

COMPUTATIONAL METHODS FOR ASSESSING CHEMICAL RISK

*Focusing on Toxicokinetic Modelling
in Zebrafish (Danio rerio)*

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Abstract

New chemicals are constantly produced and large data gaps exist on hazards of currently used industrial chemicals, stressing the need for rapid, ethically sound and cost-efficient hazard assessment methods. Traditional methods for effect assessment based on animal testing, do not meet these requirements and thus the toxicology field has been moving towards the development of new approach methodologies which include *in vitro* approaches but also computational methods. The current work has mainly focused on computational tools but also employed *in vitro* and *in vivo* methodologies for the development and validation of the *in silico* approaches.

We firstly explored chemical variation of emerging chemicals as a basis for selecting subgroups of per- and polyfluoroalkyl substances (PFASs) and bisphenols for Papers I and II. These compounds can be used for future testing and as case study compounds for *in silico* tools development. The PFASs selection showed compounds with large differences in structure and highlighted the lack of knowledge for large parts of the PFASs chemical domain. This likely is the main driver of the low predictive accuracy of some current fate models and the need for expanding their applicability domains.

In Paper II we investigated the toxicokinetics of selected bisphenols in a commonly studied model organism, the zebrafish (*Danio rerio*), and developed a physiologically-based toxicokinetic model. Novel data for fish biotransformation was derived and showed lower rates than those measured in humans, providing valuable insight for both model parameterization and for chemical safety assessment using fish. The model also demonstrated the ability to predict and rank hazard of these bisphenols in terms of organ-specific bioaccumulation making it a useful tool for chemical screening and prioritization efforts. The results indicate that bisphenols AP, C and Z as well as tetrabromo bisphenol A may have larger potential for bioaccumulation than the widely used bisphenol A (BPA), indicating that these compounds do not constitute safer industrial substitutions.

Lastly, we present in Paper III the development of a toxicokinetic model for the zebrafish embryo life-stage. Since the zebrafish embryo test is widely applied in toxicology research, the developed model provides a tool to better understand how varying testing conditions may affect dose at target thus providing a means to compare internal effect concentrations. Additionally, we applied the model in combination with data on estrogenic activity in order to rank the relative hazard of investigated bisphenols, which showed that bisphenols AF, C, B and Z may be more hazardous than BPA.

Overall the developed computational tools showed good predictive performance and improvements in parameterization, thus providing tools for understanding dose at target and toxicokinetic variation of emerging substances. Furthermore, the thesis presents novel data and findings for per- and polyfluoroalkyl substances and bisphenols, which are environmental pollutants of emerging concern of relevance for future hazard assessments and substitution processes.

Enkel Sammanfattning på Svenska

Industrikemikalier som hamnar i miljön är i fokus för modern toxikologi och miljöforskning. Framförallt egenskaper som bioackumulering och hormonstörande egenskaper är viktiga områden. Nya kemikalier utvecklas i snabb takt och det finns ett stort behov av att bedöma deras faror med snabba, etiskt sunda och kostnadseffektiva metoder. Traditionella metoder för effektbedömning baserade på djurförsök uppfyller inte dessa krav och därför har toxikologin gått mot utvecklingen av nya metoder som inkluderar cellbaserade metoder men också beräkningsmetoder. Arbetet i denna avhandling har huvudsakligen fokuserat på utveckling av beräkningsmodeller men även använt cellförsök och djurförsök för att förbättra och validera beräkningsmetoder.

Vi använde först beräkningsmetoder för att utforska kemisk variation av nya kemikalier och som grund för att göra strategiska urval av per- och polyfluorerade alkylsubstanter (PFAS) och bisfenoler för Papper I och II. Dessa föreningar kan användas som framtida fallstudieföreningar för utveckling av nya beräkningsmodeller. Urvalet av PFAS omfattade föreningar med stora skillnader i kemisk struktur och visade att det finns stora brister i kunskaper om PFAS. Dessa dataluckor är till stor del orsaken till varför vissa beräkningsmodeller presterar dåligt för denna substansklass. Det är därför viktigt att förbättra modellerna baserat på ny data från ett strategiskt val av substanser.

I Papper II undersökte vi toxikokinetik för utvalda bisfenoler i zebrafisk (*Danio rerio*). Vi utvecklade en fysiologiskt baserad toxikokinetisk modell som beskriver hur kemikalerna fördelar sig i zebrafisk. Vi bestämde metabol nedbrytning av valda ämnen i ett fiskbaserat system och data visade lägre nedbrytningshastighet än de som tidigare uppmätts hos människa. Dessa fynd är viktiga både för utveckling av modellen och för bedömning av kemikaliesäkerhet baserat på fisktester. Modellen visade också förmåga att förutsäga och rangordna faror för studerade bisfenoler genom att beräkna hur mycket som ackumulerar i olika organ. Resultaten indikerar att bisfenolerna AP, C och Z samt tetrabromobisfenol A har större potential för bioackumulering än bisfenol A (BPA), vilket indikerar att de ej är säkrare alternativ.

Slutligen presenterar vi i Papper III utveckling av en toxikokinetisk modell för zebrafiskembryo. Eftersom embryotestet för zebrafisk används i stor utsträckning inom toxikologisk forskning kan den utvecklade modellen användas för att bättre förstå hur olika testförhållanden påverkar dos av kemikalier i embryo. Den kan därför utgöra ett viktigt verktyg för att jämföra koncentrationer i zebrafiskembryo som kan leda till negativa effekter. Vi använde också modellen tillsammans med mätningar av östrogen aktivitet för att rangordna den relativa risken för undersökta bisfenoler, vilket visade att bisfenolerna AF, C, B och Z kan vara mer farliga än BPA.

Sammantaget visade de utvecklade beräkningsverktygen god prediktiv förmåga, vilket ger oss nya möjligheter att förstå dos vid målorgan och toxikokinetik för substanser som kan finnas i miljön. Dessutom presenterar avhandlingen nya fynd för miljöföröreningsgrupperna PFAS och bisfenoler av relevans för framtida riskbedömningar och substitutionsprocesser.

List of Publications

The thesis is based on the following papers and referred to by their roman numeral presented below.

- I. Chelcea Ioana C., Ahrens Lutz, Örn Stefan, Mucs Daniel, Andersson Patrik L. Investigating the OECD database of per- and polyfluoroalkyl substances – chemical variation and applicability of current fate models. *Environmental Chemistry* 2022 17, 498-508.
- II. Ioana Chelcea, Stefan Örn, Timo Hamers, Jacco Koekkoek, Jessica Legradi, Carolina Vogs, and Patrik L. Andersson. Physiologically Based Toxicokinetic Modeling of Bisphenols in Zebrafish (*Danio rerio*) Accounting for Variations in Metabolic Rates, Brain Distribution, and Liver Accumulation *Environmental Science & Technology* 2022 56 (14), 10216-10228
- III. Ioana Chelcea, Carolina Vogs, Timo Hamers, Jacco Koekkoek, Jessica Legradi, Maria Sapounidou, Stefan Örn, Patrik L. Andersson. Physiology-informed toxicokinetic model for the zebrafish embryo test – a case study of bisphenols. Submitted for publication January 2023

Author Contributions

- I. Author had a major role in planning and writing of the paper. Additionally, author performed all the computational work and analysis.
- II. Author had a major role in planning the experimental work and writing the paper. Author had the main role in performing the *in vivo* exposure and sampling of organs as well as analysing the data. All modelling and literature data extraction was performed by author.
- III. Author had a major role in planning and performing the embryo exposure and sampling of the embryos as well as analysing embryo data. Author also planned and performed the computational modelling and data extraction

Abbreviations

AD	Applicability domain
ADI	Acceptable daily intake
ADME	Absorption, distribution, metabolism and elimination
AOP	Adverse outcome pathway
AR	Androgen receptor
AUC	Area under the curve
BAF	Bioaccumulation factor
BCF	Bioconcentration factor
BP-2	Benzophenone 2
BPA	Bisphenol A
BPA-GA	Bisphenol A glucuronic acid
BPAF	Bisphenol AF
BPAF-GA	Bisphenol AF glucuronic acid
BPAP	Bisphenol AP
BPB	Bisphenol B
BPC	Bisphenol C
BPF	Bisphenol F
BPS	Bisphenol S
BPZ	Bisphenol Z
CI	Credible interval
Cl	Clearance
C _{max}	Maximal concentration
EC ₅₀	50% effect concentration
EDCs	Endocrine disrupting compounds
ER	Estrogen receptor
GA	Glucuronic acid
GIT	Gastro-intestinal tract
HC	Hierarchical clustering
hpf	Hours post fertilization
HPG	Hypothalamic-pituitary-gonadal
HPT	Hypothalamic-pituitary-thyroid
IATA	Integrated approach to testing and assessment
IVIVE	<i>In vitro</i> to <i>in vivo</i> extrapolation
KE	Key event
KER	Key event relationship
K _{oc}	Organic carbon to water normalized sorption coefficient
K _{ow}	Octanol-water partition coefficient
K _p	Permeability rate
LC-MS	Liquid chromatography coupled to mass spectrometry
LFER	Linear free energy relationship
LOEC	Lowest observed effect concentration
Log <i>D</i>	Log of octanol-water partitioning adjusted for pH
MCMC	Markov-Chain Monte-Carlo
MIE	Molecular initiating event
ML	Machine learning
MW	Molecular weight

NAMs	New approach methodologies
NOAEL	No observed adverse effect level
NRMSE	Normalized root mean squared error
ODEs	Ordinary differential equations
OECD	Organisation for Economic Co-operation and Development
PBK	Physiologically-based kinetic
PBPK	Physiologically-based pharmacokinetic
PBTK	Physiologically-based toxicokinetic
P_{bw}	Blood-water partition coefficient
PC	Principal component
PCA	Principal component analysis
PCBs	Polychlorinated biphenyls
PCPs	Personal care products
PEC	Predicted effect concentration
PFASs	Per- and polyfluoroalkyl substances
PFHxS	Perfluorohexane sulfonic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonic acid
pK_a	Acid dissociation constant
PNEC	Predicted no observed effect concentration
POPs	Persistent organic pollutants
PPT	Poorly perfused tissue
qAOP	Quantitative adverse outcome pathway
QIVIVE	Quantitative <i>in vitro</i> to <i>in vivo</i> extrapolation
QSAR	Quantitative structure-activity relationship
QSPR	Quantitative structure-property relationship
RA	Risk assessment
RMSE	Root mean squared error
RPT	Richly-perfused tissue
RT	Rainbow trout
SULT	Sulfonyltransferase
S_w	Water solubility
T	Temperature
$t_{1/2}$	Half-life
TBBPA	Tetrabromo bisphenol A
TK	Toxicokinetic
TTR	Transthyretin
UGT	UDP-glucuronosyltransferase
V	Volume
V_p	Vapor pressure
VRC	Variance ratio criterion
VTG	Vitellogenin
<i>vtg1</i>	Vitellogenin 1 gene
WoE	Weight of evidence
ZFE	Zebrafish embryo

1 Introduction

1.1 Environmental Pollutants

Anthropogenic chemical pollution has been a long-time issue for environmental and human health, with the general public becoming more aware of it after the publication of Rachel Carson's *Silent Spring* in 1962¹. Scientific research on the toxicity of pollutants has led to the ban of many hazardous substances and to the founding of numerous environmental protection agencies and international agreements aimed at safeguarding the environment including wildlife and humans from the harmful effects of chemical pollution²⁻⁵. There are many chemical properties that may be concerning from a risk assessment perspective. These properties include persistence, bioaccumulation^{2,6}, toxicity such as endocrine disruption⁷ and even mobility⁸. Although there are numerous classes of chemicals with these concerning properties, recent research on persistent organic pollutants (POPs) has been heavily focused on poly- and perfluoroalkyl substances (PFASs) while in the case of endocrine disruption, bisphenol A (BPA) and its structural analogs have become a focal point.

POPs have been recognized as an environmental threat for many years with the Stockholm Convention established as an international treaty aimed at protecting human health and the environment from their effects². Persistent compounds are transformed or degraded slowly or not at all via biotic processes, such as enzymatic biotransformation or abiotic processes such as photolysis or hydrolysis⁹⁻¹¹. The goals of the Stockholm Convention are to restrict or eliminate certain POPs based on their persistence, but also on their effects on both on human health and the environment. The list of POPs proposed by the Stockholm Convention covers polychlorinated biphenyls (PCBs), Aldrin, Chlordane, DDT, or Heptachlor to name a few. Recent additions to the list include perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA) and perfluorohexane sulfonic acid (PFHxS), which belong to the group of PFASs².

Endocrine disrupting chemicals (EDCs) in the environment have been identified as a matter of toxicological concern for human health as well as other organisms⁷ and a guidance to evaluate these compounds has only recently been revised by the Organization for Economic Co-operation and Development (OECD)¹². In broad terms, endocrine disruptors are compounds that can alter and disrupt normal functions of the endocrine system and cause adverse outcomes as a consequence of specific endocrine mechanism of action as opposed to general toxicity. Endocrine mode of action can refer to activation or inhibition of receptors such as the estrogen or androgen receptor (ER or AR), binding to hormone distributors such as transthyretin (TTR) or any other disruption of the hypothalamic-pituitary-gonadal (HPG) and -thyroidal (HPT) axes⁷. Estrogen signaling is part of the HPG axis which is highly conserved in vertebrates and its disruption has been associated with various adverse effects on reproduction and embryonal development in many species¹³⁻¹⁶. Endocrine effects have been observed at very low levels with e.g., the developing embryos showing high sensitivity to long-term effects, which become apparent later in life. It is therefore important to assess the risk of environmental pollutants that can disturb the endocrine system and thus may exert adverse effects. Bisphenol A (BPA), has been the subject of much toxicological and environmental research, in part due to its ability to bind to the ER^{17,18} and disrupt estrogen dependent pathways.

Due to the potential hazards and abundance in the environment of both PFASs and bisphenols, the current work has a particular focus on these chemicals and their environmental risk.

1.1.1 Per- and Polyfluoroalkyl Substances (PFASs)

PFASs are a broad group of chemicals with numerous different properties and applications with over 4000 of them being registered in various chemical databases^{19,20}. These compounds are being used for various industrial applications and can be found in consumer products such as non-stick coating in pans, water- or stain resistant fabrics, or fire-fighting foams^{21,22}. The wide-spread application of certain PFASs can be attributed to their chemical stability making them resistant to most degradation processes. This in turn, however, make some PFASs highly persistent in the environment. Although a number of PFASs can be degraded via biotic and abiotic processes, some of the resulting transformation products have shown higher persistence than their parent compounds^{23–25}. PFASs have been detected in various environmental matrixes such as soil, sediment, ground and surface waters, biota and human food^{26,27}. Especially shorter chain PFASs have also been shown to be very mobile in some cases, making these compounds ubiquitous in the environment²⁸. Additionally, previously studied PFASs have shown toxic properties in humans as well as various organisms. Observed adverse effects include immunotoxicity, hepatotoxicity, nephrotoxicity, metabolic and thyroid system disruption as well as developmental and reproductive toxicity^{29–31}. Although toxicity data at environmentally relevant concentrations or in wildlife is limited, adverse effects have been observed in laboratory animals and epidemiological studies^{29,31,32}.

Due to their persistence, bioaccumulation, mobility and possible adverse effects in humans and wildlife, some PFASs display concerning properties both in terms of hazard and exposure^{20,29–31,33–35}. Therefore, restricting PFASs as a group of chemicals has been recently proposed to ECHA by several member states³⁶. There are however considerable knowledge gaps regarding the majority of PFASs with only a few PFAS being investigated in more detail, such as PFOS, PFOA and PFHxS. Ankley et al. 2021²⁹ lists current needs for advancing risk assessment of PFASs which included improvements in exposure and hazard assessment through better environmental monitoring, measuring bioaccumulation and toxicity testing of a wider group of PFASs.

1.1.2 Bisphenols

BPA is used in many industries for the production of polycarbonate plastics, epoxy resins, in thermal paper inks and food packaging^{37–39}. Due to its endocrine disrupting properties, this compound has been replaced in many of these applications with other bisphenols such as bisphenol S (BPS) and F (BPF)^{37,40,41}. Additionally, other structurally similar compounds such as bisphenol AF, AP, Z, C or B (BPAF, BPAP, BPZ, BPC, BPB) are used in various industrial applications, which can lead to human exposure through leakage from packaging, insulation, polymer coating and personal care products (PCPs)^{37,40–42}. The endocrine properties of some of these structural analogs are still not thoroughly understood but many of them show similar capabilities to activate ER like BPA⁴³. Several bisphenols have been detected in environmental matrixes such as soil and water, as well as in various organisms including humans and fish^{44–51}. When comparing environmental occurrence of various bisphenols, BPA has been detected most frequently followed by BPS, BPF and BPAF^{51,52}. Additionally, BPAF, BPZ and BPAP showed higher bioaccumulation in fish than BPA indicating potential for higher internal exposure⁵³. Several bisphenols have shown adverse effects in adult and embryo vertebrates including fish, rats and humans^{45,54–58}.

Although BPA has been widely studied in literature, knowledge on the potential of other bisphenols to bioaccumulate and their toxicity in various organisms is still lacking. Thus, further research is needed to understand the potential risk of these compounds.

1.2 Risk Assessment Approaches

Assessing the risk of environmental pollutants requires knowledge on both their hazardous properties and their exposure potential to organisms. The risk assessment (RA) process usually starts with hazard identification, where potentially hazardous properties are investigated. Such an inherent property however, only poses a risk if exposure at a certain level also occurs. Thus, the next steps in a RA are exposure assessment followed by effect assessment that are evaluated in the risk characterization step^{59,60}.

In order to assess exposure, information is needed about compound-specific fate properties so as to determine the distribution of the chemical in the environment, which can be further employed to determine exposure pathways. This analysis should consider mobility via transport through air or water or transformation through biotic and abiotic processes⁵⁹. Such processes influence exposure by affecting how quickly a compound distributes in environmental matrixes and how long it remains in them. Environmental monitoring studies have been traditionally performed to investigate the concentrations of various pollutants in environmental matrixes such as soil or water, which give an indication of potential exposure to studied chemical^{61,62}. However, identifying compounds in the environment and their fate properties is only an indirect measure of exposure as it does not consider uptake and elimination processes in various organisms. Absorption, distribution, metabolism and elimination (ADME) properties of compounds are all crucial for understanding the internal exposure and are therefore important considerations in the RA process. Assessing internal exposure can be done by analyzing the concentration in organisms through biomonitoring⁶³. Such measurements have been done for humans in blood, serum or urine samples or for other species such as for example fish^{64,65}. These studies require expensive analytical methods, which are generally targeted thus only detecting compounds that were looked for^{66–68}. Although non-target screening methods have shown great advancements in recent years, they are still technically difficult and costly approaches which show large uncertainties⁶⁹.

Effect assessment aims to investigate whether a compound causes adverse effects in organisms⁵⁹. Although more data on effects are available for high production volume compounds as required by various legislations⁵, very little is known about many low volume chemicals which can still end up in the environment and lead to effects in exposed organisms. Traditional effect assessment has been based mainly on *in vivo* experiments in order to identify possible adverse effects. Such experiments are then used to derive a quantitative measure of hazard in the form of a no observed adverse effect level (NOAEL) or a predicted no observed effect concentration (PNEC) using assessment factors^{60,61}. Various model organisms have been established in laboratories for this purpose based on practical aspects but also on biological relevance such as similarity in physiology, genetics or pathology with humans. One of these models is the zebrafish (*Danio rerio*), which is a widely used model for investigating developmental, reproductive and neurotoxicity in both adult and embryonic life stages^{14,70–72}. The zebrafish embryo (ZFE) in particular has been studied extensively since the ZFE test represents a somewhat higher throughput and easier method to screen potential toxicants than the adult stages. This life-stage is considered a non-animal method in European legislation

until the start of free feeding at around five days post fertilization. Zebrafish share many genetic homologies with humans and various vertebrates^{73,74} and belong to one of the largest families of vertebrates⁷⁵, the cyprinids, allowing for extrapolation of adverse outcomes to other fish species as well. This species can thus help elucidate bioaccumulation potential and also endocrine effects of EDCs, thus constituting a versatile model for studying effects and risks of environmental pollutants^{14,70}.

Once both effect and exposure are known, risk characterization can be performed to establish safety levels in the form of a quantitative acceptable daily intake (ADI) for human safety and the predicted effect concentration (PEC)/PNEC for environmental protection. This information can be used to inform risk management decisions such as restriction or replacement of unsafe chemicals or environmental remediation efforts⁶¹.

The Green Deal initiative by the European Commission aims at achieving a toxic-free environment and proposes strategies towards this goal⁷⁶. Considering the increasing number of chemicals on the market, it is however not possible to test all compounds and perform individual risk assessment for them with traditional toxicology methods due to financial, ethical and time considerations. Thus, there is a demand for cheaper, high-throughput methods that can aid in the effect and exposure assessment of environmental pollutants. Current efforts are being made to incorporate other data from new approach methodologies (NAMs) such as *in vitro* and *in silico* data in a weight of evidence (WoE) approach for risk assessment^{42,77,78}.

1.2.1 New Approach Methodologies (NAMs)

NAMs aim to move away from *in vivo* studies for risk assessment and towards more ethical, faster and less costly methods^{78–80}. NAMs can include *in vitro* methods to assess toxicity based on cell assays such as the hERG assay for assessing cardiotoxicity⁷⁹ or by measuring receptor activation and inhibition in a variety of cell-lines⁸¹. It can also include *in vitro* methods to assess toxicokinetics including metabolism using primary hepatocytes⁸², cell lines such as HepaRGTM or cell fractions such as S9⁸³ or absorption through intestine using Caco-2 cells⁸⁴ or through skin using excised skin⁸⁵. NAMs also include *in silico* approaches which are becoming of increasing interest for toxicology in regulatory, environmental and pharmacological risk evaluations⁷⁸. Both *in vitro* and *in silico* data can be used within an integrated approach to testing and assessment (IATA) in order to evaluate risk. IATA can additionally include *in vivo* data or omics data and aims to integrate a variety of information sources for risk evaluation^{80,86,87}.

A tool for hazard and effect assessment that has been proposed for use within the IATA framework, is the adverse outcome pathway (AOP) concept^{79,87–89}. AOPs are chemical-unspecific pathways that link a molecular initiating event (MIE) to an adverse outcome via various key events (KE) and key event relationships (KER). These KEs represent quantifiable biological responses at different levels of biological organization that sequentially lead to an adverse effect^{90,91}. The AOP framework then provides a way to structure the various data sources from IATA in order to evaluate whether a compound can lead to an adverse effect based on the measured or predicted MIE or KEs. AOPs also provide a way to identify knowledge gaps or the need for new NAMs to be developed in order to understand how specific MIEs can lead to an adverse outcome^{88,89}. Data on the capacity of a compound to trigger MIEs or KEs can again be sourced from *in vivo*, *in vitro* or *in silico* experiments.

In silico tools are widely used NAMs, which can provide a fast and inexpensive way to screen chemicals and identify potential risk for organisms including humans. These types of approaches can be used to prioritize chemicals for further testing, predict various hazardous properties or biological activity, improve understanding of doses at target organ, aid in extrapolating from *in vitro* to *in vivo*, and integrate many data sources in order to provide a better picture of the potential risks associated with various compounds. *In silico* methods include tools such as multivariate regression methods, machine learning and toxicokinetic models which can be applied for grouping chemicals, read-across, and predicting effects or kinetics of untested chemicals. Multivariate analysis such as principal component analysis (PCA) and non-supervised machine-learning (ML) methods such as hierarchical clustering can be used for exploration of chemical space⁹² and selection of representative subsets of chemicals⁹³. Read-across methods have been used to group chemicals as well as to extrapolate properties and potential risk from one chemical to another based on structural similarity^{86,94}. ML has additionally been employed in supervised methods known as quantitative structure-property/activity relationship models (QSPR/QSAR)^{95–97} in order to predict chemical properties or receptor activities based solely on chemical structure^{80,94,98}. Such predictions can then be used for hazard identification, to identify compounds which trigger specific MIEs or KEs in order to be integrated in IATA, or they can be used as parameters in toxicokinetic models^{78,94}.

A downside with QSARs when it comes to predicting adverse effects is that they do not account for the dose at target organ in an organism i.e. the internal exposure. However, *in silico* tools describing biokinetics and toxicokinetics can be used to understand internal exposure within an organism or in cells^{78,86}. Toxicokinetic knowledge is crucial since it provides information about the dose at biological targets whether it is within cells tested *in vitro* or inside an organ when performing *in vivo* studies. To address this, models can be developed to account for free compound in the case of *in vitro* studies or to account for ADME in a biological system as well as in a population. Addressing *in vitro* biokinetics can be done using simple instant-equilibrium models or toxicokinetic models with only a few compartments^{99–101}. ADME can be modelled by developing physiologically-based toxicokinetic (PBTK) models for various species or parts of organisms^{102–104}. PBTK models can be used for quantitative *in vitro* to *in vivo* extrapolation (QIVIVE), extrapolation between individuals, between species as well as between environmental and internal concentrations or vice versa by forward or reverse dosimetry^{78,105,106}. Additionally, toxicokinetic and toxicodynamic modelling can be incorporated together with AOPs to create quantitative AOPs (qAOPs)¹⁰⁷.

1.3 Aims

In this work we aimed at developing NAMs in order to increase our understanding of chemical prioritization and hazard identification in terms of toxicokinetic properties of environmental pollutants with focus on PFASs and bisphenols. Computational methods were the focal point of the studies although novel experimental *in vivo* and *in vitro* data were also derived for model development and validation.

Firstly, we aimed to explore the chemical space and evaluate the variation within PFASs and bisphenols in terms of chemical structure and physico-chemical properties (Paper I and II). Additionally, we aimed at selecting small but structurally representative and environmentally relevant sub-groups of chemicals from each chemical space for future effect testing and NAMs development. This work is presented in Section 2 of the thesis.

Secondly, we aimed at investigating the toxicokinetic behavior, organ-specific distribution and bioaccumulation potential of selected sub-group of bisphenols in the model organism *Danio rerio* by developing a PBTK model (Paper II). Although all ADME properties were considered, metabolism as well as distribution to liver, gonads and brain were a major focus. This study is presented in Section 3 and Paper II.

Lastly, we aimed at developing a toxicokinetic model for the zebrafish embryo test including consideration of both physiology and experimental conditions (Paper III and Section 4). For this method we intended to incorporate literature data as well as own measurements of bisphenol kinetics, and to rank bisphenol hazard based on *in vitro* ER activity as well as predicted ZFE concentrations.

2 Exploring Chemical Variation, Grouping and Physico-Chemical Properties of PFASs and Bisphenols

In this section we describe the investigation of PFAS and bisphenol chemical space and evaluate the variation within these two compound classes in terms of chemical structure and physico-chemical properties. Additionally, we detailed the selection of sub-groups for both chemical classes and the investigation of reliability of property prediction models.

2.1 Introduction

Investigating the chemical space for groups of compounds can yield a better understanding of their structural and property variation and can therefore aid in prioritization of compounds of concern. Identifying chemical candidates for toxicity testing and biomonitoring prioritization is becoming an important step in hazard identification^{20,63,93}. Such prioritization can be done using expert-based approaches requiring data on exposure, effects or industrial application or using *in silico* predictions on environmental fate and effects^{20,63,108,109}.

Expert-based selection can for example consider knowledge on environmental occurrence such as levels in drinking water, soil, household dust or air and therefore aid in selecting compounds that are of high relevance for environmental exposure. Similarly, information about use of chemicals in various consumer products and production volumes can also be accounted for, thus identifying compounds with high exposure risk to humans or which are likely to be present in the environment. In addition to exposure consideration, one can prioritize compounds with measured hazardous properties. Expert-based selection approach may yield a less structurally diverse but highly relevant sub-group in the context of evaluating risk.

Information for expert-based selection is often-times unavailable, thus prioritization efforts have also incorporated QSAR/QSPR predictions^{109,110} or structural categories²⁰. Alternatively, methods based on chemical space such as multivariate analysis using PCA or unsupervised ML such as clustering have also been explored^{93,111}. A downside is that many freely available QSAR and QSPR models such as EpiSuite^{TM112} lack applicability domain (AD) assessment which can lead to unreliable predictions and erroneous hazard estimation based on such predictions^{113–115}. A means to tackle this uncertainty is to include larger, more heterogenous data sets in model training to cover a larger chemical space and thus result in a larger AD for the model or to build different models covering specific areas of the chemical space. PFASs for examples may be outside the AD of some currently available QSPR models and it is unclear whether the predictions are reliable¹¹³.

2.2 Aims

Our first goal was to identify structural information in order to investigate the chemical variation within PFASs and bisphenols. We aimed to curate the PFASs database in order to explore its chemical space and identify sub-groups based on identified structures. Additionally, in the case of PFASs, we intended to select diverse representatives spanning a large portion of the chemical space for future hazard testing and *in silico* tool development. Such a selection would allow to gain as much information from as little testing as possible. For bisphenols,

which is a smaller, better studied group of compounds, the goal was to select a small sub-group that is likely to be present in the human exposome and environmental matrixes based on their use in various products. Different selection approaches were therefore necessary to meet these goals.

Lastly, in order to investigate properties of PFASs and bisphenols, we collected experimental data when available and complemented those with predictions for several physico-chemical properties of the selected compounds. Additionally, we investigated uncertainties related to applicability domain for PFAS in the case of these property predictions.

2.3 Materials and Methods

2.3.1 Chemical Inventories of Bisphenols and PFASs

Bisphenols in this study were defined as molecules with two phenol rings connected by a bridge made up of one to nine carbons or a single other atom. Branching on the bridge or additional ring substitutions were also included in the definition. The majority of structural data was collected from the Swedish Chemicals Agency^{39,116} which included a total of 214 bisphenols that are used within the European Union. Further bisphenols that may be available outside the European market, were searched in scientific literature leading to a final list of 239 bisphenols. This is a wide definition of bisphenols as it includes compounds such as benzophenones in the case of benzophenone-2 (BP-2) or brominated flame retardants such as tetra-bromo bisphenol A (TBBPA).

The Organization for Economic Co-operation and Development (OECD) released a comprehensive list of PFASs chemicals in 2018 which includes over 4700 entries out of which only 1200 had structural information provided^{19,116}. In this database, PFASs were defined as chemicals that have three or more perfluorinated carbons alternatively, two or more perfluoroalkylether carbons. This definition has been recently revised to include all compounds with at least one perfluorinated methyl or methylene carbon thus including more compounds than previous definition¹¹⁷. However, the previous definition was applied in Paper I. In order to select a sub-group for testing prioritization, structures had to be first obtained based on information such as name or CAS number.

2.3.2 Selection of Bisphenols

A sub-group of bisphenols was selected based on criteria regarding their environmental occurrence, identification in human plasma and urine, their industrial use information and their potential interaction with ER. This selection was in large part based on information from the Swedish Chemical's Agency^{39,116}. Details on the selection process can be found in the supplement of Paper II.

2.3.3 Selection of PFASs

We identified structures and curated the database of over 4700 entries resulting in a list of 3363 for which chemical descriptors were generated. We applied PCA on the PFASs data set with 59 chemical descriptors in order to reduce the dimensionality to a total of 5 significant principal components (PCs). We used hierarchical clustering to divide the PFASs dataset into a total of 12 clusters. In this case an agglomerative algorithm using Euclidean distances was applied and the variance ratio criterion (VRC) was used to inform the decision of cluster

number. The final subsets for testing were selected based on the most central compounds in each cluster thus ensuring representation of a wide span of the chemical domain. The workflow and selection process are described in Paper I.

2.3.3.1 *Chemical Space*

Chemical descriptors are quantitative features representing the chemical structure and can be seen as dimensions placing each molecule somewhere in a large multi-dimensional chemical space^{92,118}. Although exploration of this space has been focused on discovering drug-like compounds^{92,118,119}, it has also been investigated for industrial chemicals^{93,111}. The chemical space has the dimensionality of the chosen number of descriptors which may vary from a single dimension to hundreds depending on the purpose. The chosen descriptors in current study have been previously proposed as suitable for investigating industrial chemicals^{93,111}. However, using a large number of dimensions poses a challenge as it increases needed computing time and power, therefore requiring dimensionality reduction techniques for further investigation of the data^{92,120}. Many suitable techniques exist such as PCA¹²¹, t-distributed stochastic neighbor embedding, locality preserving projections, self-organized maps, projection pursuit, generative topography mapping and more^{92,120}.

2.3.3.2 *Principal Component Analysis*

PCA is an unsupervised, linear technique that has been widely used to explore chemical space^{93,111,118,119}. Additionally, this method has been used to define AD of QSAR and QSPR models^{122,123}. This technique aims to capture as much of the data variance as possible in the multi-dimensional space by fitting lines or hyperplanes, called principal components (PC), to data. The PCs are fitted by minimizing the least squares. Each PC is orthogonal and uncorrelated to the previous, thus the first PC describes most of the variance^{98,124}. PCA can therefore be employed to summarize a large proportion of data variance in only a few principal components i.e., it reduces the dimension to the number of PCs that are significant. This method is often-times used for pre-processing data before the use of either unsupervised or supervised machine learning as many algorithms require lower dimensionality as well as uncorrelated descriptors⁹⁸.

2.3.3.3 *Clustering*

Although PCA is a useful tool for capturing data variance, other techniques are required to make use of this information. Clustering approaches have been previously used in chemistry to understand chemical similarity or dissimilarity and select sub-groups of data based on structural information^{93,125,126}. The purpose of the selection could be to select compounds with similar properties or structures which can be employed in drug development to select desired compounds, or in hazard identification to select compounds that may be similarly toxic to a well-studied compound^{127,128}. Additionally, one can use clustering to select dissimilar compounds in order to cover a large chemical space^{93,125,126}.

Hierarchical clustering (HC) is a method for grouping based on distance in multivariate space^{129,130}. In this study, where PCA was applied as pre-processing, this refers to the distance between PC values of the different PFASs. Either agglomerative or divisive algorithm can be used to create a dendrogram with HC^{126,129}. The agglomerative algorithm starts with each compound in the data set as a separate cluster and merges the two most similar entries together until a single cluster with all the data is formed while the divisive algorithm performs the same process in reverse i.e. starts with all data in one cluster and separates the two most dissimilar groups. Additionally, various distance measures can be used for HC such as Euclidean,

Manhattan or Mahalanobis distance¹³¹. Regardless of algorithm and distance measure, the end-result is a dendrogram which needs to be “cut” at a specific level in order to obtain an interpretable number of clusters. Choosing the number of clusters may depend on the purpose of the grouping, thus it can be based on the modelers requirements or it can be informed by mathematical optimum criteria like the variance ratio criterion (VRC)^{129,132}.

2.3.4 Physico-Chemical Properties

Physico-chemical properties were collected from literature when available experimentally and otherwise predicted using QSPR models for both bisphenols and PFASs. These included environmentally and toxicologically relevant properties such as the log octanol-water partition coefficient ($\log K_{ow}$), water solubility (S_w) and acid dissociation constant (pK_a) for bisphenols and PFASs along with the organic carbon to water normalized sorption coefficient (K_{oc}), vapor pressure (V_p) and bioconcentration factor (BCF) for only PFASs. We then assessed whether PFASs were within the applicability domain of the model based on molecular weight (MW), number of fluorine atoms, and whether used model adjusted for aliphatic fluorine fragments.

2.4 Results and Discussion

2.4.1 Bisphenol Selection and Properties

Previous research on bisphenols have mainly focused on a narrow chemical group, not allowing substitutions on the rings, but a strict definition of “bisphenol” is not clearly described in most publications^{133,134}. Other work has focused on BPA replacements with same use in products rather than structural similarity^{135,136}. Kitamura et al. 2005⁴³ tested 19 structural analogs of BPA which included compounds with a single phenolic ring thus using a broader definition than employed in this work. However, previous studies presented relatively small groups of compounds and were not aimed at compiling a comprehensive list of bisphenols as starting point for prioritization. Current work compiled an inventory of 239 bisphenols, out of which 11 were selected with likely human exposure risk, environmental occurrence and measured or predicted ER activity as a criterion of hazard. The only exception being Bimox M, selected as a potentially high exposure compound due to its application, but with no predicted ER activity, in order to have a negative control with similar chemical structure for future *in vitro* testing. There is some bias in the selection process as it favors compounds for which more research is available or that have been detected in targeted screening approaches. This is oftentimes the case with expert-based approaches. However, the advantage is that there is more certainty in the environmental and human health relevance of the selected compounds. Keminer et al. 2020¹³⁶ applied similar criteria in terms of prioritizing compounds that bind to endocrine receptors and that are used in applications leading to potential environmental exposure. However, the focus of their selection was BPA substitutes thus included much more variation in selected chemical structures as it was not limited to bisphenols. Nonetheless, there was overlap with our selection and previous studies with most of them including BPC, BPAP, BPZ, BPS, and BPF.

The selected bisphenols (Figure 1) were deemed to be of high or medium exposure risk due to their uses in consumer products^{37,39,45,137}. Most of the selected compounds such as BPA, BPS, BPF, BPAF, BPAP, BPC, BPB, and BPZ are registered for use in polymeric synthesis and as coating resins. Notably, BPB, BPC, and BPS are present in food packaging while BP-2 is found in cosmetics as a UV-blocker. TBBPA is a well-studied brominated flame retardant employed

for fire-proofing of furniture, carpets and other products. Additionally, all these compounds with exception of Bimox M, have been detected in both human samples, such as urine and serum and in environmental matrixes including water where they can affect aquatic organisms^{37,39,45,137,138}.

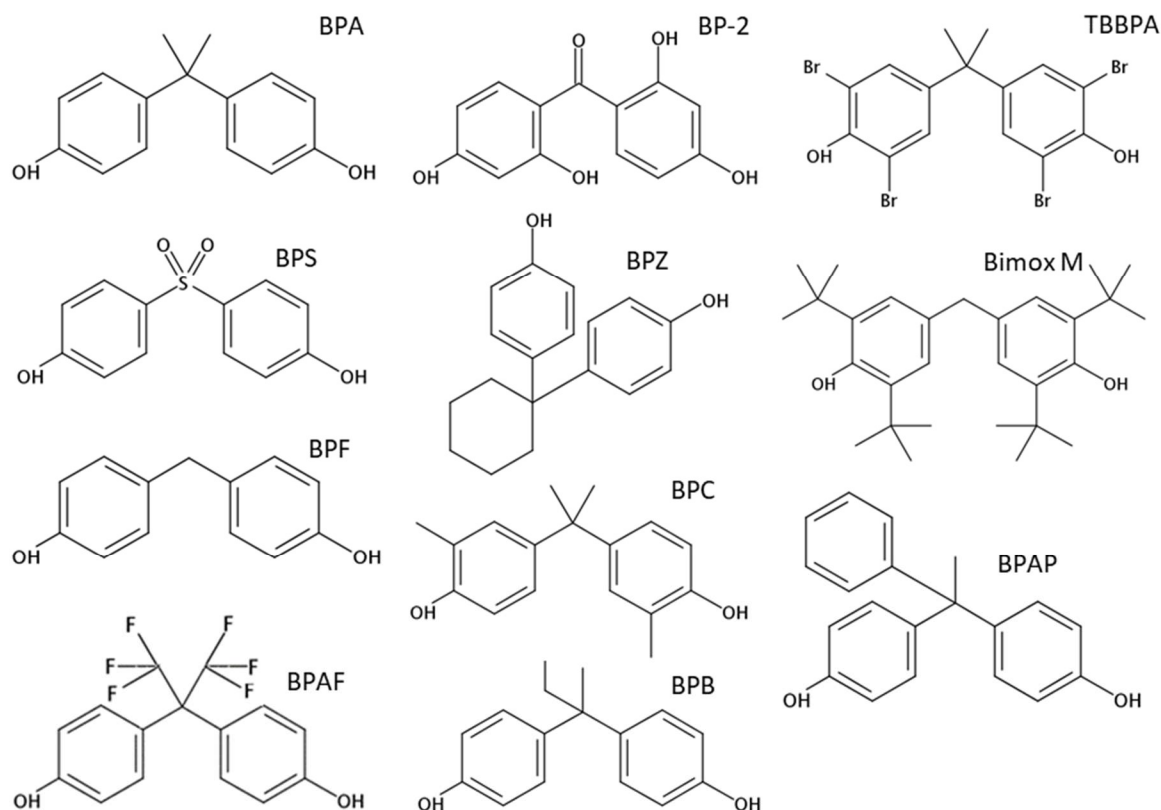


Figure 1. The molecular structures of bisphenols selected for future investigations and deemed of high environmental relevance.

2.4.1.1 Physico-Chemical Properties

Physicochemical properties of selected bisphenols are presented in Table 1. Unlike PFASs, bisphenol properties have a higher likelihood to be accurately predicted as similar compounds are generally included in training data sets of predictive models. BPA for examples is part of the training data for various EpiSuite™ models¹¹². In this study measured data were considered of higher reliability than predicted ones, thus we collected measured data when available for $\log K_{ow}$ of BPA¹³⁹, BPAF, BPS¹⁴⁰ and TBBPA¹⁴¹. The $\log K_{ow}$ in these studies was measured at pH 7 and is referred to as $\log D$ as it represents the pH-dependent octanol-water distribution. Although $\log K_{ow}$ and $\log D$ are the same for neutral compounds, they may differ for ionizable compounds, thus the measured $\log D$ at pH 7 is considered more environmentally relevant¹⁴². However, since many tissue partitioning models needed for PBTK model in Section 3, are developed based on $\log K_{ow}$ as opposed to $\log D$, the first was used when no measured data was available. The $\log K_{ow}/\log D$ of the selected bisphenols vary from 2.1 for BPS to 4.75 for TBBPA with the exception of Bimox M which has a much higher value of 9.1. All of them are hydrophobic (with a $\log K_{ow}$ above 1) and thus have the potential to partition into organisms^{143–146}.

Table 1. Selected sub-group of eleven bisphenols and their physico-chemical properties

Abbreviation	Name	CAS	MW (g/mol)	log K_{ow} ^a	S_w ^b	pK _a ^c
BPA	Bisphenol A	80-05-7	229	3.4 ^d	1.7*10 ²	9.8
BPAF	Bisphenol AF	1478-61-1	336	4.7 ^e	4.3	9.1
BPAP	Bisphenol AP	1571-75-1	290	4.5	3.8	10
BPB	Bisphenol B	77-40-7	242	3.9	29	10
BPC	Bisphenol C	79-97-0	256	4.3	7.5	10
BPF	Bisphenol F	620-92-8	200	2.9	5.4*10 ²	9.8
BPS	Bisphenol S	80-09-1	250	2.1 ^f	3.5*10 ³	7.4
BPZ	Bisphenol Z	843-55-0	268	4.3	3.78	9.8
BP-2	Benzophenone-2	131-55-5	246	2.7	4*10 ²	6.8
BM	Bimox M	118-82-1	425	9.1	1*10 ⁻³	11
TBBPA	Tetrabromo bisphenol A	79-94-7	544	4.75 ^g	1.7*10 ⁻⁴	9.4 ^h

^aMedian prediction CompTox, ^bWater solubility predicted by EpiSuite Wskowwin v 1.42; ^cpK_a predicted by Jchem for Excel v 19.21.531 (ChemAxon) ^dlog D from Staples et al 1998¹³⁹, ^ePredicted using Jchem for Excel v 19.21.531 from ChemAxon¹⁴⁷, ^flog D from Choi and Lee 2017¹⁴⁰, ^glog D from Kuramochi et al.2008¹⁴¹ Note in Paper II, this value was wrongly given for pH 3 instead of 7, current value is given for pH 7. ^hMeasured value from ECHA¹⁴⁸

Considering Lipinski's rules of 5 on bioavailability, most of the selected bisphenols have a MW below 500, a log K_{ow} below 5, less than 5 H-bond donors and less than 10 H bond acceptors suggesting potential for internal exposure in organisms¹⁴⁹. However, in order to assess hazard, it is important to understand effects as well as internal dose, i.e. the dose at target of toxicity. Since internal concentrations at target organs are related to physiology as well as to chemistry, this can be further investigated using PBTK modelling as discussed in Sections 3 and 4. Hazardous properties in terms of ER activity can be investigated *in vitro* for these compounds and is presented in Section 4.

2.4.2 PFASs Selection and Properties

2.4.2.1 Chemical Space Exploration

The original OECD database of 4730 PFASs compounds constitutes of 19% chemical mixtures or polymers, which were filtered out. Such entries require different approaches for modelling and descriptor generation than single, non-polymeric molecules. Although there are some approaches for using ML on both polymers and mixtures, these methods are more complex and generally have narrower applicability¹⁵⁰. Mixture approaches are similar to single compound methods, but using for example mixture descriptors based on individual compounds and the mixture together^{151,152}. Nonetheless, these models either have the AD of a single mixture but at different compositions or only a 1:1 ratio of two compound mixtures^{150,153}. Polymers have only been modelled using monomeric units and mainly applied to model biological responses rather than physico-chemical properties of the polymers^{154,155}. Thus, robust and more varied approaches need to be developed in order to better address mixtures and polymers and these were therefore filtered out in Paper I.

The PFAS entries of the OECD database were curated resulting in a list of 3363 PFAS which were highly diverse in size (150-3217 Da), molecular functional groups (e.g. linear, branched, containing aromatic rings, ketones, acids, esters etc.) and number of fluorines in each molecule (5-102). A principal component analysis was performed using 59 chemical descriptors which

resulted in five significant principal components (PCs) with the first one (PC1) explaining 45% of data variance. Although the PCs summarize the variance across many descriptors, comparing the weights of various descriptors for each PC can provide indications about which of these are more critical for describing data variance. PC1 was generally related to descriptors related to molecular surface and size including the Wiener path number (wienerPath)¹⁵⁶, the area of van der Waals surface (vdw_area) and the first kappa shape index (Kier 1)¹⁵⁷. The second PC describing 17% of the variance had high weights on density, number of fluorines and aromaticity.

Cheng and Ng 2019 presented a curation of the same database using similar approaches as in Paper I for structure generation and obtained 3486 structures by using the chemical identifier resolver (CIR) tool to generate structural information in the form of Simplified Molecular Input Line Entry System (SMILES)^{158,159}. This study additionally collected data from other databases and employed different curation of structures, hence the differing number of structures. Similarly, they found that a large proportion of the database were short chain PFASs.

Clustering approach was used in Paper I as suggested by Rännar & Andersson 2010⁹³ to select a highly diverse sub-group of PFAS. Five principal components were used as the basis for performing clustering aimed at splitting the highly diverse data set into more homogenous clusters. Data was split in a total of 12 cluster based on the VRC as well as consideration on even distribution of compounds between clusters. Six of these clusters, contained at least one of the 34 well-studied PFASs and included mainly small to medium-sized, highly fluorinated and mostly linear structures (Paper I). In this context well-studied PFASs were defined as those with more than 10 citations according to Wang et al. 2017¹⁶⁰. These PFAS have been detected in environmental matrixes such as water^{161,162}, air¹⁶³ and soil¹⁶⁴. In other words, half of the clusters do not contain any well-studied PFAS, highlighting the large knowledge gaps covering mainly larger, branched, and highly polar or aromatic compounds. Additionally, the well-studied PFASs may not necessarily be representative for their corresponding cluster, especially if they are mainly at the “edges” of these chemical spaces.

These findings of knowledge gaps in PFASs chemical space are also in line with measurements of environmentally occurring PFASs. Several studies analyzing extractable organofluorine in environmental matrixes showed large proportions of unidentified fluorine containing molecules even after targeted analysis of well-studied PFASs^{66,165}. Björklund et al. 2021⁶⁶ found that 88% of extractable fluorine was not accounted for even after targeted analysis of 34 known PFASs, while Koch et al. 2019¹⁶⁵ found >92% of unknown extractable organic fluorine in some samples. Although some of these can be compounds that are not necessarily in the category of PFASs, it still highlights the knowledge gaps when it comes to environmental occurrence in fluorinated compounds.

2.4.2.2 *PFASs Sub-Group Selection*

Experimental studies of a representative sub-group based on the 12 PFASs clusters would address existing knowledge gaps efficiently as it would cover a large area of the chemical space thus allowing for extrapolation between similar compounds. In order to identify such a sub-group, 5% of the most central compounds in each cluster were selected. Centrality was based on Euclidean distances in the five dimensions of the PCs for each cluster. Picking PFASs based on the center of each cluster instead of the edges ensures that the sub-group is still representative for each cluster, spans the majority of the chemical domain but does not include any extreme outliers. This selection, denoted as theoretical training set, contained 165

chemicals presented in the SI of Paper I with the most central compound in each cluster presented in the Paper I.

However, realistically, a large part of the database may not be commercially available as the OECD database contains registered compounds that are not necessarily being produced. For this purpose, we propose the selection of a procurable training set instead. Procureability was assessed based on test set suggested by Patlewicz et al. 2019²⁰, the Norman suspect screening list¹⁶⁶ and an inventory provided by the Swedish Chemicals Agency¹¹⁶. This resulted in the selection of a procurable test set containing 23 PFASs spanning over all 12 clusters. This training set offers a more practical alternative to the theoretically selected one while still capturing the large variability within the PFASs database.

Patlewicz et al. 2019²⁰ proposed a selection of 75 PFASs based on considerations of procureability as well as 53 expert-based category definitions suggested by Buck et al. 2011²¹. This selection was done based on 271 PFASs which were available at the time while considering structural diversity but also more practical considerations such as volatility, solubility and *in vivo* data availability. This selection was recently expanded to a second set of 75 PFASs with further considerations of varying structural categories¹⁶⁷. These studies also aimed to select diverse PFASs for the purpose of future hazard assessments of this emerging group of chemicals.

2.4.2.3 *Applicability of Predictive Models for PFASs Properties*

In order to assess applicability domain of current environmental fate models, we proposed three simple approaches based on the parameters molecular weight ranges, number of fluorine fragments and number of fluorine atoms. More advanced methods are typically used for assessing AD in predictive models, however these methods generally need to be applied during the process of model training and development⁹⁵. AD assessment requires structural information of training data which is often provided, but it more importantly requires the descriptor set and values used for training each model which are not given for all the investigated models in current study. One of the simplest methods to assess AD is using the parameter ranges of the descriptors in the training data thus resulting in a multi-dimensional bounding box¹⁶⁸. PCA is commonly used for AD assessment in combination with descriptor ranges thus resulting in a multidimensional bounding box based on the PCs^{123,169}. Alternatively, distance from the centroid of the PCA-processed multi-dimensional training set can be accounted for¹¹⁵. Various distance measures can be used as presented for the clustering. A downside with these approaches is however that they do not consider empty spaces in the descriptor ranges¹⁶⁸. Probability density distribution-based methods can account for empty spaces and these are thus considered more accurate and robust but are more restrictive and less commonly used¹⁷⁰. Nonetheless, AD assessment has been proposed by the OECD as an important aspect of reliable QSAR modelling¹¹⁴.

When looking at fluorine fragments or number of fluorines, below 1% of the database would be considered within domain for log K_{ow} , S_w , log D and log K_{oc} models. Interestingly, the Vp model included perfluorinated compounds in the training data, thus a large portion of the database was considered within domain. A previous study investigating the AD of EpiSuiteTM models based on descriptor ranges, and suggested that some fluorinated compounds are within the applicability of the model¹⁶⁹. It is important to note that AD would be assessed differently in case of models that do not contain training and test sets. Models based on statistical thermodynamics, rather than empirical data, are likely to predict more accurately. This is the

case for example for COSMOtherm which has been shown to perform better than EpiSuite™ in predicting K_{ow} for perfluorinated compounds^{113,171}.

Collected experimental data from literature was used to assess predictive performance of current environmental fate models for PFASs. The R^2 between predicted and observed data was calculated for each of the models and this assessment showed that $\log K_{ow}$, $\log D$ and $\log K_{oc}$ are predicted fairly accurately ($R^2 > 0.5$). A previous study by Arp et al. 2006¹¹³ found that $\log K_{ow}$ predictions using EpiSuite showed up to 5 orders of magnitude error of prediction for highly fluorinated compounds. The model has however been updated since¹¹³. A more recent study compared the predictive accuracy of COSMOtherm, EpiSuite™, OPERA models from CompTox Chemical Dashboard and Linear Solvation Energy Relationships (LSERs) for physico-chemical properties of 25 PFASs¹⁷¹. They investigated the majority of properties presented in current study and showed that EpiSuite™ performed reasonably well for $\log K_{ow}$ and V_p , and performed poorly for S_w and air-water partitioning. COSMOtherm and OPERA performed well for all investigated properties, with COSMOtherm showing the most accurate S_w predictions. These findings are in line with Paper I when it comes to EpiSuite™ predictions. It is important to note however, that some of these models show good performance for only a limited set of small and highly fluorinated PFASs and does not reflect the predictivity of the whole database.

The BCF predictions, in contrast, yielded a negative R^2 . This was the case for the EpiSuite™ BCFBAF model as well as for 3 other BCF models in VegaHub¹⁷², highlighting that prediction of this property is challenging for PFASs. This lack of predictivity could be in part explained by the fact that current BCF models are based on $\log K_{ow}$ and do not account for species-specific ADME properties. PFASs have been shown to bind strongly to plasma proteins and their long half-lives in humans are believed to be caused by selective active re-uptake in the kidneys¹⁷³. More complex biological models such as PBTK models would likely be more suitable for predicting such properties of PFASs¹⁷⁴.

Presented work is the first to investigate AD of EpiSuite™ for such a comprehensive list of PFASs and thus highlights the large uncertainties of using commonly utilized fate models for predictions of PFASs. It is apparent that most PFASs are outside these domains and thus the models cannot be used for accurate predictions or testing prioritization. One solution is to develop PFASs specific models, which consider the unique chemical properties of these compounds. This has been done by Wang et al. 2015 for predicting gas-particle partitioning¹⁷⁵, by Cheng and Ng 2019 for predicting bioactivity¹⁵⁸ or by Le et al. 2021¹⁷⁶ for predicting fate properties. Another option would be to expand the training set of current models and include data on PFASs in order to expand the AD. This could be achieved by incorporating compounds from the selection presented in Paper I into experimental screening efforts which in turn would provide additional training data.

3 Modelling Toxicokinetics of Bisphenols in Adult Zebrafish

In this section we describe the development and performance of a zebrafish PBTK model aimed at investigating the toxicokinetics and organ-specific distribution of selected bisphenols. Additionally, we present measurements of fish-specific biotransformation and *in vivo* organ-distribution of BPZ in *Danio rerio*. More details can be found in Paper II.

3.1 Introduction

ADME are the key components in understanding the dose that reaches biological targets of toxicity, thus activating MIE and possibly leading to adverse effects^{78,103,177}. Additionally, ADME properties are the drivers of bioaccumulation in various species. Thus, compounds with same hazardous properties can have different risks depending on these processes. Investigating ADME and bioaccumulation *in vivo* can be done by measuring compound concentration in organs or whole body of various model species. Alternatively, PBTK modelling can be used to model these processes. Existing *in vivo* measurements as well as *in vitro* data, physico-chemical properties measurements and QSPR predictions can be integrated within these models^{102,104,178}. PBTK models are a type of toxicokinetic (TK) models which simulate ADME with focus on physiology. In literature, PBTK, physiologically-based kinetic (PBK) and physiologically-based pharmacokinetic (PBPK) models all refer to the same approach. PBTK models are composed of different compartments, which can represent one or several organs or parts of organs as seen in Figure 2. A series of time-dependent ordinary differential equations (ODEs) are used to simulate kinetic processes by estimating the amount of a chemical in each compartment and predicting internal concentrations in target tissues^{60,177}. PBTK models are species and compound specific, thus require both species and chemical specific information for development.

Since bisphenols have been frequently detected in waters, developing fish PBTK models would be of relevance to assess environmental exposure and dose at target^{103,106}. Fish species commonly used for studying ADME of environmental pollutants include rainbow trout (*Oncorhynchus mykiss*), fathead minnow (*Pimephales promelas*), three-spined stickleback (*Gasterosteus aculeatus*), common carp (*Cyprinus carpio*), Japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*)^{103,105,179}. Out of these, zebrafish has been one of the most commonly studied species for endocrine research, thus understanding ADME properties better for zebrafish could aid in future extrapolation of effect data from this model organism to others. PBTK models for several fish species have been previously developed but focusing mostly on other compounds than bisphenols^{105,180–184}. One zebrafish model and one generic fish model adapted for five fish species using validation data for BPA have been developed previously^{179,185}. However, these models did not consider other bisphenols and do not include more recently published *in vivo* zebrafish data for validation. Additionally, these models were designed to be compound unspecific thus their accuracy is within a 10-fold error or higher as typically seen for generic models^{102,178,179,186}.

Understanding organ-specific distribution of various bisphenols in especially liver, gonads and brain would help in processes of chemical prioritization and hazard identification. These organs are likely targets of both endocrine disruption and other adverse effects caused by

bisphenols. Although the liver is not part of the HPG axis, it is relevant for estrogenic effects in fish due to being a site of ER expression. The activation of ER in fish hepatocytes has been shown to induce production of the egg-yolk protein vitellogenin (VTG)⁵⁴. Furthermore, changes in VTG levels have been proposed to be a key event following ER activation as part of an AOP which is currently under development for fish¹⁸⁷. Lastly, several bisphenols including BPA, BPAF, BPF, TBBPA and BPS have been shown to alter VTG response in zebrafish indicating that liver may be an important target organ for investigating risk and ADME of bisphenols^{188,189}. The role of adult zebrafish gonads in the toxicity of bisphenols is not entirely clear. Both ovaries and testes are part of the HPG axis and alteration of function or morphology in these organs has been found upon bisphenol exposure^{190,191}. However, in female fish, compounds reaching the ovaries can potentially transfer to developing eggs and thus end up in the embryo after fertilization. Studies on ZFE exposed to various bisphenols have shown adverse effects on development making exposure via maternal transfer a relevant consideration when assessing risk^{56,192}. Lastly, adverse outcomes in zebrafish related to brain have been identified in previous studies upon exposure to bisphenols^{193–195}. Such effects have been observed for both embryos and adults making the brain another possible target of toxicity for bisphenols.

3.2 Aims

We aimed to develop a PBTK model to better understand ADME properties and bioaccumulation in adult zebrafish of the previously selected bisphenols. The goal of such model was to predict dose of the various bisphenols at suspected target organs of toxicity, namely liver, brain and gonads. In order to parameterize and calibrate this model, we measured fish-specific biotransformation *in vitro* as well as *in vivo* organ distribution of BPZ. Details on the study can be found in Paper II.

3.3 Materials and Methods

3.3.1 *In Vivo* Zebrafish Experiments

Toxicokinetics of BPZ were studied *in vivo* in female zebrafish so as to calibrate the model on an additional bisphenol and also to investigate time-course distribution to brain, which was not previously studied in zebrafish for bisphenols. Additionally, we measured distributions to the liver and gonads as potential target organs of toxicity.

3.3.2 *In Vitro* Biotransformation

Biotransformation rates for bisphenols other than BPA in fish were not available in literature and were therefore measured *in vitro* to parameterize the model. We measured intrinsic clearance of bisphenols in rainbow trout (RT) S9 by quantifying the disappearance of parent compound over time. Metabolic clearance can be investigated *in vitro* as a proxy for the *in vivo* system using either whole hepatocytes, S9 sub-cellular fractions, microsomal proteins or even isolated enzymes^{83,196–199}. This can be done by either measuring disappearance of parent compound over time or the appearance of specific metabolites over time in order to calculate a total metabolic clearance rate or rate of specific metabolic processes, respectively. In the case of fish biotransformation, OECD guidance have been published describing protocols for measuring intrinsic clearance using either hepatocytes (RT-HEP) or S9 sub-cellular fraction (RT-S9) in rainbow trout (*Oncorhynchus mykiss*)^{82,83}. The S9-sub-cellular fraction contains

both enzymes of phase I and phase II metabolism, making it possible to identify the combined rate of multiple metabolic pathways rather than specific ones^{83,196}. Additionally, S9-fraction measurements have been identified as a suitable parameter of hepatic clearance in generic PBTK models¹⁷⁸.

3.3.3 Zebrafish PBTK Modelling

In this study we expanded a previous zebrafish model by Grech et al. 2019¹⁷⁹ in order to improve model predictions for the previously selected bisphenols and their main metabolites. We incorporated the process of egg laying and an egg compartment in order to model maternal transfer. Metabolism was modelled to occur in the liver compartment using measured *in vitro* clearance rates for parameterization. Additionally, metabolite kinetics were also modelled for BPA glucuronic (BPA-GA) acid and BPAF glucuronic acid (BPAF-GA). The model structure for female fish is presented in Figure 2 while the one for males is available in SI of Paper II.

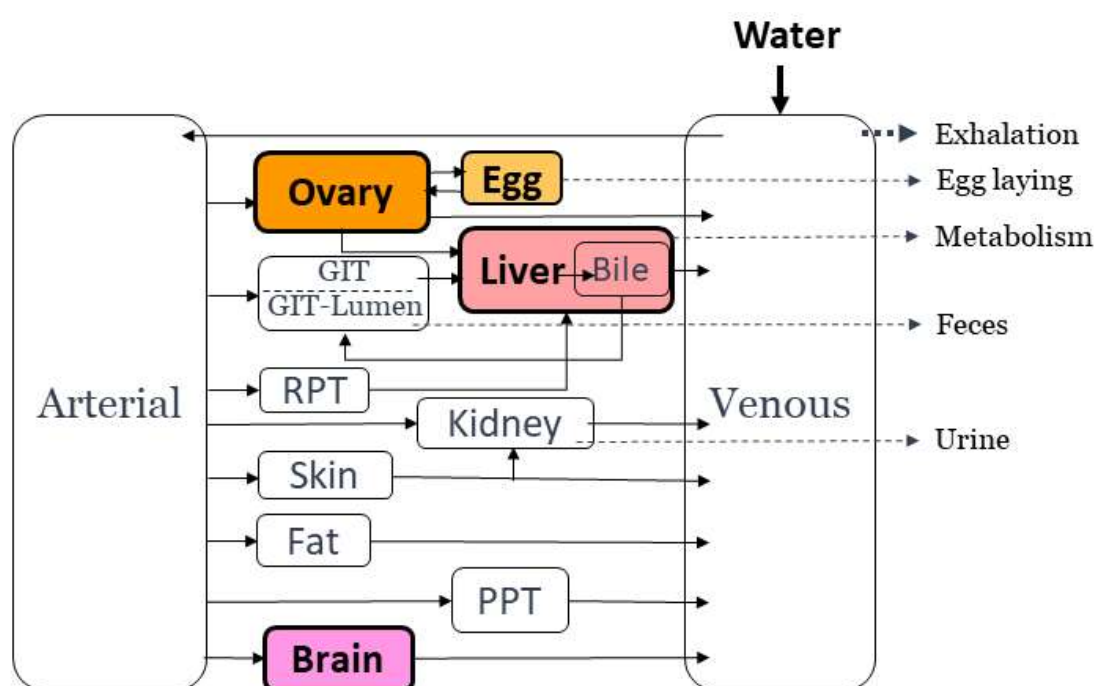


Figure 2. Female zebrafish PBTK model structure. Solid lines represent mass flow of compound and dotted lines represent elimination routes. Coloured compartments represent organs of toxicity for which measured data was available. GIT = Gastro-intestinal tract; RPT = richly-perfused tissue; PPT = poorly-perfused tissue.

Measured bisphenol-specific zebrafish tissue partition coefficients were not available in literature but QSPR models have been developed and validated on data from larger fish species previously^{200–202}. Although these QSPR models were not developed for zebrafish, they were considered a more relevant option than the use of QSPR models developed on mammalian data. A model for predicting fish blood-water partitioning (P_{bw}) developed by Fitzsimmons et al. 2001²⁰² was used to predict P_{bw} based on $\log K_{ow}$ and was adjusted for unbound fraction as suggested in previous PBTK models^{179,185}. Secondly, the tissue-blood partition coefficient (P_{tb}) was predicted based on water and lipid content in tissue using the model from Bertelsen et al. 1998²⁰⁰. The P_{tb} QSPR model was developed using compounds with $\log K_{ow}$ ranging from 0 to 8, thus Bimox M was considered outside the AD and therefore unlikely to give a reliable prediction. Since *in vivo* data was more abundant for some compounds, some of the partition coefficients were fitted on experimental data using Nelder-Mead fitting algorithm if QSPR predictions did not agree with measured organ concentrations. These included the liver-

partitioning for BPA and BPAF while brain-blood partitioning was fitted on BPZ data and used to parameterize all of the compounds due to lack of any other brain partitioning data.

3.3.3.1 Model Performance and Sensitivity

Predicted and observed data from literature as well as own *in vivo* data were compared by calculating area under the curve (AUC), maximal concentration (C_{\max}), half-life ($t_{1/2}$) and bioconcentration factor (BCF). The normalized root mean squared error (NRSME) was additionally computed for performance assessment. For QSAR/QSPR models the root mean squared error (RMSE) is generally calculated for this purpose²⁰³. However, the RMSE is scale dependent, thus in the case of PBTK model predictions which can have varying dosing concentrations, the RMSE requires a normalization for concentration. In this study we normalized by the C_{\max} in order to obtain a scale-adjusted value, i.e., the NRMSE²⁰⁴.

Lastly, global sobol sensitivity analysis²⁰⁵ was performed by varying the parameters within a uniform distribution by $\pm 20\%$. Sensitivity analysis is a method for assessing how changes in parameter value influence the outcome²⁰⁶. The general approach is to randomly sample parameter values of one or multiple parameters within a given distribution and use each sample to run the model. The obtained model output, is then used to calculate a sensitivity index describing the sensitivity of the chosen model output to changes in that parameter. Sensitivity analysis is commonly classified as either local or global where local sensitivity analysis varies a single parameter randomly while the other are kept fixed while global sensitivity analysis varies all parameter values at the same time^{207,208}. Although local sensitivity analysis is simpler and computationally less intensive, it does not consider that parameters may covary²⁰⁷. In contrast, global sensitivity analysis can account for these correlations between parameters and is therefore considered a more reliable approach for sensitivity analysis of PBTK models^{208,209}. There are a few available algorithms which can be employed for sensitivity analysis including sobol, extended fourier amplitude sensitivity test (eFAST) and Morris^{208,209}.

3.4 Results and Discussion

3.4.1 *In Vivo* Zebrafish Experiments

A mean BPZ water concentration of 17 $\mu\text{g/L}$ was quantified over the exposure duration. Although this is a marginally higher concentration than employed in other bisphenol studies, it is still below some environmental water measurements of BPA which have been reported with values as high as 28 $\mu\text{g/L}$ ²¹⁰. Unlike the other measured organs, liver showed large variations between replicates in this study. This trend has however been observed previously *in vivo* zebrafish studies^{211,212}. This could be due to inter-individual differences in metabolic capacity which has been previously observed both for CYP1A^{213–215} as well as for phase I and phase II enzymes²¹⁶ in zebrafish. Additionally, liver is a challenging organ to sample in such a small fish species, thus experimental error may also be a factor. When looking at the distribution of BPZ in different organs, liver and gonads showed higher BCFs than brain although the differences were not significant. Experimental whole body BCF was higher for BPZ than for BPA, which is consistent with measurements in carp⁵³.

3.4.2 *In Vitro* Biotransformation

Measured *in vitro* clearances in this study showed little variation between most bisphenols with the majority of values within one order of magnitude. The only exceptions were BP-2, TBBPA and Bimox M with the first two showing higher metabolic rates while no biotransformation was identified for the last. When it comes to Bimox M the low solubility and high lipophilicity may have caused the compound to precipitate and therefore no rate could be measured.

Interestingly, the rates for RT-S9 measured in Paper II showed values one order of magnitude lower than those in reported for humans²¹⁷ for BPA, BPAF, BPAP, BPB, BPC and BPF. In contrast, BPZ, BP-2 and TBBPA showed rates within the same magnitude as those reported for humans in CompTox Dashboard. This indicates that using human rates to parameterize other species such as fish may lead to over-estimation of clearance and therefore under-estimation of risk of some bisphenols. It is important to note that in this study we assumed that rainbow trout and zebrafish metabolic rates are similar. However, since rainbow trout and zebrafish are phylogenetically closer related to each other than to humans or rats, these values likely provide a more accurate estimate than using mammal data.

For BPA, measured biotransformation rates are available in literature for a single isoform UDP-glucuronosyltransferases (UGTs) namely UGT1A1¹⁹⁹ and a single isoform of sulfonylesterases (SULTs)¹⁹⁸. *In vivo* comparison of the sulfonic acid and the glucuronic acid (GA) conjugates of BPA show a two order of magnitude higher concentrations of the latter after exposure to BPA, making the sulfonic acid conjugate levels negligible in comparison²¹⁸. This suggest that the rates measured for SULTs are likely not representative of the *in vivo* clearance of bisphenols. Additionally, although single isoform rates are highly relevant for better understanding metabolic pathways, they may not be suitable for parameterizing liver clearance in PBTK models. Measurements on isolated phase II enzymes, such as UGTs or SULTs can underestimate the metabolic rates as they do not account for the influence of phase I metabolism on phase II reactions. In contrast, the S9 sub-cellular fraction used in current study encompasses multiple isoforms from both phases^{83,196}. Since disappearance of parent compound was measured, the measured rate is an estimate of the total liver clearance and therefore more suitable for parameterization. One downside, however is that we cannot distinguish whether phase I, II or both are the drivers of the observed biotransformation. Additionally, it is not possible to calculate Michaelis-Menten kinetics with this data, but only linear rates.

3.4.3 PBTK Model Predictions

When considering all available adult zebrafish data measured for bisphenols^{211,212,218,219}, the majority of concentrations were predicted within a 2-fold error (Figure 3). Below, model performance per compartment is discussed further.

3.4.3.1 *Whole Body*

The AUC and C_{max} for whole body BPZ and BPAF were predicted within 2-fold of the values calculated using experimental data while for BPA this was the case for the study by Chen et al.2017²¹¹ but not for the study by Lindholm et al. 2003²¹⁸ However, the majority of whole body and carcass concentration data points were predicted within a 5-fold error for BPA, BPAF and BPZ and 50% of them were within a 2-fold error (Figure 3). BPA studies varied more than one order of magnitude between highest and lowest doses, showing that the model is capable of making fairly accurate predictions within a large dosing range. Notably, the high dose exposure

of 97.5 $\mu\text{g/L}$ was over-predicted which could be an indication for saturation of kinetic processes in the *in vivo* situation that is not accounted for in the model.

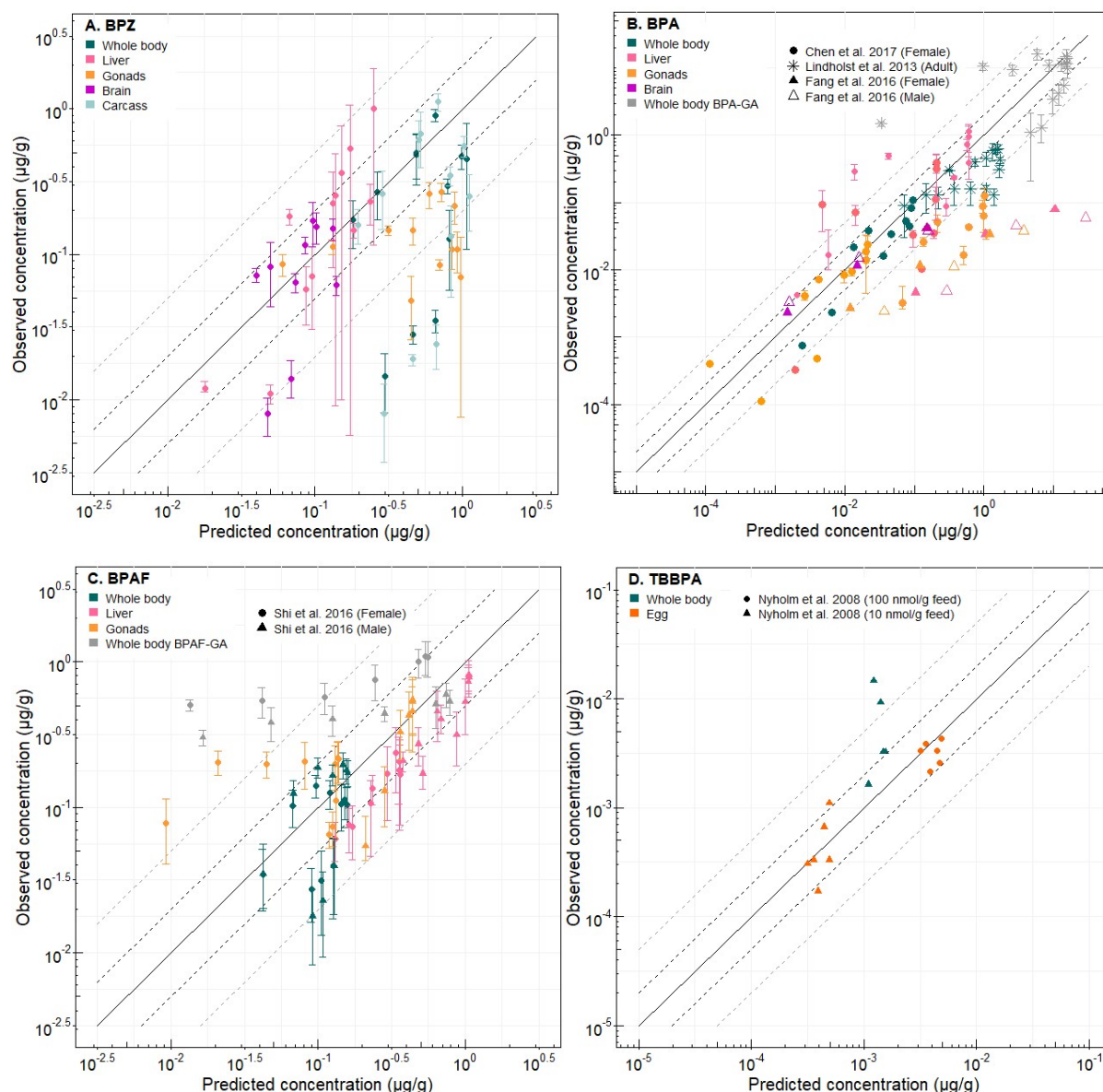


Figure 3. Observed data from literature (B, C, D) as well as own *in vivo* studies (A) compared to predicted by developed PBTK model presented in Paper II for BPZ (A), BPA and BPA-GA (B), BPAF and BPAF-GA (C) and TBBPA (D). Solid line shows perfect fit while dotted lines show 2-fold (black) and 5-fold (grey) errors. Error bars represent measurement standard deviation. BPZ NRMSE = 0.7; BPA NRMSE = 0.3; BPAF NRMSE = 0.4; TBBPA NRMSE = 0.5; Data used for fitting was also included in the figure but not in the NRMSE calculation. Data for metabolites was not included for NRMSE calculation. Figure was adapted from Paper II.

When comparing difference in kinetics between genders, Fang et al. 2016²²⁰ reported similar degree of bioaccumulation in male and female zebrafish organs upon exposure to same dose of BPA. However, there is some uncertainty in this measured data since this study showed large variation in water concentrations and therefore unstable dosing conditions which were not compared between genders. Shi et al. 2016²¹² showed stable water concentrations for BPAF and reported higher measured concentrations in males, suggesting there may be differences in absorption or elimination processes between males and females. The PBTK model presented in Paper II for female includes elimination via egg-laying in addition to other routes, but still

over-predicts the concentrations of bisphenols in females (Figure 3A, 3C). Another possible explanation for the observed gender-differences could be dissimilarity in metabolic capacity. Levels of the metabolite BPAF-GA have been reported to be higher in females than males exposed to the same dose of BPAF, thus supporting this hypothesis²¹². On the contrary, expression of UGTs has been reported to be higher in male than in female zebrafish¹⁹⁹. Our metabolic rates measurements used for parameterization cannot account for gender-differences as the S9 fraction was extracted from a mixed homogenate of both male and female fish.

TBBPA data were underpredicted by the model (Figure 3D). Unlike the studies on other bisphenols which were dosed through water exposure, TBBPA was dosed through feed²¹⁹. Oral absorption could not be accurately parameterized as multiple data sets were not available for this exposure route leading to large uncertainties in model predictions. Additionally, feed exposure leads to higher uncertainty in the measured data since different individuals may ingest differing amount of feed. However, if such data was available it is possible to model zebrafish oral absorption as demonstrated previously^{221,222}.

3.4.3.2 *Metabolites*

The model predicted the majority of measured BPA-GA and BPAF-GA concentrations within a 5-fold error and the AUC as well as C_{\max} within a 2-fold difference. Data for BPA-GA was however used for fitting so performance cannot be assessed in a non-biased way. Although the glucuronic acid conjugates of bisphenols have not been reported to show hazardous properties or ER activity^{223,224}, they are still relevant to model for better understanding of metabolite fate. Furthermore, studies on mammals have shown that BPA-GA can be de-conjugated in the intestine and re-absorbed leading to enterohepatic recirculation and prolonging the half-life of parent compound^{225,226}. This process has not been investigated in fish, but is highly warranted as the measured concentrations of BPAF-GA and BPA-GA were much higher than their respective parent compounds in exposed zebrafish.

3.4.3.3 *Muscle*

Recent data published after the publication of Paper II by Han et al. 2022²²⁷ investigated the accumulation of BPZ, BPC, BPF and BPS in muscle tissue of adult male zebrafish. Since the majority of the poorly perfused tissue (PPT) in the developed PBTK model is represented by muscle, this compartment was compared to the observed data (Figure 4). As for the whole-body data presented earlier, there was a tendency to over-predict high concentrations which were used in this study with medium and a high dose groups in this study ranging from 40 to 3000 µg/L and 200 to 15,000 µg/L, respectively. These over-predictions could be due to saturation processes of the absorption and distribution not being considered in the model. Additionally, the study design by Han et al. 2022 did not include feeding of the fish for 13 days which could result in volume concentration in muscle as well as much lower adipose tissue fraction than the mean value used for modelling. Nonetheless, predictions were within a 5-fold error for 41% of data-point and 10-fold error for 83% of data showing reasonably good performance of the model (Figure 4).

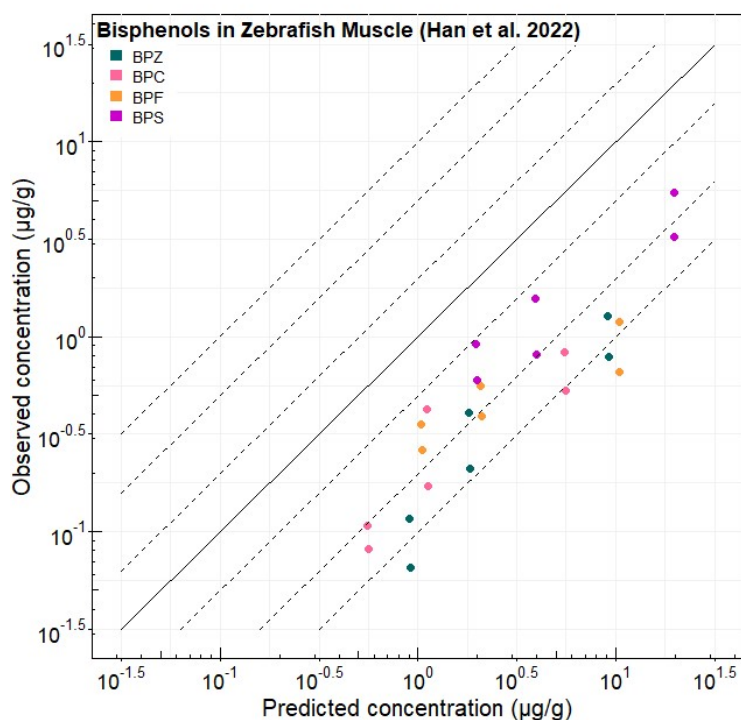


Figure 4. Observed data from Han et al. 2022 compared to predicted by developed PBTK model presented in paper II for BPZ, BPC, BPF and BPS. Solid line shows 1:1 fit while dotted lines show 2-fold, 5-fold and 10-fold errors. The observed compartment was muscle while the predicted was poorly perfused tissue.

3.4.3.4 Liver

Liver AUC and Cmax were predicted within a 2-fold error of measured data for BPA, BPAF and BPZ. Additionally, all BPAF and 92% of BPA liver concentration predictions were within a 5-fold difference from measured ones while 53% of BPZ predictions were within a 2-fold error (Figure 3). Previous model¹⁷⁹ under-predicted liver concentrations for BPA and in order to improve model performance we re-fitted the liver partitioning for BPA based on organ data by Chen et al. 2017²¹¹ This *in vivo* study was however performed in mixture, thus there is some uncertainty regarding influence of other compounds on ADME properties of BPA. Although current model over-estimated liver accumulation of BPA dosed alone as opposed to in mixture, it still constitutes an improvement compared to previous model¹⁷⁹ with lower prediction error and considering that over-predictions may lead to more protective measures following the precautionary principles.

3.4.3.5 Gonads and eggs

Gonad concentrations were predicted within a 2-fold difference for the majority of BPA and BPAF data (Figure 3) with male gonad predictions showing greater accuracy. The measured BPZ data in female gonads were generally over-predicted but still mostly within a 5-fold error. Egg concentrations were only available for TBBPA²¹⁹ and showed good performance with majority of predictions within a 2-fold error (Figure 3D). The lower accuracy for female data compared to males could be in part explained by the larger variation in female gonad volumes and composition at reproductive age depending on when in the spawning cycle they were sampled. Since female zebrafish can spawn as often as once a day, there are differences between individuals in the amount of eggs and therefore the size of this compartment which can lead to volume dilution due to the rapid growth⁷⁰.

3.4.3.6 General Performance

Previously published generic model by Grech et al.¹⁷⁹ predicted whole body concentrations within 3 to 10-fold error while liver concentrations were within 10-fold error for one study but showed much higher error for data by Chen et al. 2017 (see Paper II SI for comparison)²¹¹. Both brain and gonad concentrations were predicted at higher than 10-fold error. A recently published multi-species fish model by Mit et al. 2022²²⁸ for BPA and BPA-GA shows an improved performance to the Grech model for zebrafish with exception of whole-body prediction which showed 10 fold or higher error. Thus, our model shows better predictive performance for zebrafish than previous literature. This improved performance can in part be explained by the novel data on metabolic clearance in fish, which will likely be used for parametrization in future fish models and which was previously lacking for bisphenols. Additionally, time-course distribution to brain of zebrafish had not been previously published, thus our BPZ measurement offered both unique calibration data for brain but also validation for a less studied bisphenol. Interestingly, the accuracy of all predictions in all organs increased when only considering exposure and not depuration phase and the half-life was consistently under-predicted for all bisphenols. This indicates that better understanding and parameterization of the depuration phase would improve model predictions. Modelled depuration showed a slower decrease than that of measured data (Paper II), indicating that extra-hepatic clearance or additional elimination routes may be present. When it comes to biotransformation, gills, gonads and muscle have been previously reported to be metabolically active in fish and the inclusion of extra-hepatic biotransformation could lead to more accurate estimates of concentrations²²⁹. Furthermore, biliary excretion could also play a role in elimination of bisphenols in fish. In a survey of fishes from rivers and markets of China, Wu et al. 2016²³⁰ showed that BPA is bioaccumulated in dissected bile separate from liver. Similarly, a study by Pettersson et al. 2006²³¹ showed high concentrations of parent BPA in the bile of juvenile rainbow trout exposed to effluents containing BPA.

3.4.4 Bioconcentration of Bisphenols

BCFs at steady-state were estimated using the developed PBTK model for 10 selected bisphenols in various organs and was compared with experimental studies if available (Figure 5). The majority of whole body as well as organ-specific BCFs were predicted within a lower than 2-fold error for measured data on BPZ (Figure 5A), BPA (Figure 5B) and BPAF (Figure 5C). The only exception were muscle BCFs which were predicted within a 6-fold error (Figure 5D).

When ranking the different bisphenols in terms of BCF, predictions (Figure 5E) were generally in agreement with previously observed data in various fish species^{49,53,227,232}. A study by Han et al. 2022²²⁷ on BPZ, BPF, BPC and BPS in ZF showed muscle BCFs highest for BPZ followed by BPC, BPF and BPS thus showing the same trend as the predictions for PPT (Figure 5, D). Wang et al. 2020⁵³ observed highest accumulation of BPAF followed by BPAP, BPZ and BPC and lowest for BPS in carp exposed to a mixture of bisphenols. Similar accumulation was observed for BPC and BPZ, then lower for BPAF, BPF, BPA and lowest for BPS in lake water fish⁴⁹. Although BPAF doesn't follow this trend for our predictions, previous zebrafish studies suggest that BPAF has similar accumulation as BPA. This discrepancy for BPAF may be caused either by species differences or mixture effects since the carp study dosed using a mixture of bisphenols and wild lake fishes are exposed to mixtures of pollutants.

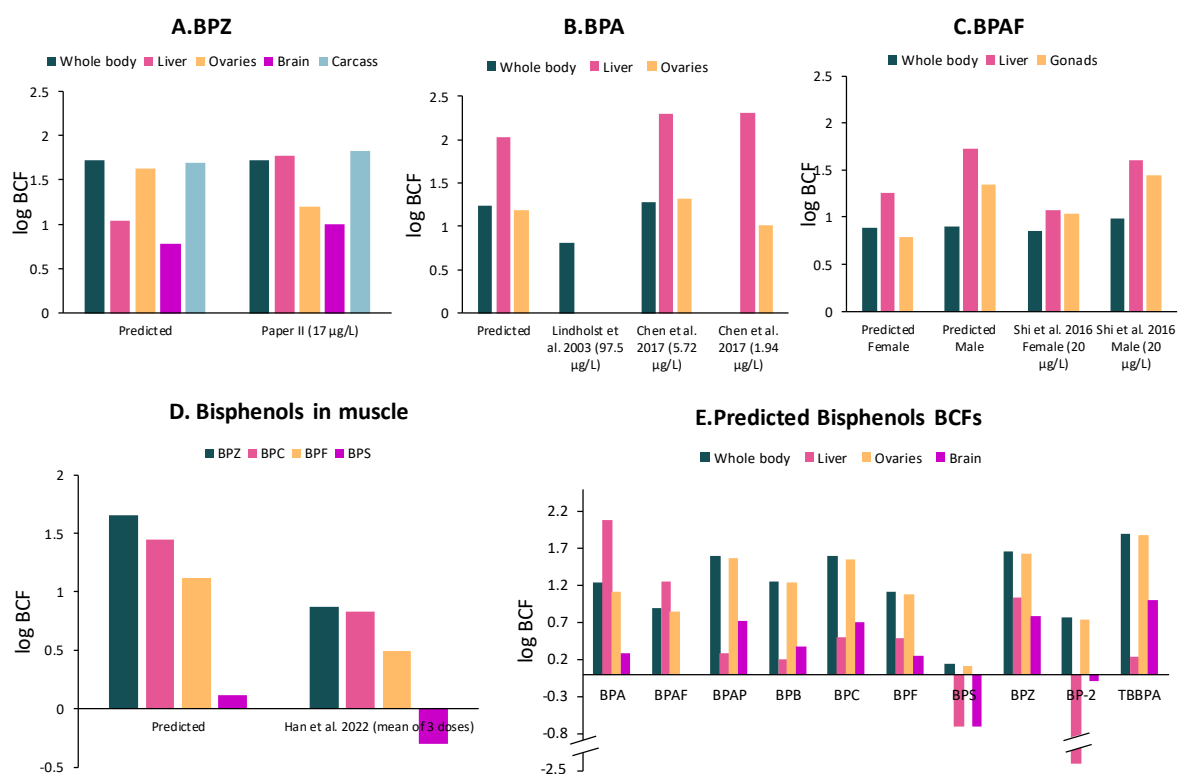


Figure 5. Predicted and observed log bioconcentration factors (BCFs) for BPZ (A), BPA (B), BPAF (C), BPZ, BPC, BPF and BPS (D) and comparison of predicted log BCFs between ten bisphenols in female zebrafish (D) organs.

4 Modelling Toxicokinetics in Developing Zebrafish Embryos

In this section we present the development of a TK model for the zebrafish embryo incorporating both literature and own experimental measurements of bisphenols in ZFE. Additionally, own *in vitro* ER activity measurements and predicted concentrations in ZFE using developed model are used in order to rank relative hazard of bisphenols. This work is presented in more detail in Paper III.

4.1 Introduction

As much toxicology research is moving away from animal methods and towards use of NAMs there are important aspects of *in vivo* research that are still difficult to capture with *in vitro* methods. The complexity of both compound specific toxicokinetics and toxicodynamics can lead to observations of adverse outcomes which are not detectable *in vitro*. However, *in vivo* tests are low throughput, ethically questionable and expensive. Therefore, the ZFE has become a widely studied test system which represents a compromise between the complexity of an organism and the practical aspects of *in vitro* testing^{233–235}. Although it is not a true replacement of an animal test, the ZFE represents a refinement of adult fish testing²³⁶. According to the European Parliament²³⁷, ZFE are not considered an animal method before free feeding which starts around 120–140 hours post fertilization (hpf) depending on temperature (T)²³⁴. The ZFE has been investigated for the purpose of environmental RA²³³ as well as to study developmental effects caused by EDCs^{238,239}, making it a suitable organism for a variety of toxicology research. Concordance between mammalian and ZFE data has been shown for effects of opioids, indicating potential for species extrapolation²⁴⁰. Additionally, high correlation has been observed between data from ZFE testing and *in vivo* fish toxicity, making it a valuable future test for reducing the use of adult fish^{235,236,241}.

Although OECD guidance has been published for acute toxicity testing in ZFE²⁴², testing other endpoints of toxicity has been performed with varying protocols. This includes varying dosing regimes, water renewal, temperature, water volume to embryo ratio as well as observing endpoints at differing times of development. This variation in methodologies has made it difficult for researchers and regulators to compare ZFE studies and has led to heterogeneous reporting of chemical hazard both in terms of differing EC₅₀ or BCF values^{56,57,188,192,243–247}. Some of these differences may be due to inter-individual variation but another more influential factor could be variation in toxicokinetics caused by differing experimental set-ups that results in differences in internal concentrations and therefore effects.

As seen in the previous section, toxicokinetics play an important role *in vivo* and can be modelled using PBTK. ADME are critical to consider for *in vitro* systems as well and can be modelled similarly with partitioning or TK models, also referred to as biokinetic models^{78,101}. Some of these models consider binding to well materials including plastics, evaporation of compound, binding to serum constituents or partitioning into cells^{99,101,248–252}. The majority of these models describe the distribution assuming instant equilibrium and employ partitioning models for each phase^{99,248,250}. This approach is suitable for compounds which reach equilibrium quickly in assays using a cell monolayer with low or no metabolic capacity but may not be suitable in other scenarios¹⁰¹. However, equilibrium may not be reached in the case of

quickly metabolized compounds or only slowly reached in the case of more complex systems such as sandwich cultures¹⁰¹. For these cases, TK models have been developed, which do not assume instant equilibrium and describe time-dependent kinetic or diffusion processes *in vitro*^{249,251,252}. Since ZFE tests are performed in wells, like cell systems, and have been shown to be metabolically competent^{243,245,253}, the approaches used for these *in vitro* TK models could therefore be applied to ZFE modelling. In this section we will refer to the ZFE model as a TK model since it doesn't describe physiology to the same extent as a PBTK model, which usually includes various organs and blood flows to them. The model still incorporates physiological parameters however, and can thus be considered physiologically-informed. Additionally, some principles from *in vitro* TK models can be applied for describing the ZFE test system, such as plastic binding or diffusion and permeability driven flow of compound into the biological system.

A ZFE TK model would help with comparison of internal concentration reached in ZFE in different studies and assess the influence of various study-conditions on this internal dose. Furthermore, such a model could provide a reliable way for future extrapolation from internal embryo concentrations to *in vivo* adult zebrafish. These potential applications have therefore led to the development of ZFE models in recent years^{254–261}. Existing models are fitted to specific compound data and thus cannot be applied for extrapolation to new compounds unless novel calibration data for these compounds is obtained. Furthermore, only limited amount of physiological data has been applied in ZFE models. The model developed in Paper III aims to incorporate important TK processes, such as epiboly, blood circulation, metabolism and surface area changes which have not previously been accounted for. Lastly, the majority of previous models^{254,256,259} employed fitting algorithms which do not account for parameter covariances. This presents an issue for TK models which contain highly correlated parameters. An exception are the models by Siméon et al. 2019 and Billat et al. 2022 that employed Bayesian approaches for parameter calibration^{255,257}. Thus, employing such an approach in the model development would yield more reliable parameter estimations.

4.2 Aims

In this study we aimed to develop a TK model for ZFE focusing on bisphenols as model compounds and to validate it on data not used for calibration. The goal with this novel ZFE model was to incorporate available physiological data on processes such as epiboly, metabolism and temperature-dependent development and employ Bayesian inference for unknown parameter calibration. Lastly, we aimed to apply the model to rank hazard of bisphenols based on predicted ZFE concentration and measured ER potency. Details on methodology can be found in Paper III.

4.3 Materials and Methods

4.3.1 ZFE Internal Concentration Measurements

Although there are measured literature data on concentrations of bisphenols in ZFE after dosing, most data constitute single time-points, thus do not provide information on the time-dependent kinetics of the rapidly developing ZFE^{56,189,243–247,262–267}. Such data is useful for validating a TK model but is not suitable for parameter calibration which would require more data points. We therefore measured concentration in ZFE dosed with either BPA, BPAF or BPZ, at five different time points during development as well as corresponding water concentrations with and without ZFE.

4.3.2 ZFE Modelling

The ZFE TK model describes kinetics between compartments of plastic, water, embryo and yolk as well as for chorion (encompassing the perivitelline space) before hatching and the schematic is shown in Figure 6. As for PBTK models in general, each compartment was assumed to be well-stirred and homogenous. Since this model represents a developing organism, volumes were modelled to change over time and rates were temperature dependent as observed for ZFE experimentally²⁶⁸. Mass flow of compound was described as driven by diffusion and limited by permeability thus including considerations of surface area and permeability rate. These equations were set up as done by Nichols et al.1996²⁶⁹ for fish skin and the flow is referred to as permeability limited in this study, but it also incorporates principles of Fick's law of diffusion within the definition.

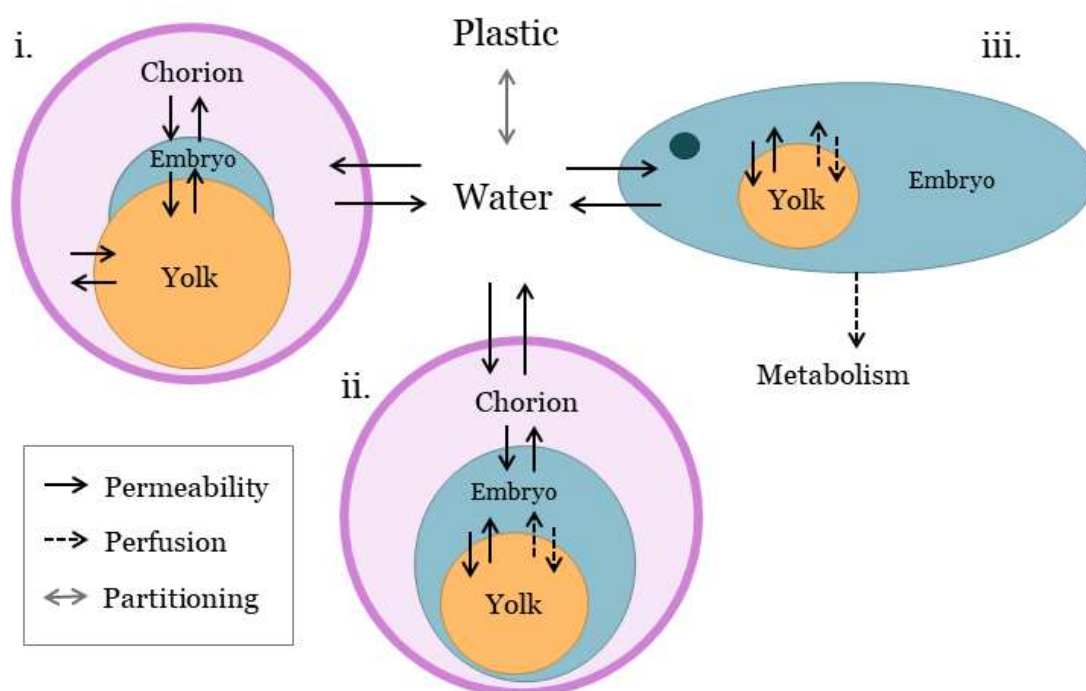


Figure 6. ZFE TK model schematic: i. Structure before 100% epiboly, where flow occurs between chorion, yolk and embryo; ii. Structure after 100% epiboly (around 10 hpf) and start of blood flow (36 hpf) but before hatching (60 hpf), where embryo has fully engulfed the yolk, thus there is no flow between chorion and yolk; iii. Structure after hatching (60 hpf) and start of metabolism (72 hpf) where chorion has been removed and there is direct flow between water and embryo.

Partitioning between water and plastic was modelled assuming instant equilibrium using equation by Kramer 2010 for water-plastic partitioning¹⁰⁰. At 0 hpf the permeability limited flow of compound was modelled to occur between chorion, embryo and yolk as well as between chorion and water. After 100% epiboly, when embryo body fully covers the surface of the yolk^{70,268,270,271}, flow of compound between chorion and yolk was removed. After 36 hpf, mass flow of compound between embryo and yolk was modelled to occur also via blood perfusion (Figure 6, ii) incorporating measured cardiac output in ZFE^{272–276} which was scaled for size as done for adult fish PBTK models previously (Paper II)^{179,185}. In this case however the blood was assumed to be part of the embryo as opposed to a separate compartment. At 72hpf, saturable metabolism was introduced as part of the embryo compartment based on rates measured for adult fish in Paper II.

4.3.2.1 *Parameterization and Model Calibration*

Physiological parameters were obtained from literature if available and set as mean values between studies including parameters such as hatching time, start of blood circulation, embryo growth rate, yolk consumption rate, surface area growth rate, epiboly rate, Arrhenius T, volumes (V) of perivitelline space, of embryo and of yolk at 0 hpf and cardiac output^{188,192,255,257,260,268,270–281}. Yolk-chorion partition coefficient was assumed to be equal to yolk-water and was calculated using a linear free energy relationship (LFER) model by Ulrich et al.²⁰²⁰ developed for ZFE yolk²⁸². Parameters with unknown values or with large inter-study differences were calibrated using Bayesian inference. This included parameters such as the permeability rates (K_p) (mm/h) across the membranes of yolk, embryo and chorion, the substrate concentration at half the maximal velocity (V_{max}) of biotransformation (K_m), the fraction of cardiac output going to yolk, the starting time of biotransformation and the partition coefficients between different compartments with exception of yolk-water and plastic-water. Only measured data for BPZ was used for parameter calibration with Bayesian inference while the rest was used for validation. In order to extrapolate between compounds, the calibrated partition coefficients were assumed to be correlated to $\log K_{ow}$ and were adjusted for this property of each bisphenol. Lastly sobol sensitivity analysis was performed to identify sensitive parameters.

Parameter fitting for TK models can be performed with algorithms for minimizing sum of squares of error, such as Nelder-Mead or Levenberg-Marquardt^{251,283} or with Bayesian inference approaches^{106,284,285}. The issue with the former option is that, unlike Bayesian, they do not consider the joint distribution of all parameters resulting from each individual parameter variability and uncertainty, thus it has been proposed that Bayesian approaches are more suitable for TK parameter estimation^{284,285}. Bayesian inference allows for estimation of parameter distributions rather than fixed values and for incorporating prior knowledge on both uncertainty and variability of each parameter^{284,285}. A Markov-Chain Monte-Carlo (MCMC) approach is used for sampling from prior distribution in order to calculate posterior distributions using a user-defined likelihood function. The likelihood describes the probability of observing the measured data given the sampled parameter values for each chain. After reaching convergence, the obtained posterior distributions of model parameters can be used to simulate a 95% credible interval (CI) for TK model predictions thus incorporating variability and uncertainty of model parameters. Although this approach shows great promise in the field of TK modelling, it is computationally demanding. Additionally, if very little measured data is available for both priors and for likelihood calculations, the method will not reach convergence. Therefore, many published TK models using Bayesian inference including the one presented

in Paper III, fix some of the known or insensitive parameters and only use Bayesian inference for calibration of unknown or sensitive parameters^{183,255,257,286}.

4.3.3 Hazard Ranking

In order to assess hazard of bisphenols in terms of estrogenicity, we measured ER activity of the eleven bisphenols presented in Section 2 with the ER-LUC assay using VM7Luc4E2 cells (previously called BG1luc4E2)²⁸⁷. The potency was presented in terms of 50% effect concentration (EC_{50}) and also in terms of free EC_{50} which represents the concentration adjusted for the unbound compound in media. Free EC_{50} was calculated using the model by Honda et al. 2019²⁸⁸ which was based on the Armitage et al. 2014²⁴⁸ model. We then calculated a ration between the predicted C_{max} in embryo body and both the nominal and free EC_{50} . We compared the ranking to literature compiled lowest observed effect concentrations (LOEC) for *vtg1* induction in ZFE.

4.4 Results and Discussion

4.4.1 ZFE Model

The measured ZFE internal concentrations followed similar trends as in literature with an increase until around 72 hpf, followed by a steady decline, thus not showing steady-state levels during the studied time window (Figure 2 in Paper III). Predictions of the newly developed ZFE model follow a similar trend and incorporates previously not considered data on biotransformation and blood circulation. The decline in observed compound concentration could be both due to volume dilution and biotransformation. Several studies have found ZFE to metabolize BP-2, BPA, BPF, BPS and TBBPA early in development^{243,245,253}. Additionally, metabolites of valproic acid and paracetamol have been measured in exposed ZFE and increased UGT expression has been observed after 72 hpf further supporting that ZFE are metabolically competent^{199,261}. The Bayesian calibration estimated a median start of metabolism at 72 hpf for BPZ, which is in line with these studies.

Blood circulation has also been observed in early ZFE with most studies suggesting blood flow and cardiac output are measurable between 24-48 hpf thus the mean of 36 hpf was used for parameterization^{273,275,276,278}. Additionally, high yolk perfusion has been reported previously, which is consistent with the Bayesian estimated median value for fraction of cardiac output supplying the yolk of 0.85.

4.4.1.1 Prediction Accuracy

The ZFE model predictions showed good performance for BPA, BPAF, BPF and TBBPA with the majority of data for BPA and TBBPA as well as all data for BPAF and BPF being predicted with a 5-fold error (Figure 7). Additionally, 41% of data for these four compounds was predicted within a 2-fold error. These predictions encompass data from 14 different studies with varying experimental conditions thus showing the model is capable to adjust for varying experimental design. Prediction error was also comparable to measured data variation. For example, two studies using the same experimental design and doses for BPA exposure measured internal ZFE concentrations differing 2-fold^{56,264} while another study²⁴³ using same dose and similar experimental design as in Paper III showed differences in ZFE BPA concentrations of 2-4 fold.

Predictions for BPS and BP-2 are however not as accurate as for the other bisphenols (Figure 7). This is likely due to the fact that the currently developed model does not account for

ionization and both of these bisphenols have a pK_a of 7.4 or below (Table 1) thus are ionized to a large extent in exposure water and cannot permeate the ZFE as easily. Future addition of ionization based on for example $\log D$ adjustment could help expand the AD of the ZFE model. Currently however, bisphenols which are to a high extent ionized in water are considered outside the AD of the ZFE model and were therefore not included in the hazard ranking presented below.

Despite over-all good performance, the model under-predicts a large portion of the data. A likely explanation for that is that biotransformation was parameterized based on adult data due to lack of measurements in embryos. However, previous studies suggest that although metabolically competent, the ZFE has lower metabolic activity than adult zebrafish which increases throughout development^{199,253}.

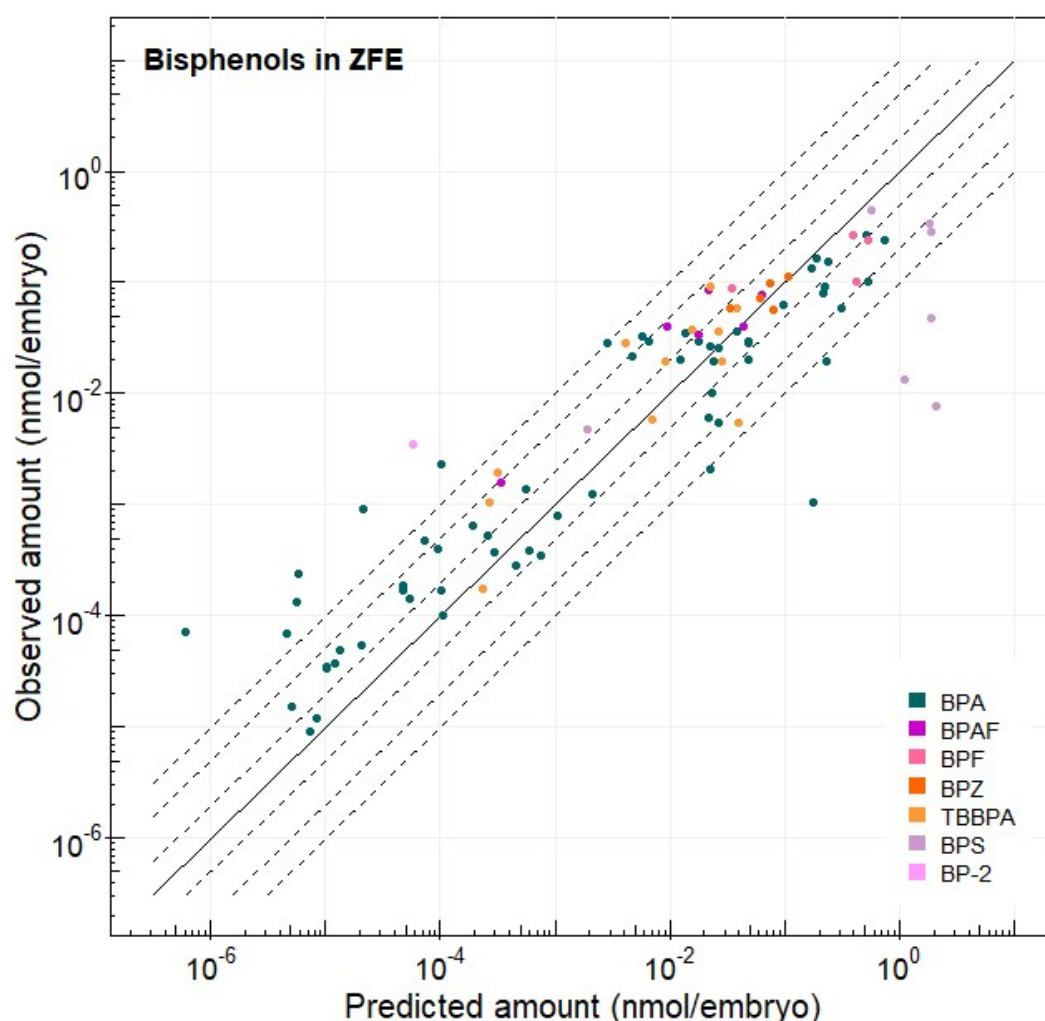


Figure 7. Observed and predicted internal concentration for BPA, BPAF, BPF, TBBPA, BPZ, BPS, and BP-2. Solid black line represents 1:1 correlation while dotted lines represent 2-fold, 5-fold and 10-fold errors. BPZ data was used for model calibration. Adapted from Paper III.


4.4.2 Hazard Ranking

As an example of model application potential, we ranked bisphenols based on a relative hazard ratio calculated using the EC_{50} for ER potency and the predicted C_{max} in ZFE. This was done so

as to consider both hazard, in terms of EC₅₀, and internal exposure, in terms of embryo body (excluding yolk or chorion) C_{max}. This notably only considers hazard in terms of estrogenicity thus other toxicities are not considered. Additionally, the C_{max} represents a worst-case scenario and does not reflect the time-dependent exposure.

There are some uncertainties with the use of VM7Luc4E2 cell assay for the EC₅₀ since it expresses human ER. Comparison with a zebrafish ER cell line such as the ones presented by Le Fol et al. 2017¹⁸⁹ would be more suitable. Nonetheless, ERs are generally conserved in vertebrates²⁸⁹. Both nominal and free EC₅₀ were used for the relative hazard ratio calculation in order to investigate whether changes in the relative ranking would be observed. Since the ZFE model did not consider free concentration, using nominal concentration may be more comparable to developed assay. However, a recent study comparing toxicity *in vitro* and in ZFE found that free EC₅₀ for toxicity is more suitable for this comparison²⁹⁰. The obtained relative hazard ratios were compared to literature data on *vtg1* induction in ZFE by bisphenols as a biomarker for ER agonism in ZFE^{192,291–293}.

Table 2. Hazard ranking of bisphenols based on measured estrogenicity (μM), calculated relative hazard ratio and lowest observed effect concentration (LOEC) for *vtg1* induction in ZFE

Most hazardous	Nominal EC ₅₀ (μM) ^a	Relative hazard ratio (C _{max} /nominal EC ₅₀)	Relative hazard ratio (C _{max} /free EC ₅₀) ^b	LOEC for <i>vtg1</i> induction in ZFE (μM) ^c
	BPAF	BPAF	BPAF	BPAF
	BPC	BPC	BPC	BPB
	BPB	BPB	BPB	BPA
	BPA	BPZ	BPZ	BPF
	BPZ	BPA	BPA	BPAP ^d
	BPF	BPF	BPF	BPC ^d
	BPAP	BPAP	BPAP	BPZ ^d
Least hazardous				

^aRanking based on EC₅₀ measured in Paper III. ^bRanking based on ratio between maximal concentration predicted in embryo body and the free concentration in cell media at EC₅₀ predicted using a model by Honda et al. 2019. ^cRanking based on mean of lowest observed effect concentration in ZFE from literature ^{192,291–293}. ^dNo LOEC data available.

Ranking based on relative hazard ratio did not differ whether nominal or free concentration were used, but they differed from the ranking based on solely nominal concentration indicating that internal exposure plays a role in hazard (Table 3). Ranking based on *vtg1* induction was similar to the one using the calculated ratios, although with some missing data. Although LOECs may not be entirely comparable to the EC₅₀, they give an indication of effect and a confirmation that some bisphenols indeed reach their biological target inside the embryo.

All of the ranking approaches indicate that BPAF and BPB may be of higher concern than BPA. The ranking was also the same when using either free EC₅₀ or the relative hazard ratio (Paper III). Although in this example application adjusting for internal ZFE amounts did not change the ranking as compared to using free EC₅₀, this may not be the case for other compound or toxicities. An example is PFASs where accumulation in ZFE explained differences in toxicity to a larger extent than intrinsic property²⁵⁴. Thus, a similar hazard ranking could be performed focusing on other toxicity endpoint than ER.

5 Discussion and Concluding Remarks

The current work presents development and use of various NAMs for prioritizing and assessing hazard of environmental pollutants. Core principles of NAM development are accuracy, transparency, and understanding of their limitations and applicability domain⁷⁹. Although these principles have been in part addressed in the papers and previous sections, these are discussed in more detail in this section.

5.1 QSPR Models

As shown in Paper I some PFASs properties are not predicted accurately by certain publicly available models, such as EpiSuiteTM, which may be due to many PFASs lying outside the AD since few or no PFASs were included in training of the models. Thus, incorporating transparent AD assessment within QSAR/QSPR modelling would allow users to better understand uncertainty in predictions. For these models a chemical AD could be calculated with any of the methods discussed in Section 2. This would evidently not improve model performance but rather transparency in presentation of model limitations, thus allowing for more informed decisions with presented results.

Additionally, chemical AD of these *in silico* models can be expanded using data from well selected chemicals spanning a larger or complementary chemical domain. That would increase the chemical variation in the training set and thus increase the models AD and reliability for that chemistry. Addition of varied, representative training sets such as those presented in Paper I, would present an efficient way to move forward. Thus, the PFASs selection presented in Paper I, provides a basis for future testing and model development aimed at expanding ADs. The selection approach presented in Paper I could also be employed on a more heterogenous group of compounds such as registered industrial chemicals, which would provide a means to expand the AD of models in a way relevant for assessing environmental pollutants.

5.2 PBTK and TK Models

Applicability domain of PBTK models has only recently been discussed in the literature^{102,104,186} with the recent OECD guidance on PBTK modelling, highlighting the need for considering model applicability²⁹⁴. There are however no established methods to assess AD for PBTK models since the assessment is complex and needs to include considerations of physiology, chemistry and experimental conditions. Generally, PBTK models presented in literature are considered as either specific or generic with the latter having a broader chemical AD but generally lower predictive accuracy than more specific ones¹⁰². Generic PBTK models have been regarded as acceptable with predictions within a 10-fold error^{102,179} while chemical-specific model predictions are considered adequate at up to 2-fold error²⁹⁴. Both models presented in Paper II and III are capable of predicting the majority of data points within a 2- to 5-fold error for adult zebrafish (Paper II) or embryo (Paper III), thus demonstrating better performance but narrower chemical applicability (bisphenols) than generic fish models^{179,185}.

5.2.1 Physiological Considerations

Physiology dependent applicability of PBTK models may consider for example gender, tissues, life-stage, and species. As seen in Paper II, models for male and female zebrafish differed in terms of structure, compartments and physiological parameters. This model showed good capability to predict data for both male and female zebrafish with similar performance. We also noted the importance of including muscle as a tissue since the predictive accuracy for PPT, as an estimate for muscle, was lower than for the other discussed tissues. Exposure studies in fish often report levels in muscle and thus such a model improvement would be feasible. Another important consideration is that data used for calibrating existing PBTK models are oftentimes obtained from juvenile or young adult animals which poses uncertainties when it comes to extrapolation to younger and older life-stages. Overall, most generic PBTK models are developed for simulating adult life-stages¹⁸⁶. As presented in Paper II and III, modelling approaches differed between adult and embryos in terms of structure but also in terms of kinetic processes despite modelling the same species and compounds. Adult zebrafish absorption were modelled via gill and food intake as these are believed to be major routes for compound intake while embryo intake was exclusively modelled via permeability and diffusion through the skin since gills are not fully developed at that life-stage. Furthermore, volume dilution is likely to play a more crucial role in a quickly growing embryo rather than in an adult, thus requiring incorporation of volume changes in the model, as done in Paper III. Performance of whole-body predictions from the ZFE model (Paper III) was comparable although on average lower than that for the adult zebrafish PBTK (Paper II). This may in part be due to the better physiological information available for parameterizing the adult model as opposed to the ZFE model. These considerations highlight that physiological applicability of PBTK models needs to be clearly defined in terms of life-stage, gender and species for an accurate use of these NAMs. There are additional important biological considerations such as tissue compositions, transporters or metabolic enzymes which are mainly important due to their interactions with chemicals and are therefore discussed under chemical considerations below.

5.2.2 Chemical Considerations

PBTK models need to consider chemical specific parameters covering those that are describing chemical interactions with the biological system. As such, chemical specific parameters in PBTK models are dependent on both the biological system and the chemistry and can include absorption, metabolism and elimination rates, tissue partitioning, active transport across membranes or permeability rates. Chemical space coverage of a large number of published PBTK models has recently been investigated and show many gaps²⁹⁵. Additionally, tissue partition coefficients, biotransformation and unbound fraction have been addressed in recent papers as the major needs of improvement for PBTK and biokinetic models^{78,102,104,178}. Notably, the parameters identified as sensitive for both adult and ZFE models (Paper II and III), included partition coefficients, biotransformation parameters and plasma fraction unbound (only considered in Paper II).

AD of tissue partition coefficient models needs to consider both chemical and biological applicability. In terms of chemical applicability, only a small number of mainly low molecular weight, neutral, and moderately hydrophobic compounds have been employed for model development suggesting a narrow applicability domain with ionizable compounds or extremely hydrophobic compounds usually not falling within domain¹⁷⁸. When considering biological applicability such as tissue or species, few tissue partition coefficient models have been trained

on fish data^{200–202} as opposed to mammalian data^{296–298}. Additionally, fish specific tissue partitioning models have been trained on fewer compounds than some mammalian ones, therefore having a narrower chemical AD. There are large uncertainties with applying mammalian models to predict fish parameters for the purpose of covering a larger chemical AD, since this application would be outside the biological AD. We therefore applied a fish specific partitioning models for Paper II, which showed good predictions for some tissues. However, partitioning to liver for BPA and BPAF as well as the brain partitioning did not perform well as well as other organs, and had to be calibrated based on *in vivo* zebrafish data. This may be due to active transport in the case of liver or the blood-brain barrier in the case of brain. However, it could also indicate that the tissue partitioning QSPR models for fish may require further improvements. Alternatively, a future study could investigate whether mammalian QSPR models could yield accurate predictions of tissue partitioning even when applied on other species such as fish. If these predictions show good performance, it would be possible to use tissue-partitioning models developed for data-rich species and apply it to those where little measured data is available. Schmitt 2008²⁹⁸ showed good predictions across a variety of mammalian species indicating that such predictions may be used across species. This indicates it could be possible in the future to develop a generic partition coefficient QSPR model with a wide AD when it comes to both chemicals but also species and tissues. Such general QSPR models could be developed by accounting for partitioning to specific types of lipids and proteins as recently presented by Endo et al. 2013²⁹⁹ and Schmitt 2008²⁹⁸. This approach however, requires information on content of specific lipids and proteins as well as fractions of interstitium and cells in tissues of each species of interest, which is not available for many species. Thus, obtaining such data for species of interest may be of great use for improving future PBTK modelling efforts. In addition, QSPR models tailored for tissue partition coefficients often only consider a small set of chemical descriptors such as $\log K_{ow}$, $\log D$ and pK_a ^{200,298}. However, properties such as MW and polar surface area have shown to influence cell permeability³⁰⁰ and thus could influence partitioning into tissues. It would therefore be of interest to develop generic QSPR models that include more chemical descriptors in addition to accounting for lipids and proteins as described above.

No QSPR model for water to embryo partition coefficient predictions has been developed previously. The method applied in Paper III for parametrization of partitioning based on Bayesian calibration and adjustment for $\log K_{ow}$ showed reasonable performance in the narrow chemical domain of bisphenols but is likely not applicable to compounds that are very different structurally. A previous ZFE model by Billat et al. 2022²⁵⁷ used a cell-partitioning model⁹⁹ for ZFE partitioning parameterization as an alternative. This application included an adjustment factor that varied across each compound and was fitted based on measured data²⁵⁷, thus not allowing for extrapolation to other compounds unlike the approach developed in Paper III. In order to expand the applicability of the ZFE TK model to support chemical extrapolation, development of a partitioning model would be required. This has been in part addressed by the recent development of a yolk-water partitioning model²⁸², which was applied in Paper III. However, partitioning to embryo body and perivitelline space components has not been investigated yet. A general partitioning model like presented for tissues, could potentially be applied to embryo. However, it would have to be adjusted to partition from water rather than plasma as commonly done for tissues. Additionally, the composition of the ZFE rapidly changes over time³⁰¹ and therefore, new composition data and predictions of partitioning would be required at small time intervals for increased accuracy.

Another crucial partitioning process which has been discussed for PBTK models is plasma protein binding¹⁷⁸. In Paper II we parameterized the unbound fraction in plasma using measured data in human plasma or *in silico* predicted data from CompTox Dashboard^{217,302}. The chemical AD of the model used for this parameter has not been provided while the biological domain only considers human plasma. In general, models predicting unbound fraction in plasma are developed on human-specific data or *in vitro* measurements using human albumin^{123,303,304} and it is therefore uncertain whether such data can be applied for fish since serum proteins differ. Zebrafish serum for example contains a large amount of apolipoproteins and no albumin unlike humans, where it constitutes almost half the plasma protein content³⁰⁵. Additionally, a third of female zebrafish total serum protein mass is made up of various VTG types which are present at much lower concentrations in male plasma suggesting that different approaches for plasma protein binding may be necessary for different genders^{220,305}. VTGs could affect distribution of compounds and may contribute to the gender-specific differences in kinetics discussed in Paper II. An investigation into plasma protein binding of other species and development of non-human or non-albumin binding models would be useful for investigating TK of chemicals for environmental applications.

When it comes to *in vitro* TK models, partitioning into cells as well as binding to serum constituents or well walls has been modelled using the concept of free fraction calculations^{101,248,288}. The free fraction of compound in *in vitro* systems has been considered to be more relevant for *in vitro* to *in vivo* effect extrapolations than using nominal concentration³⁰⁶, and was therefore applied in Paper III. Incorporating free fraction in ZFE would likely make the ZFE predictions more comparable to the calculated free fraction *in vitro*. However, free fraction in ZFE blood was not parametrized due to lack of validation data. Addition of this parameter in the ZFE model would improve the understanding of effect concentration at target since only free compound is able to bind to target receptors, such as ER.

Biotransformation is considered one of the most sensitive parameters both for PBTK and TK modelling but also in the context of assessing exposure and hazard of environmental pollutants^{104,186,197}. Although metabolic clearance is measured for a few compounds in human and rat models, it is generally not available for other species including fish. Biotransformation is especially relevant in the case of bisphenols which have been shown to be quickly metabolized^{212,218,226}. Paper II presents novel *in vitro* data of biotransformation rates of bisphenols using the RT S9 fraction bioassay. These data show lower metabolic clearance rates for fish than for humans, highlighting the need for species-relevant data when it comes to PBTK model parameterization. The applicability of these rainbow trout measurements as a parameter for zebrafish adult present uncertainties due to species differences, which could be addressed by future studies comparing metabolic rates between S9 fractions of commonly used model organisms as well as their metabolic enzyme expression profiles. Such studies could elucidate whether the rainbow trout measurements could be employed directly in models for other species or as basis for developing predictive models. Additionally, it would clarify the applicability domain of the RT S9 *in vitro* assay, potentially making this OECD guidance⁸³ more applicable for investigating TK properties in a variety of species. The clearance rates presented in Paper II were also employed in Paper III, adding additional uncertainty due to life-stage dependent processes as discussed in the paper. Thus, further quantitative investigation of differences between adult and embryos is required for more accurate parameterization. A simple way to incorporate such future measurements into the ZFE model is by using a scaling factor where embryo metabolic rates represent only a specific fraction of

the adults. However, this would not address potential differences in expression of various enzymes which could be addressed by incorporating specific enzyme rates rather than total clearance.

Accounting for ionization has been previously discussed as an important consideration for both PBTK and *in vitro* partitioning models since ionization influences the partitioning of compounds and therefore the free fraction and absorption through phospholipid membranes^{99,102,298}. The developed models in Paper II and III do not adjust for ionizable compounds such as BPS and BP-2. In the case of BPS, the adult model shows predictions within a 5-fold error while the ZFE model predicts 10-fold or higher indicating that considering ionization may be more crucial for the ZFE model. A potential approach to address this, would be to incorporate ionic fraction and only consider the non-ionized fraction available for partitioning into organisms as seen in recent literature^{99,255}. Additionally, a tissue partitioning model which accounts for ionization as done by Schmitt 2008²⁹⁸ could be employed. Such additions would expand the applicability domain of developed models to also include ionic compounds.

An important consideration for PBTK and TK model applicability domain, is the dose range for which predictions are accurate. This is especially an issue at high doses where saturation of various processes may occur. In general, few PBTK models describe saturation of elimination or absorption processes while saturation of metabolic processes has been incorporated in some models^{102,186}. Models presented in Paper II and III do not incorporate saturation with the exception of metabolism in the ZFE model. Both models also show better performance in the dose ranges of the calibration data, meaning in the case of the adult model that high doses showed a trend of overprediction while in the case of the ZFE model, low doses resulted in underpredictions. For the adult model, overpredictions at high doses may indicate that elimination and metabolism processes may have reached saturation. In the case of ZFE data, many studies employ doses above environmental concentrations including the work presented in Paper III, which could mean that some processes have already been saturated. Using such studies to calibrate TK models to extrapolate to low environmental exposure concentrations can therefore lead to underpredictions as observed in Paper III. Thus, incorporating saturation of kinetic processes in the modelling would allow for extrapolation between studies with different dosing scenarios that would expand the applicability domain of ZFE models. This could be done by obtaining data on both maximal reaction velocity (V_{max}) and the concentration of compound at which half the V_{max} is reached (K_m) for each individual process. However, this would require *in vitro* testing at multiple doses and for each individual chemical. Although data on saturable kinetics would be necessary for modelling high exposure scenarios, they may not be as relevant for parameterizing fish PBTK models developed for hazard identification of environmental pollutants as these are usually present at low doses. However, it is important to consider whether saturation may have been reached.

An additional consideration which many PBTK and TK models lack including the ones presented in this thesis, is accounting for active transport. The mass flow of compound in compartments is generally modelled via passive processes such as diffusion or partitioning. Including active transport in kinetic models requires knowledge on rates and affinity but such data are not available for many compounds and transporters with exception of some pharmaceuticals. Incorporating active transport may be more relevant for some compounds. For example, active transport of PFASs via organic anion transporters has been demonstrated to affect half-life and shown to be necessary for human PBTK modelling of this class of chemicals.³⁰⁷ In the case of bisphenols, efflux pumps such as ATP-Binding Cassette

transporters^{308,309}, may influence the degree of accumulation in different organs and could explain some of the compound-specific differences in liver accumulation observed in Paper II.

5.2.3 Experimental Considerations

Experimental or environmental conditions such as exposure routes, temperature or well-plate materials, should also be considered in the AD of PBTK and TK models. As observed in Paper II there was a large difference in model accuracy between aqueous and feed exposure while skin absorption was not considered at all, thus the PBTK model cannot be applied accurately for oral or skin absorption and requires further development to cover these processes. Another important consideration when modelling kinetic processes in fish, is the effect of temperature. Both adult and ZFE models developed in the papers, adjust for the effects of temperature on various processes such as cardiac output and various growth rates. However, more processes such as metabolism may also be affected by temperature that could potentially be accounted for in the future. Considering temperature in models aimed for investigating hazard of environmental pollutants is highly relevant in the context of climate change as well since changes in the environment may influence the ADME and thus the risk of these compounds to organisms³¹⁰. When it comes to *in vitro* systems another important aspect is the well material. Binding to polystyrene well plate plastic has been modelled previously and was included in Paper III^{100,250}. The ZFE model however does not consider other types of plastics or glass. Additionally, the plastic binding model was previously developed for polycyclic aromatic hydrocarbons presenting some uncertainty when it comes to application for bisphenols.

5.3 Conclusions and Future Perspectives

The papers discussed in this thesis present novel developments in the field of NAMs but also highlight needs for improvement in order to increase their accuracy and reliability. We explored the chemical space showing large variation among PFASs compounds and selected a representative subset for future research in Paper I. The presented methodology can be applied to other compound classes in order to select sub-groups of compounds that can be tested for expanding applicability domains of QSAR and QSPR models. Additionally, we selected an environmentally relevant sub-set of bisphenols which were then used for further NAM development in Papers II and III. In Paper II, the organ-specific distribution and bioaccumulation potential of selected bisphenols was investigated through development of an adult zebrafish PBTK model which showed improved accuracy compared to previous models. We also measured *in vitro* metabolic clearance rates for these compounds, thus providing a valuable dataset for future modelling efforts in fish and showed that these values differ from human-specific rates for most bisphenols. Model development was additionally aided by the novel data on BPZ distribution, which had previously not been studied and showed higher bioaccumulation potential than BPA. Additionally, measured BPZ data in brain suggests lower accumulation in this organ than previously predicted. The PBTK model suggested that in terms of whole-body bioaccumulation; TBBPA, BPZ, BPC, BPAP and BPB may possess more concerning properties than BPA. Lastly, we present a novel approach for modelling TK in ZFE incorporating more measured physiological parameters than previously done and showing good predictive performance. We demonstrated that the model presented in Paper III is capable of predicting a large variation of experimental conditions making it possible to compare internal concentrations rather than nominal. In terms of combined data on

accumulation potential and ER activity, BPAF, BPZ, BPC and BPB show more concerning properties than BPA which is similar to the conclusions in adults.

Most PBTK models are developed based on *in vivo* data for calibration and validation¹⁰⁴. This approach has been referred to as top-down model development. This approach has been partially employed in Paper II for BPZ since data was integrated from multiple sources, but *in vivo* testing was also performed for model validation and calibration. NAMs however aim to move away from *in vivo* methods and use exclusively *in silico* or *in vitro* data⁷⁹. Although Paper III used data from a non-animal method from a legal stand point, the ZFE test is often not viewed as a true replacement. Recent developments in the field however aim to develop PBTK models which follow the bottom-up principle where models are developed based on exclusively *in vitro* or *in silico* data¹⁰⁴. The main uncertainties identified in this thesis were related to AD, biotransformation and partitioning, which could to some extent be addressed with *in vitro* approaches to inform either the parameters directly or the development of *in silico* models aimed at predicting these properties. Thus, future developments in PBTK modelling for these parameters may not require further *in vivo* studies but rather well targeted development of *in vitro* and *in silico* methods. Although suggestion for additional *in vivo* studies have been described in this thesis, they should be aimed for the improvement and development of QSPR models for parameterization of PBTK models rather than additional *in vivo* TK investigations of specific compounds.

Lastly, development efforts on NAMs have been mainly focused on human risk⁷⁹ and thus many of these methods are mainly applicable to human hazard identification with few papers being focused on environmental hazard³¹¹. Additionally, the chemical applicability domains of many NAMs are narrow and often mainly cover drug-like compounds. Although the TK models presented in this thesis focus on a single fish species (*Danio rerio*), many of the presented principles can be used for extrapolation to other fish species given the availability of physiological parameters, making the current work of relevance for environmental toxicology research. Thus, the work presented in this thesis provides developments of new approach methodologies which bring the field a step closer to the goal of animal-free chemical safety assessments.

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