species-specific transient reporter gene assays to be used in ecotoxicology. We emphasised the potential pitfalls of transient transgenesis, leading to non-specific effects, and devised strategies to deal with the latter. TP cross-talk has been identified as a factor of uncertainty in regards to the assessment of specific-toxicity. Additionally, we employed fish cytotoxicity assays in acute exposure scenarios and utilised forward dosimetry toxicokinetic modelling to derive bioavailable effect concentrations that correlated significantly with low-tier *in vivo* data. As discussed, the results advocate an intensified use of fish *in vitro* assays in integrated testing strategies. Alternatively, our approach can be unified with reverse dosimetry *in vivo* toxicokinetic modelling, as available in the literature, to aim for the first generation of QIVIVE models in ecotoxicology. Additionally, the approach can also be transferred to modelling bioavailable concentrations in assays of specific toxicity, instead of assays of non-specific-toxicity. Finally, we utilised our designed assays in the testing of environmental samples and discussed species-centred environmental screening in the context of the emerging literature. Additionally, this thesis discusses how emerging technologies (3D cell cultures and CRISPR/Cas) and scientific frameworks (Tox21 and AOP) will evolve the field in the future, in order to deal with the rising challenges of the Anthropocene epoch.

7. Outlook & future perspective

The discussion outlined how novel technologies, such as 3D cell culture and CRISPR/Cas, have the potential to enhance our opportunities to design robust *in vitro* methods, substantially without the current disadvantages and pitfalls. Taken together, this is a step towards developing suitable test systems that can deliver appropriate predictions of *in vivo* scenarios. Further, the conceptual platforms of AOP networks and qAOPs, in connection with qHTS, can build a future framework that is capable of the holistic assessment of multidimensional toxicology. In parallel to other disciplines, contextual *in silico* techniques can be complemented by AI and self-learning algorithms and thereby exponentially increase their potential. Clearly, existing frameworks can be very resource-demanding, in scientific, energetic, and economic perspectives. It will be interesting to see if the "digital age" will be able to live up to its promises. History has seen the rise and fall of several trends in science and society, such as the atomic age, or exaggerated hopes alongside the decryption of the human genome. Contextually, it may be thrilling to ask what Tox31 will look like. Will we be able to predict *in vivo* effects accurately from utilised NAMs or will we be back to testing lethal apical endpoints?

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Popular science summary

Chemical engineering has changed the world in an unprecedented fashion. Within our everyday life, we are surrounded by a myriad of human-made compounds. Some of these are designed to have specific effects on organisms, such as pesticides and pharmaceuticals. Others, such as industrial chemicals, may have undesirable effects that manifest afterwards. Nevertheless, all of them eventually might end up in the environment. Given that some compounds might have unwanted adverse health effects on humans and animals, their application and potential prohibition have to be controlled appropriately. This can be evaluated via "environmental risk assessment". Law enforces the outcomes of the environmental risk assessment.

However, given the sheer amount of compounds released into the environment, classic toxicity testing, as employed for environmental risk assessments, is challenged, given its lengthy and costly procedures. Classic toxicity testing utilises *in vivo* techniques (e.g., animal models) which also raises animal welfare and related ethical issues. Novel scientific frameworks, such as "toxicity testing in the 21st century" (Tox21), are instead promoting the use of alternative or so-called "new approach methods" (NAMs), comprising *in vitro* ("in a test tube") and *in silico* (computer models) techniques. These techniques are suggested to be cheaper, faster, and reduce the number of animals needed for research and testing purposes.

Various NAMs have been established in the field of human toxicology. Currently, most bioanalytical tools in use are based on cells of either mammalian or bacterial origin. It has been questioned whether such test systems are also representative for aquatic species, for example, fish, as they

represent the actual protection goal within ecotoxicology. In this context, we proposed the design and establishment of transient reporter gene assays of fish-cell origin. "Transient transgenesis" allows for a quick and easy alteration of genetic material, thus resulting in recombinant (genetically altered) cell lines. Thereby, the investigator is introducing specific genetic information (plasmid DNA) temporarily into the cells. Genetically alternated cells express a quantifiable signal upon exposure to toxic stress.

This thesis describes how to design, establish, and scrutinise transient reporter gene assays in fish cell lines, namely of the oxidative stress response pathway (Nrf2/Keap1/ARE) and the xenobiotic metabolism pathway (Ahr/ARNT/XRE). After their development, we tested the established assays using positive controls, a variety of compounds, and environmental samples. Further, we investigated the issue of artificial effects that can be introduced by the transient transgenesis procedure and suggested strategies on how to circumvent such effects. Finally, we conducted a study to evaluate if cell-based toxicity tests could be a suitable alternative to toxicity testing in fish embryos. We used *in silico* methods (chemical distribution models) to calculate the bioavailable concentrations in fish cell lines (*in vitro*) and fish embryos (low-tier *in vivo*) and then compared the toxicity of several compounds in these two test systems. When using the modelled bioavailable concentrations instead of the nominal concentrations, we could show that the toxicity observed in the two test systems was correlating strongly (almost to the point of absolute unity).

Taken together, the results showed and discussed in this thesis prove NAMs to be at least adequate alternatives to classic *in vivo* testing. Nevertheless, further method development is needed, as technologies are progressively evolving. Accordingly, we discuss in the thesis how the onset of 3D cell culture and sophisticated gene engineering tools (CRISPR/Cas) will empower the scientific community to design better test systems in the near future. Finally, the establishment of high-throughput screening facilities applying *in vitro* techniques and the conceptualisation of big data *in silico* models ("quantitative adverse outcome pathway networks") will pave the way for a new kind of toxicology; potentially capacitating the toxicological community to test the totality of all problematic chemical compounds surrounding us, in regards to their potential adverse health effects.

Populärvetenskaplig sammanfattning

Innovationer och produkter som baseras på produktion av syntetiskt framställda kemikalier har förändrat världen på ett sätt som saknar motstycke. Mängder av dessa kemiska föreningar omger oss i vår vardag. Några av dessa är utformade för att ha specifika effekter i organismer, såsom bekämpningsmedel och läkemedel. Andra kemikalier, till exempel industrikemikalier, kan ha oönskade effekter som upptäcks först efter att kemikalierna börjat användas. Gemensamt för alla dessa ämnen är att slutligen kan hamna i miljön. Eftersom vissa föreningar kan ha negativa hälsoeffekter på människor och djur, måste produktion och användning av sådana kemikalier regleras på ett lämpligt sätt. Arbetet med att uppskatta och värdera riskerna med dessa substanser kallas riskbedömning (”environmental risk assessment”), vilken sedan ligger till grund för myndigheternas regulatoriska beslut om hur en viss kemikalie från produceras och användas.

Det stora antalet syntetiska kemiska föreningar som förorenar miljön utgör en stor utmaning för de klassiska toxicitetstesten som används för bedömning av miljörisker, eftersom det kräver omfattande resurser och tar lång tid att toxicitetstesta varje kemikalier. Dessutom har sådana klassiska toxicitetstester ofta baserats på in vivo-tekniker, eller djurmodeller, som följaktligen leder till etiska frågeställningar. Under senare år har flera strategidokument publicerats, såsom ”Toxicity Testing in the 21st Century”-rapporten, som istället förespråkar en mer omfattande användning av så kallade NAMs (”new approach methods”), bland annat innefattande in vitro-tekniker (”i ett provrör”) och in silico-tekniker (datormodeller). Syftet med dessa tekniker är att göra det möjligt att toxicitetstesta ett större antal kemiska föreningar, genom att erbjuda testsystem som är mindre kostsamma och

dessutom snabbare. Dessutom har dessa NAM en stor potential att minska antalet djur som används för forskningsändamål. Cellbaserade in vitro-modeller som kan användas för toxicitetstestning utgör en viktig grupp av sådana NAMs. Dessa modeller benämns ofta som bioanalytiska verktyg.

Olika NAMs har etablerats för att undersöka kemikaliers toxiska effekter på människa. För närvarande är de flesta bioanalytiska verktyg som används baserade på antingen däggdjursceller eller bakterier. Det har ifrågasatts om sådana testsystem är representativa även för vattenlevande arter, till exempel fiskar. Det är viktigt att använda testsystem som är representativa för vattenlevande organismer, eftersom dessa utgör en viktig del av det som skyddas då syntetiska kemikaliers toxicitet utvärderas och riskbedöms. För att bidra till utvecklandet av testsystem som är representativa för vattenlevande organismer har vi i denna avhandling använt odlade fiskceller för att utveckla testmetoder baserade på en teknik som kallas för reporter gene assay och transfektion. Transfektion är en teknik som möjliggör introduktion av nytt genetiskt material i de odlade cellerna, vilket resulterar i rekombinanta (genetiskt förändrade) cellinjer. Med hjälp av transfektionen kan vi introducera ett DNA-material som gör att cellerna producerar ett lätt detekterbart protein då de utsätts för en viss typ av toxiska ämnen. Genom att mäta mängden av detta lätt detekterbara protein som cellerna producerar kan vi få ett mått på hur toxisk den kemikalie vi studerar är.

Denna avhandling beskriver utvecklandet och utvärderandet av testmetoder för reporter gene assay i fiskcellinjer, för att studera två viktiga typer av toxicitet; oxidativ stress respektive aktivering av aryl hydrokarbonreceptor (AhR). Oxidativ stress har studerats genom etablerandet av en cellinje som kan upptäcka aktivering av signalleringsvägen Nrf2/Keap1/ARE och aktiveringen av AhR har studerats genom etablerandet av en cellinje som kan upptäcka då AhR aktiveras av in ligand (signalleringsvägen Ahr/ARNT/XRE). Efter utvecklandet testade vi de etablerade metoderna med positiva kontroller, olika kemiska föreningar samt prover tagna i miljön. Vidare undersökte vi problemet med artificiella effekter som kan uppstå genom transfektsprocessen och föreslog strategier för hur sådana effekter kan undvikas. Slutligen genomförde vi en studie för att utvärdera om cellbaserade toxicitetstester kan vara ett lämpligt alternativ till toxicitetstester i fiskembryon. Vi använde in silico-metoder för att beräkna

de biotillgängliga halterna av kemikalierna i fiskcellinjer (in vitro) och fiskembryon (låg nivå in vivo) och jämförde sedan toxiciteten hos ett antal föreningar i dessa två testsystem. När vi använde de beräknade biotillgängliga koncentrationerna istället för de nominella koncentrationerna kunde vi visa att den toxicitet som observerades i de två testsystemen korrelerade starkt.

Sammantaget visar resultaten som presenteras och diskuteras i denna avhandling att NAMs har stor potential att komplettera, och i vissa tillämpningar även ersätta, klassiska in vivo-tester. Det krävs dock ytterligare utveckling av metoderna, eftersom tekniken successivt utvecklas. I avhandlingen diskuteras även hur etablering av 3D-cellkulturer och sofistikerade gentekniska verktyg (CRISPR/Cas) kommer att ge vetenskapen möjlighet att utforma bättre testsystem inom en snar framtid. Slutligen diskuteras hur anläggningar för högeffektiv screening, som använder in vitro-tekniker, samt användning av av in silico-modeller för stora mängder data (“quantitative adverse outcome pathway networks”) skulle kunna bana väg för en ny typ av toxikologi där alla problematiska kemiska föreningar som omger oss kan testas i sin helhet med avseende på deras potentiella negativa hälsoeffekter.

Acknowledgements

This thesis was funded by the Swedish research council (grant no. 2015-03404) and financially supported by the VH faculty and the BVF department. The thesis was conducted at the department for biomedicine and veterinary public health (BVF), SLU Uppsala.

Additional funding bodies that need to be mentioned: Lennart Hjelm fund, Knut and Allice Wallenberg foundation, GS-VMAS mobility support, and SETAC mobility support for their generous contribution to my postgraduate studies and travels. Also embracing praise to the DAAD-Rise program for supplying me with competent undergraduate student.

First of all, I want to display my gratitude to my supervisors **Agneta Oskarsson** and **Johan Lundqvist** for my recruitment, their guidance, instructions, help, and collaboration throughout my PhD studies. Especially, I am thankful to Johan for always being open minded to new ideas and impulses and always being supportive in financial terms and during internal review processes.

Further, I want to show appreciation to my co-supervisor **Stefan Örn** for his humoristic ways, yoga class instructions, and initiating a snus addiction.

Many praises go out to my co-authors **Johan Lundqvist, Agneta Oskarsson, Carolina Vogs, Yuxin Han, Gunnar Carlsson, Maximiliane Montag, Kim Frieberg, Geeta Mandava, Foon Yin Lai**, and **Lutz Ahrens** for vice-versa contributions.

Henner Hollert, for accepting the invitation to act as an opponent during my thesis defence.
Adam Lillicrap, Björn Brunström, and **Maria Jönsson**, for being part of the examining committee.

Maria Yu and **Matilda Stein Åslund**, for proofreading the thesis manuscript.

In addition, I feel much obliged to all my colleagues and co-workers at the toxicology group, the BVF department, and the VH faculty. Especially, the ones I will forget to name in the following.

Anders Glynn, for bringing new impulses and fruitful discussions.

Geeta Mandava, for always keeping me well nourished.

My former colleague **Anna Kjerstine Rosenmai**, mostly for being a crazy Dane.

My former colleague **Jacob Mensah-Attipoe**, for his friendly ways during our unfortunately suddenly interrupted colleagueship.

All my undergraduate students, whom I had the pleasure to supervise, for their conducted work, but also for giving me the opportunity to mature as a scientist and person: **Yuxin Han, Maximiliane Montag, Alexander Spiegelhalter, Friederike Nintzel**, and **Kim Frieberg**.

My fellow PhD students of the toxicology group and the BVF department, who recently started to multiply exponentially: **Johannes Pohl, "Mr. Li", Erica Selin, Maria Yu, Kim Frieberg, Pontus Öhlund, Stina Hellman, Jennifer Nyström**, and **Sebastian Pineda**.

My dear friend and lunch partner **Karin Alvåsen**.

Other colleagues of the BVF department: **Elisabet Ekman, Carl Ekstrand, Peder Eriksson, Bernt Hjertner, Ivar Vågsholm, Magnus Åbrink**, and **Lisa Lindström**.

The VHC technical staff, for always offering a helping hand and being the only people actually "working": **Lena Olsson**, **Ann-Sofie Waxin**, and **Lee Farmer**.

The organic chemistry group, for our unexpected collaboration (and for me repairing your pipes after flooding the lab): **Lutz Ahrens, Foon Yin Lai, Oksana Golovko, Vera Franke, Frank Menger, Malin Ullberg**, and **Karin Wiberg**.

My international friends, co-workers, and supervisors in ecotoxicology, who have always been a great source of inspiration: **David Saunders, Patrick Heinrich, Katharina Heye, Florian Zindler, Christopher Fassbender, Thomas Braunbeck, Markus Hecker**, and **Nicholas Foulkes**

The "**Tecan M1000 infinite**" plate reader, for the mutually shared time and only starting to fall apart after I have finished my experiments.

Beyond, I want to appreciate all my childhood, youth, and study friends, who occasionally visited, or at least supported me mentally from a long distance. Especially, members of the infamous **"Keller-crew" and hangarounds**.

Irma, for being a lousy, lazy, but cute dog.

My relatives, as they are spread all over the world in Romania, Germany, Spain, and Canada.

Annika, for her love and understanding.

Matilda, for her love, companionship, multiple proofreading, "bra tipsen", and instructions on "how to fall up the wall".

My loving parents **Elena** and **Victor**, whom I have to thank for basically everything.

Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2020:69

Modern ecotoxicology requires new approach methods to ensure proper environmental risk assessment in an economically and ethically feasible manner. This thesis reports on the development of *in vitro* cytotoxicity and reporter gene assays (oxidative stress response and xenobiotic metabolism toxicity pathways) in zebrafish cell lines. Chemical distribution models complemented these systems, resulting in high positive correlations to low-tier *in vivo* assays (zebrafish embryos). Conclusively, the established assays show great potential in future toxicity testing.

Sebastian Lungu-Mitea received his doctoral education at the Department of Biomedical Sciences and Public Health, Swedish University of Agricultural Sciences. He obtained his B.Sc. and M.Sc. degrees from the University of Heidelberg, Germany.

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Online publication of thesis summary: http://pub.epsilon.slu.se/

ISSN 1652-6880
ISBN (print version) 978-91-7760-656-7
ISBN (electronic version) 978-91-7760- 657-4