UMEÅ UNIVERSITY

Development of *in silico* methods to aid chemical risk assessment

Focusing on kinetic interactions in mixtures

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“All models are wrong, but some are useful.”

– George E. P. Box

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Abstract

The environment and biota are constantly exposed to numerous chemicals through contaminated food, soil, water, and air. These chemicals can be taken up and distributed to reach sensitive tissues where they may cause various effects. Many of these chemicals lack data on their environmental and human health effects. Traditional toxicological tests relying on animal experiments are today being phased out in favor of cell-based and computational methods for early hazard detection and exposure assessment. This thesis focuses on developing computational tools for various stages of chemical risk assessment with a particular focus on bisphenols and per- and polyfluoroalkyl substances (PFAS).

In **Paper I**, quantitative structure-activity relationship (QSAR) models covering molecular targets of the thyroid hormone (TH) system were developed and applied to two data sets to prioritize chemicals of concern for detailed toxicological studies. In **Papers II** and **III**, experimental and computational approaches were combined to study toxicokinetics and maternal transfer in zebrafish. Our main focus was to study potential mixture effects on administration, distribution, metabolism, and elimination (ADME) processes, i.e., to reveal if co-exposed chemicals impact each other's ADME. Physiologically based kinetic (PBK) mixture models were developed to allow translation of external exposure concentrations into tissue concentrations and modelling plausible mechanisms of chemical interactions in a mixture.

Main findings of this thesis are summarized as follows:

- Application of QSAR models (**Paper I)** to two chemical inventories revealed that chemicals found in human blood could induce a large

range of pathways in the TH system whereas chemicals used in Sweden with predicted high exposure index to consumers showed a lower likelihood to induce TH pathways.

- Two zebrafish experiments (**Paper II** and **Paper III)** did not reveal statistically significant mixture effects on ADME of chemicals.
- In **Paper II,** a PBK mixture model for PFAS accounting for competitive plasma protein binding was developed. The model demonstrated good predictive performance. Competitive plasma protein binding did not affect the predicted internal concentrations.
- In **Paper III** we developed a binary PBK model parametrized for two bisphenols and PFOS showing that competitive plasma protein binding has an effect on ADME of bisphenols at PFOS concentrations at μg/L levels. At these levels internal concentrations of bisphenols were shown to decrease, implying that PFOS outcompeted bisphenols from studied plasma proteins resulting in higher excretion rates.

Developed QSAR models showed good predictive power and the ability to identify and prioritize chemicals of concern with confidence. Additionally, PBK models aid in hypotheses testing and predicting exposure concentrations at which co-exposed chemicals could potentially influence each other's ADME properties. These tools will provide overall early tier data on exposure and effects using non-testing methods in assessment of risks of chemicals.

Enkel sammanfattning på svenska

Miljö, djur och människa är ständigt utsatta för många kemikalier genom förorenad mat, jord, vatten och luft. Dessa kemikalier kan tas upp och spridas för att nå känsliga vävnader där de kan orsaka olika effekter. Många av dessa kemikalier saknar data om deras miljö- och hälsoeffekter. Traditionella toxikologiska tester som förlitar sig på djurförsök fasas idag ut till förmån för cellbaserade och beräkningsmässiga metoder för tidig upptäckt av faror och exponeringsbedömning. Denna avhandling fokuserar på att utveckla beräkningsverktyg för olika stadier av kemisk riskbedömning med särskild fokus på bisfenoler och per- och polyfluoroalkylsubstanser (PFAS).

I **artikel I** utvecklades kvantitativa struktur-aktivitets relations (QSAR) modeller för molekylära mål till sköldkörtelhormonsystemet (TH) och tillämpades på två databaser för att prioritera kemikalier för mer detaljerade toxikologiska studier. I **artiklarna II** och **III** kombinerades experimentella och beräkningsmetoder för att studera toxikokinetik och tidig exponering hos zebrafiskar. Vår huvudfokus var att studera potentiella blandningseffekter på processer relaterade till administration, distribution, metabolism och eliminering (ADME) av kemikalier, dvs. att avslöja om samtidigt exponerade kemikalier påverkar varandras ADME. Fysiologiskt baserade kinetiska (PBK) blandningsmodeller utvecklades för att möjliggöra översättning av extern exponeringskoncentration till vävnadskoncentration och modellering av tänkbara mekanismer för kemiska interaktioner i en blandning. Huvudresultaten från denna avhandling sammanfattas som följer:

• Tillämpning av QSAR-modeller (**artikel I**) på två databaser visade att kemikalier som finns i mänskligt blod kan inducera en stor mängd

mekanismer i TH-systemet medan kemikalier som används i Sverige med predikterad hög exponeringsindex för konsumenter visade en lägre sannolikhet att inducera TH-systemet.

• Två zebrafiskexperiment (**artikel II** och **artikel III**) uppvisade ej statistiskt signifikanta blandningseffekter på ADME av kemikalier.

• I **artikel II** utvecklades en PBK-blandningsmodell för PFAS som tar hänsyn till effekter vid proteininbindning i blod. Modellen visade god prediktiv prestanda och effekten av kompetitiv proteininbindning påverkade inte de predikterade interna koncentrationerna.

• I **artikel III** utvecklade vi en binär PBK-modell anpassad för två bisfenoler och PFOS, vilket visade att kompetitiv proteininbindning har en effekt på ADME av bisfenoler vid PFOS-koncentrationer på μg/L-nivåer. Vid dessa nivåer minskade de interna koncentrationerna av bisfenoler, vilket antyder att PFOS utkonkurrerade bisfenoler från studerade plasmaproteiner med högre utsöndringshastigheter som följd.

Utvecklade QSAR-modeller visade god prediktiv kraft och förmåga att identifiera och prioritera potentiellt skadliga kemikalier. Dessutom kan PBK-modeller hjälpa till vid hypotesprövning och förutsäga exponeringskoncentrationer under vilka samtidigt exponerade kemikalier potentiellt kan påverka varandras ADME-egenskaper. Dessa verktyg kan generera tidig data om exponering och effekter med hjälp av icke-testmetoder vid riskbedömning av kemikalier.

List of Publications

All the following papers are provided as appendices.

I. **Dracheva, E.**; Norinder, U.; Ryden, P.; Engelhardt, J.; Weiss, J. M.; Andersson, P. L., *In Silico* Identification of Potential Thyroid Hormone System Disruptors among Chemicals in Human Serum and Chemicals with a High Exposure Index. Environ Sci Technol 2022, 56, (12), 8363-8372.

II. **Golosovskaia, E.**; Örn, S.; Ahrens, L.; Chelcea, I.; Andersson, P. L., Studying mixture effects on uptake and tissue distribution of PFAS in zebrafish (Danio rerio) using physiologically based kinetic (PBK) modelling. Sci Total Environ 2024, 168738.

III. **Golosovskaia, E.**; Örn, S.; Leonards, P.; Koekkoek, J.; Andersson, P. L., Studying interaction effects on toxicokinetics in zebrafish combining experimental and modelling approaches. *Submitted manuscript*.

Author Contributions

Paper I

The author had a major role in planning the study and was responsible for data analysis, implementation of conformal prediction method and manuscript writing.

Paper II

The author had a major role in planning and performing *in vivo* experiment, data analysis, development of physiologically based kinetic models and was responsible for writing the manuscript.

Paper III

The author had a major role in planning *in vivo* experiment, data analysis, development of physiologically based kinetic models and was responsible for writing the manuscript.

Abbreviations

AD	Applicability Domain
ADME	Administration, Distribution, Metabolism and Excretion
AhR	Aryl Hydrocarbon Receptor
AO	Adverse Outcome
AOP	Adverse Outcome Pathway
BPA	Bisphenol A
BPAF	Bisphenol AF
BPBP	Bisphenol BP
BPM	Bisphenol M
BPP	Bisphenol P
BPZ	Bisphenol Z
CA	Concentration Addition
CAR	Constitutive Androstane Receptor
CP	Conformal Prediction
CRA	Chemical Risk Assessment
DEHP	Di(2-ethylhexyl) phthalate
DEP	Diethyl phthalate
DIO1, DIO2, DIO3	Iodothyronine deiodinases 1, 2, 3
diPAP	Fluorotelomer phosphate diester
DNEL	Derived No Effect Level
ECHA	European Chemicals Agency
FOSA	Perfluorooctane sulfonamide
FPR	False Positive Rate
HBDB	Human Blood Data Base
IA	Independent Action
ICP	Inductive Conformal Prediction
IVIVE	*In vitro*-to *in vivo* extrapolation
IYD	Iodotyrosine Deiodinase
KE	Key Event
LC	Liquid Chromatography
MAF	Mixture Assessment Factor
MBP	Monobutyl phthalate
MIE	Molecular Initiating Event

MoA	Mode of Action
MRA	Mixture Risk Assessment
MS/MS	Tandem Mass Spectrometry
NAMs	New Approach Methodologies
NIS	Sodium/iodide Symporter
NOAEL	No Observed Adverse Effect Level
NOEC	No Observed Effect Concentration
PBK	Physiologically Based Kinetic
PBK-TD	Physiologically Based Kinetic – Toxicodynamic
PEC	Predicted Exposure Concentration
PFAS	Per- and Polyfluoroalkyl Substances
PFHpA	Perfluoroheptanoic acid
PFHxS	Perfluorohexane sulfonic acid
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonic acid
PNEC	Predicted No Effect Concentration
PPARD	Peroxisome Proliferator Activated Receptor delta
PPARG	Peroxisome Proliferator Activated Receptor gamma
PXR	Pregnane X Receptor
(Q)SAR	(Quantitative) Structure-Activity Relationship
RMSEP	Root Mean Squared Error of Prediction
SE-PR	Swedish Product Register
SL	Significance Level
SVHC	Substance of Very High Concern
TBBPA	Tetrabromobisphenol A
TDR	True Discovery Rate
TH	Thyroid Hormone
THDC	Thyroid Hormone system Disrupting Chemical
TPO	Thyroperoxidase
TPR	True Positive Rate
TRHR	Thyrotropin-Releasing Hormone Receptor
TRβ	Thyroid Receptor beta
TSHR	Thyroid stimulating Hormone Receptor
TTR	Transthyretin
UPLC	Ultra-Performance Liquid Chromatography

Introduction

Chemical pollution and endocrine disruption

The human population is constantly exposed to thousands of chemicals through various sources including diet, consumer products and building materials. In the environment, organisms are in constant contact with numerous environmental pollutants and their combinations through various matrices including soil, water and air. Chemical pollution has been recognized as one of the "planetary boundaries", which remain to be fully understood and quantified.[1, 2] It has recently been estimated that there are currently over 350 000 chemicals and mixtures on the market world-wide[3] and this number is constantly growing. Many of these chemicals lack data on environmental and human health effects. It is known that chemicals can produce combined toxic effects.[4-6] Lack of hazard information of chemicals and their mixtures is a problem in understanding causes of diseases, including disturbances of the endocrine system.

One of the critical endocrine related pathways is the thyroid hormone system. Thyroid hormones play a crucial role in many tissues and are involved in several processes, e.g., regulation of energy utilization, cell cycle control,[7-9] metabolism, female reproduction[10] and nervous system development[11, 12]. Thyroid hormone system disruption induced by environmental contaminants and industrial chemicals is covered in the U.S. EPA Endocrine Disruptor Screening Program (EDSP) alongside with the estrogen and androgen related systems.[13, 14]

In this thesis, the focus was mainly on two classes of chemicals: bisphenols and per- and polyfluoroalkyl substances (PFAS). Bisphenols include

chemicals of high concern due to known endocrine disruptive activity and large production volumes. PFAS are of emerging concern as they include mobile and persistent compounds and are widely used in a variety of applications.

Bisphenols

Bisphenol A (BPA) is a high volume production synthetic chemical known for its endocrine disruptive activity.[15] BPA is widely used in production of polycarbonate plastics for various applications such as plastic food containers, toys, receipts, medical devices and water pipes.[16, 17] The main source of human exposure to BPA is ingestion of BPA leak from food and beverage containers, and less amounts of BPA get into the body via inhalation or skin exposure.[18, 19] Animal studies have reported various health effects on fetus development, as well as on reproductive, immune, and nervous systems, and carcinogenicity.[20, 21] Given these health concerns, the European Chemicals Agency (ECHA) classified BPA as a substance of very high concern (SVHC) and restricted its use in thermal paper, which stimulated the search for replacements,[22] i.e., bisphenol AF (BPAF) and bisphenol Z (BPZ) among others, creating a risk of "regrettable substitution" if a substitute appears to have undesired hazardous properties.[23]

Per- and Polyfluoroalkyl Substances (PFAS)

PFAS is a large (~10.000) and broad group of organic chemicals recently redefined as substances containing at least one fully fluorinated methyl (-CF_3) or methylene carbon atom (-CF_2-) without any H/Cl/Br/I atom attached to it.[24] This new definition covers the large and diverse PFAS universe from small molecules to polymers.[25] PFAS include compounds known to be persistent and bioaccumulative and drew attention in the

early XXI century when occurrence and hazardous properties of the two "long-chain" PFAS – perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) – were discovered. Due to the useful properties such as thermal and chemical stability, PFAS are widely used in plastics, paper, textiles, electronics and consumer products.[26] Emissions of PFAS will be around 4.4 million tonnes over the next 30 years only in European Economic Area, according to ECHA estimations,[27] increasing the PFAS levels in soils, rivers, air, water and house dust. Evidence is growing concerning health effects of PFAS including disturbance of the immune system, endocrine disruptive effects, cancer and developmental toxicity.[28]

Chemical risk assessment (CRA)

Given the multitude of chemicals being released into the environment, it's crucial to estimate and, if necessary, reduce the risks posed to both humans and the environment by chemical exposure. Chemical risk assessment (CRA) is a process of estimating the likelihood of potential adverse effects in a target organism when exposed to chemicals. CRA consists of several stages: 1) hazard assessment; 2) exposure assessment; and 3) risk characterization.[29]

Hazard assessment includes hazard identification and dose-response assessment. In hazard identification, the chemical's potential to interact with biological targets and cause adverse effects is estimated. Toxicological activity can be measured *in vitro* (experiments performed in a laboratory vessel), *in vivo* (experiments on living organisms or cells) or using *in silico* (computational) methods. The dose-response assessment step estimates the relationship between the exposure level and the appearance and severity of an adverse effect. One of the purposes of the dose-response assessment is to identify reference values that are to be

used in the risk characterization step. These reference values are ratios between doses deemed to be safe, e.g., No Observed Adverse Effect Level (NOAEL), No Observed Effect Concentration (NOEC), and assessment factors. The latter are policy decisions-based values accounting for, e.g., intra-/interspecies or dose regimen differences. The obtained reference values are called, e.g., Derived No Effect Level (DNEL) in human risk assessment or Predicted No Effect Concentration (PNEC) in environmental risk assessment.

Exposure assessment aims at providing data on levels of chemicals an organism or population is exposed to including sources, duration, magnitude, and frequency. Chemical exposure can be measured via environmental monitoring by analyzing various media (e.g., water, soil, and air) or biota including humans or predicted using computational exposure models. Ideally, to relate exposure information and hazard data and obtain insights into the mode of action (MoA) of a chemical, internal concentrations, i.e., tissue levels, of chemical are needed. However, due to the lack of such data in many cases, external exposure levels are used in regulatory context.

Risk characterization integrates the previous steps and provides relative risk measures, often expressed as risk quotients. Risk quotients are ratios between exposure levels and levels deemed to be “safe” or not causing “unacceptable damage” (reference values). In human health risk assessment, a ratio between exposure and, e.g., DNEL is calculated. In environmental risk assessment, Predicted Exposure Concentration (PEC) is divided by PNEC. If these ratios are equal to or exceed one, risk is unacceptable and thus risk management decisions may be needed to reduce the risk from exposure to a chemical.

Mixture risk assessment (MRA)

Chemical exposure never occurs just to a single component; we are constantly in contact with chemical mixtures. In a mixture, chemicals can produce combined effects, which complicates risk assessment.[30] Requirement for MRA exists for intentional mixtures, e.g., pesticides, pharmaceuticals and food additives, however, for unintentional mixtures, i.e., consisting of unknown constituents, there is no such requirement.[4]

Despite acknowledged mixture exposure, risk assessment nowadays is largely based on assessing risks of individual chemicals,[4] though attempts have been made to assess mixtures using concentration addition (CA) or independent action (IA) models, which are now used as default in regulatory risk assessment of intentional mixtures.[31] The choice of an approach is based on whether chemicals have similar or different MoA. CA is applicable when mixture components have similar MoA and the total mixture toxicity is calculated as the sum of potency-corrected exposure concentrations of its constituents, whereas IA is used when compounds in a mixture have different MoAs and act at different biological targets. Under the IA approach, toxicity of mixture equals individual toxicities of its constituents.

Both CA and IA assume that chemicals in a mixture do not interact with each other.[32] Not all mixture toxicity data can be described by CA or IA models. Chemicals in a mixture can influence each other's toxicokinetics and toxicodynamics leading to mixtures having greater or lower overall toxicity than predicted by CA/IA.[31] Toxicokinetic interactions include changes in chemical's administration, distribution, metabolism and excretion (ADME) processes, e.g., modulation of uptake or excretion processes, effects on plasma protein binding or membrane transport.[31, 33, 34]

There is a tremendous need for developing tools and strategies for MRA in the scientific community as mixtures can cause a significant damage to the exposed organism even if all its constituents are at levels considered as safe from a regulatory perspective.[35, 36] The mixture assessment factor (or mixture allocation factor (MAF)) concept has been proposed to account for possible elevated mixture risks already when authorizing individual substances.[35, 37] MAF represents an acceptable contribution of a mixture constituent to a total toxicity of the mixture. It is an additional safety factor accounting for the total risk of exposure to a mixture.

Adverse Outcome Pathways (AOPs)

Adverse Outcome Pathways (AOPs)[38] are means to organize sequences of events leading to an apical adverse effect. AOPs provide a mechanistic linkage between adverse effects (AO) observed *in vivo* and a chain of events on lower organizational levels (molecular, cellular, tissue) that lead to the AO. An AOP starts with a Molecular Initiating Event (MIE) at a molecular target, then proceeds through one or several Key Events (KE) at higher organizational level to an *in vivo* observed AO measured in animal studies.[39] The AOP framework was initially proposed to be used in ecotoxicology and environmental risk assessment[38] with many examples of its application in this context,[40-44] however, the concept is used in the human risk assessment as well. AOPs do not exist as plain sequence of events, two or more AOPs can share some of their MIE, KEs or AO, and thus can be grouped into AOP networks to represent the biological complexity. Nonetheless, individual AOPs are seen as a practical unit for development and evaluation, whereas AOP networks serve as a prediction unit representing the real-world scenarios.[45]

New Approach Methodologies (NAMs)

Traditional CRA testing methods rely on *in vivo* animal testing which often involves animal suffering and sacrifice, and provides information about adverse outcome itself (apical endpoint), without giving an understanding of the underlying causes of the observation on lower organizational levels.[46] Such methods have a long history in the scientific and regulatory community and have an established confidence in derived data.[47, 48] The large and growing amount of man-made chemicals and endless amount of their combinations requires developing strategies and instruments to select priority chemicals for CRA. Toxicology of the XXI century moves towards establishing and developing New Approach Methodologies (NAMs) to inform CRA and minimize animal testing. NAMs are broadly defined as any methodology, approach, technology, or a combination of those which provides information about chemical hazard and safety assessment and does not include testing on vertebrate animals. This definition includes *in silico* approaches and *in vitro* assays.[47] NAMs intend to reduce traditional animal testing by providing the first estimations of hazard, exposure and potential effects caused by a chemical on lower organizational level. These estimations guide prioritization and risk assessment especially relevant for data-poor and new chemicals on the market.

Computational tools to aid risk assessment

(Quantitative) structure–activity relationship models ((Q)SARs)

(Quantitative) structure–activity relationship models ((Q)SARs) are mathematical models relating potency of a chemical to induce MIE to its structure and/or physico-chemical properties (descriptors). Such models

are used in the fields of drug discovery and toxicology to reveal chemicals with potential desired/unwanted potencies, thus speeding up evaluation process and prioritizing compounds for *in vitro* or *in vivo* testing. (Q)SAR models provide a fast and cost-effective approach to screen large chemical inventories and identify potential hits (drug discovery) or chemicals possessing toxic properties (toxicology) or those interacting with a particular biological target when experimental data are lacking. (Q)SARs rely on an assumption that similar structural/physico-chemical properties lead to similarities in biological activity of the compounds. QSAR models are built on quantitative descriptors while SAR models are qualitative relationships between chemical structure and activity/property. Multiple algorithms for performing this task have been developed and successfully applied, however, there is no agreement on the best performing algorithm.[49] In 2004, the organization for economic co-operation and development (OECD) reached an agreement on principles for validation of (Q)SAR models for regulatory purposes and in 2007, the guidance document on development and validation of (Q)SAR models was published.[50] The following principles were agreed upon: 1) a defined endpoint; 2) an unambiguous algorithm; 3) a defined applicability domain (AD); 4) appropriate measures of goodness-of-fit, robustness and predictivity; and 5) a mechanistic interpretation, if possible.

(Q)SAR models were previously developed for endocrine disruption for specific chemical classes[51, 52] and using large chemistry-unspecific data sets such as Tox21.[53] These models can be applied to various elements of an AOP pathway, from MIEs and KEs to apical AOs such as lethality, developmental or reproductive toxicity. In addition, such models have been developed to predict bioconcentration factors (BCFs) in organisms[54, 55] as well as parameters that can be utilized in physiologically based kinetic (PBK) models discussed in the next section. These parameters include

physico-chemical properties, e.g., octanol-water partition coefficient ($\log K_{ow}$),[56, 57] tissue:blood partition coefficients (PCs)[58-60] and ADME properties, e.g., biotransformation rates, Michaelis–Menten constant (K_m) and the maximum reaction rate (V_{max}).[61-65]

Model predictive accuracy is usually estimated with several statistical parameters. In models with quantitative response variables, root mean squared error of prediction (RMSEP) and Q^2 are generally used. Models giving qualitative outcome can be assessed with parameters such as accuracy, sensitivity (true positive rate, TPR), specificity (true negative rate, TNR), false positive rate (FPR), and precision (true discovery rate, TDR) (Figure 1).

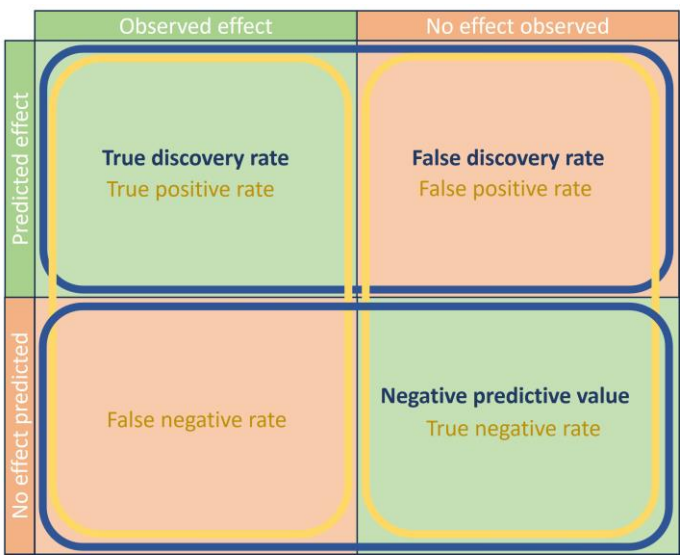

Figure 1. Common statistical measures of SAR performance used in this study. Blue shapes and text - based on predicted effect; yellow - based on observed effect.

Physiologically based kinetic (PBK) models

Physiologically based kinetic (PBK) models are a means of mathematical representation of chemical ADME processes in the body with a set of differential equations. PBK models can take on a various grade of simplification of a real species, focusing on organs of interest, represented as compartments connected via blood flow.[66] PBK models are routinely used in pharmacology for various applications, such as translation of external exposure to internal (target tissue) exposure dose and *in vitro* to *in vivo* extrapolation (IVIVE). In IVIVE, *in vitro* measured bioactivity data is translated to *in vivo* using reverse dosimetry approach by estimating the external exposure dose required to achieve levels in the target tissue equivalent to the *in vitro* measured concentration.[67, 68]

Development of a PBK model depends on selected kinetic assumption, i.e., diffusion-limited or perfusion-limited, and requires physiological parameters of species of interest (i.e., cardiac output and blood flows, tissue volumes, body weight), as well as physico-chemical parameters of modelled compound (e.g., tissue:blood partition coefficients, $\log K_{ow}$) and kinetic parameters (e.g., clearances, kinetic constants for metabolic reactions). The model's prediction accuracy depends highly on the underlying assumptions and the precision of the parameters. In the absence of experimentally determined physico-chemical parameters, PBK model can be parametrized with *in silico* predicted ones (i.e., with QSAR models). PBK models can be developed for specific individuals in a population and thus account for interindividual variability, providing a range estimation of "dose-at-target" and allowing extrapolations between populations and species.

There is a need to investigate interactions between chemicals in mixtures and account for them in predictive tools. Efforts have previously been

made to develop PBK models for mixtures from binary to complex, as reviewed previously.[31] However, applicability domain covered by developed mixture PBK models remains rather narrow in terms of chemicals, species and exposure routes, with majority of efforts put into the inhalation intake in rats exposed to mixtures of volatile organic chemcials.[31] PBK models allow not only to predict chemical kinetics in mixtures, but also to test hypotheses on how mixture compounds affect the kinetics of a specific chemical and thus elucidate the mechanism of interaction. Various ADME effects have been modelled including metabolic interactions[69, 70], direct chemical-chemical interactions (crystal formation)[71] and plasma protein binding interactions[72]. In a mixture PBK model, single chemical PBK sub-models are linked together via a compartment where their interaction is modelled, e.g., liver or blood. Development of such models requires more parameters, i.e., chemical-specific parameters for each chemical considered and parameters for modelling their suspected interaction(s).

Several platforms and coding languages are used in the scientific community to develop PBK models. Software like Simcyp, GastroPlus, MATLAB, and Berkley Madonna as well as programming languages R and Python are used for model development.[73]

Objectives of this thesis

The present work aimed at developing *in silico* tools that aid in CRA and MRA at the "hazard identification" and "exposure assessment" stages. The first goal was to develop a set of SAR models capable of identifying potential thyroid hormone system disrupting chemicals (THDCs) with confidence, immediately giving an estimation of the AD of the model (**Paper I**). We aimed at covering all thyroid hormone system MIEs which had *in vitro* data sufficient to train the models. Then, the developed panel

of models was applied to i) a data base of chemicals identified in human blood samples (Human Blood Data Base: HBDB),[74] and ii) chemicals predicted to have a high exposure index to consumers registered in the Swedish Product Register (SE-PR) in 2018[74] in order to reveal potential THDCs.

The second goal of this thesis was to give an estimation of internal exposure of selected compounds and evaluate possible kinetic interactions between them. For this, the selected model species zebrafish (*Danio rerio*) were exposed to complex mixtures of PFAS (**Paper II**) and bisphenols in combination with chemicals of different classes (**Paper III**) potentially affecting e.g., metabolism and plasma protein binding. Tissue concentrations over time data were obtained from these experiments. Mixture PBK models were then developed and validated on experimental data from **Paper II** and **Paper III**.

More specifically, **Paper II** aimed to investigate kinetic interactions between chemicals of the same group (PFAS) using modelling and experimental data. In this study, a PBK model for estimating internal concentrations of PFAS compounds exposed as a mixture was developed. Competitive plasma protein binding was considered for potential ADME mixture effects. The model can take on binary to complex chemical mixtures.

In **Paper III**, effects on chemical's ADME were studied in a complex mixture of constituents from different chemical classes at environmentally relevant concentrations. The mixture included phenols, PFAS and phthalates, and exposure concentrations were chosen considering other *in vivo* studies (e.g., BPZ, BPA and BPAF) and close to environmentally relevant levels for the rest of chemical constituents. A

binary mixture PBK model was developed to estimate at which exposure concentrations chemicals may interfere with each other's ADME.

Materials and Methods

QSARs for identification of thyroid hormone system disrupters

Following the first step in an CRA, i.e., hazard identification, data was collected from the Tox21 data base and literature data on the recently reviewed known and potential MIEs of the thyroid related AOPs[75] (**Paper I**). A powerful approach capable of using all available data and simultaneously estimating the model's applicability domain was chosen to build a panel of QSAR models with a goal to identify potential THDCs.

Data for QSAR model development

Data required to develop and validate QSAR models includes *in vitro* activity of chemicals towards certain MIEs of interest. From a total of 26 known and potential MIEs of the thyroid system AOP network presented elsewhere,[75] qualitative activity data was available in Tox21[76, 77] for eight MIEs, and data for six MIEs was obtained from scientific literature.[78, 79] This yielded 19 data sets for 14 MIEs of the TH system, with separate agonistic and antagonistic mode of action for some biological targets (Table 1). Modelled MIEs included biological targets specific to TH system as well as hepatic nuclear receptors related to metabolism of xenobiotics (called hereafter general MIEs), as their disturbance has been shown to affect the TH levels in the organism.[75, 80, 81] All collected datasets were pre-processed and made "QSAR-ready" by removing duplicated records, mixtures and structures with ambiguous activity.

The MIEs not covered by modelling in **Paper I** are those responsible for cellular TH transport, e.g., monocarboxylate transporters 8 and 10

(MCT8, MCT10), organic anion transporter polypeptides (OATPs); and some targets involved in TH synthesis, e.g., iodotyrosine deiodinase (IYD). These MIEs have limited evidence of chemical disturbances and have no high-throughput screening data available.

Table 1. Data collected for known and putative MIEs of the TH system.

MIE[1]	Mode of action	Number of active compounds (% in the set)	Source
Thyroid-specific MIEs			
TRHR	Antagonist	49 (0.07)	Tox21[2]
TSHR	Agonist	268 (4.2)	Tox21
	Antagonist	211 (3.2)	Tox21
TPO	Inhibition	292 (29.6)	[82]
NIS	Inhibition	39 (19.2)	[83]
TTR	Binding	88 (39.6)	[78]
TRβ	Agonist	34 (0.5)	Tox21
	Antagonist	319 (6)	Tox21
DIO1	Inhibition	204 (11.8)	[84]
DIO2	Inhibition	275 (16)	[84]
DIO3	Inhibition	293 (17)	[84]
General MIEs			
CAR	Agonist	863 (13.7)	Tox21
	Antagonist	144 (2.9)	Tox21
PXR	Agonist	1506 (25.1)	Tox21
AhR	Agonist	748 (11.8)	Tox21
PPARD	Agonist	86 (1.4)	Tox21
	Antagonist	63 (1.08)	Tox21
PPARG	Agonist	194 (3.1)	Tox21
	Antagonist	344 (6.1)	Tox21

[1] TRHR – Thyrotropin-releasing hormone receptor; TSHR – Thyroid stimulating hormone receptor; TPO – Thyroperoxidase; NIS – Sodium/iodide symporter; TTR – Transthyretin; TRβ – Thyroid receptor beta; DIO1, DIO2, DIO3 – Iodothyronine deiodinases 1, 2, 3; CAR – Constitutive androstane receptor; PXR – Pregnane X receptor; AhR – Aryl hydrocarbon receptor; PPARD – Peroxisome Proliferator Activated Receptor Delta; PPARG – Peroxisome proliferator activated receptor gamma

[2] Tox21 data was taken from references [76] and [77]

Balance of the model's qualitative training set has a great effect on the model performance.[85] Classification algorithms may not be able to perform well on imbalanced datasets favoring the majority (inactive) class, which results in low sensitivity for the minority (active) class.[86] This

problem has been widely recognized in the field of biological data, e.g., computer-aided early disease identification and high-throughput screening assay data.[87-90] Several techniques have been developed to minimize the effect of dataset imbalance on the model predictive ability. Such techniques include over-sampling, when entries of the minority class are sampled several times, and under-sampling, when a certain number of entries from a majority class is selected. Both techniques aim to reach a certain ratio of observations in each class. Under-sampling of the majority class may narrow down the chemical space covered by a model.[91] Conformal prediction (CP) framework is an alternative approach shown to handle severely imbalanced data sets.[92, 93]

Conformal prediction framework

Conformal prediction (CP) framework is based on a mathematical proof by Vovk et al.[94] and was designed to add a confidence measure to a prediction $\hat{y}$ of an observed label y to the traditional machine learning methods for both classification and regression tasks.[95] The confidence estimation is given with user-defined significance level (SL, or error rate, ε) denoted as $1\text{-}\varepsilon$. The CP framework can be wrapped around any method producing $\hat{y}$. This algorithm is a confidence predictor, meaning that it outputs a prediction region containing $\hat{y}$ with probability $1\text{-}\varepsilon$ instead of one label $\hat{y}$. Prediction region is an interval in regression tasks or consists of label sets in classification problems.

Defined nonconformity measure is required to derive predictive region of CP and serves to estimate how different is an example from old, already seen ones. Thus, nonconformity measure is an immediate estimation of model's AD.[96] Nonconformity scores, calculated with nonconformity measure, are used to derive so-called p-values in CP. p-values here are a fraction of old examples of nonconformity scores larger than the current

example's score, i.e., if the *p*-value is small, the example is very different from the previous ones (note that in classification, there will be several *p*-values corresponding to each class).

For binary classification task with "active" and "inactive" labels, CP's prediction region consists of four sets: {active}, {inactive}, {both} and {null} (empty). The first two sets are single-label predictions, entities put into {both} cannot be given a point prediction under the current SL, and entities in the empty set are considered outside an applicability domain of a model. To assign an entity to one of the abovementioned sets, *p*-values are calculated separately for each set and the result set label is the one where *p*-value is greater than the defined SL.

The CP framework was chosen for several reasons: 1) flexibility in the underlying machine learning method used; 2) ability to handle imbalanced datasets; 3) immediate estimation of the model's AD; and 4) possibility to set confidence level for a task.

In this work, an inductive conformal prediction setting (ICP) was used with underlying Random Forest method consisting of 300 trees (**Paper I**). In ICP, the initial training set (all data collected for a MIE) is first split into a working training set and a test set which is used for external validation. The working training set is then further split into proper training set (used for training the underlying model) and a calibration set, which is used to calculate nonconformity scores and derive *p*-values for the examples for test set entities. The final prediction was obtained by taking a median p-value from 50 ICPs (Figure 2).

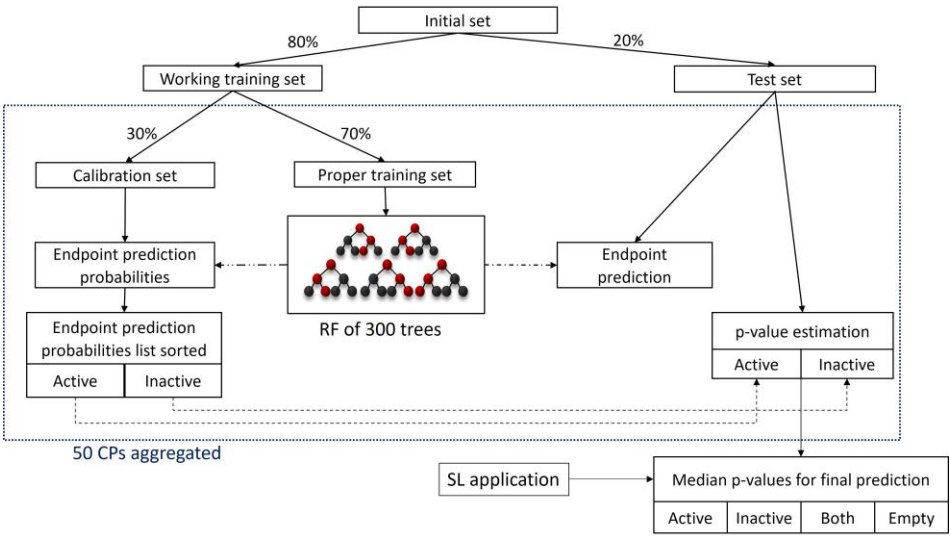

Figure 2. Conformal prediction framework used to develop SAR models in ***Paper I****.*

Model performance estimation

Performance of the 19 models developed was estimated with several commonly used statistical measures for qualitative outputs, such as TPR, FPR, TDR, in addition to statistical measures specific to CP framework, i.e., validity (error rate of predictions corresponds to the selected SL) and efficiency (rate of single label predictions). TPR and FPR were calculated only for single label predictions ({active} and {inactive} sets), while TDR was accounted for in {active} and {both} sets. Since efficiency of CP depends on the SL chosen, all models were validated with several SLs to investigate the effect of acceptable error rate on models' performance.

Toxicokinetic investigations

In vivo experiments on chemicals kinetics

Traditional estimation of internal exposure includes *in vivo* kinetic experiments, where test individuals are exposed to chemical(s) of interest

for a desired period of time (exposure period), often followed by a depuration period in environment free of studied chemicals. Tissue samples are then collected at certain timepoints during exposure and depuration periods and amounts of studied chemical are quantified. Such experiments give insights on the ADME of chemicals. Water samples taken throughout experiments account for actual water exposure concentration of chemicals of interest.

Papers II and III included *in vivo* mixture kinetics experiments with female zebrafish as a model organism with two different kinds of mixtures. In **Paper II**, a complex mixture of compounds belonging to the same class (PFAS) was investigated, whereas in **Paper III**, we considered a mixture of different compounds (bisphenols, PFAS, phenols, phthalates) (Table 2). Mixture compounds selected in **Paper III** represented chemicals potentially affecting various ADME processes, e.g., metabolism or plasma protein binding. In both **Paper II** and **Paper III**, an experimental approach was combined with PBK modelling to investigate potential mixture effects on ADME of the selected chemicals.

Table 2. Compounds selected and analyzed in toxicokinetic studies.

Study	Test compound	Acronym	Nominal concentration
Paper II	Perfluoroheptanoate	PFHpA	10 µg/L
	Perfluorooctanoate	PFOA	10 µg/L
	Perfluorononanoate	PFNA	10 µg/L
	Perfluorobutane sulfonate	PFBS	10 µg/L
	Perfluorohexane sulfonate	PFHxS	10 µg/L
	6:2 perfluorooctane sulfonate	6:2 FTSA	10 µg/L
	Perfluorooctane sulfonamide	FOSA	10 µg/L
Paper III	Bisphenol A	BPA	10 µg/L
	Bisphenol Z	PBZ	10 µg/L

	Bisphenol AF	BPAF	10 µg/L
	Pentachlorophenol	PCP	20 ng/L
	Triclosan	TCS	20 ng/L
	Tribromophenol	TBP	20 ng/L
	Heptafluorobutyric acid	PFBA	20 ng/L
	Perfluorobutane sulfonate	PFBS	20 ng/L
	Perfluorooctane sulfonate	PFOS	20 ng/L
	Diethyl phthalate*	DEP	20 ng/L
	Bis(2-ethylhexyl) phthalate*	DEHP	20 ng/L

* chemicals were not analyzed

Similar *in vivo* experiments were conducted in these two studies with female adult zebrafish (*Danio rerio*) at the aquatic laboratory of the Department of Biomedical Sciences and Veterinary Public Health in Uppsala, Sweden (Figure 3). The fish were raised under controlled husbandry at the laboratory before the start of the experiment. For each experiment (**Papers II and III**), 50 fish were held in a 40L glass aquarium. The exposure was conducted using a flow-through system where the water volume was renewed approximately 3 times per day. A stock solution with the chemical mixture was simultaneously pumped into the tank using ISMATEC 24 peristaltic pump. Fish feed used in both experiments was analyzed for presence of studied chemicals. In **Paper II**, the PFAS uptake was studied for 32 days, when most of the compounds of interest were expected to reach steady state according to previous *in vivo* experiments on these chemicals.[97-99] In **Paper III**, case chemicals (bisphenols) were shown previously to reach steady state much faster,[100-102] thus, a shorter period (12 days) for uptake was chosen, coupled with longer depuration phase of 16 days to see the elimination pattern of, e.g., PFOS, which has been shown to be bioaccumulative or have a longer half-life.[103, 104] Fish tissue samples were collected with shorter time interval in the beginning and longer ones at the end of the uptake phase in both

experiments. Four fish tissues were sampled in each study: liver, ovaries, brain and carcass (remaining body). Tissues from single fish represented a sample, and at each time point, three fish were sampled.

In addition, maternal transfer of PFAS was investigated in **Paper II**. Two groups of zebrafish were kept for reproduction: fish kept in clean water and fish exposed to the mixture of studied PFAS for 32 days. For spawning, female fish were placed in clean water without case chemicals along with an unexposed male zebrafish originating from the stock population in the lab. Fertilized eggs were then selected under stereomicroscope and embryos were raised in clean water in Petri dishes. For exposed fish, embryo samples were taken for chemical analysis immediately after spawning (day 0), and after 3- and 6-days post fertilization (dpf). Embryo samples from the control fish were taken for analysis on days 0 and 6.

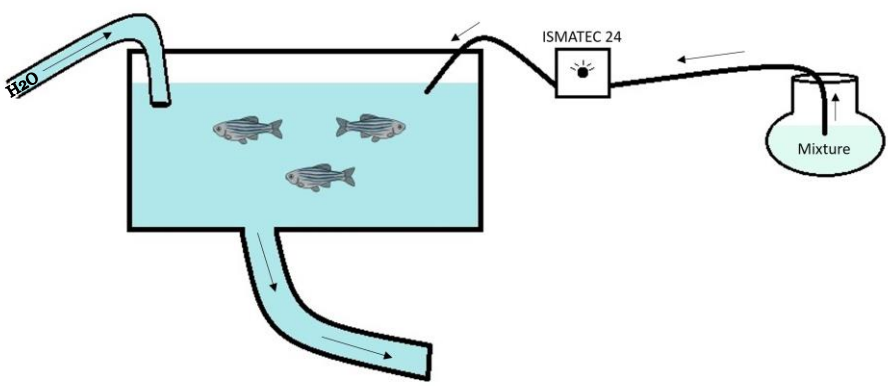

Figure 3. In vivo experimental setup

Chemical analysis

Tissue and water samples collected from zebrafish during the experiments were analyzed at SLU, Uppsala, Sweden (**Paper II**) and Vrije Universiteit Amsterdam (**Paper III**). In case of PFAS (**Paper II**), ultra-performance liquid chromatography (UPLC) coupled to a tandem mass spectrometry (MS/MS) (SCIEX Triple Quad 3500) was used for the instrumental analysis of samples. For **Paper III**, we aimed at analyzing all diverse studied chemicals consequentially with one method. For this, an analytical method able to quantify PFAS at the first step and phenolic compounds in the second step using the same extract, was developed. Liquid chromatography (LC) (ExionLC, Sciex) coupled with a Turbo V Ion source was used for derivation of PFAS compounds, and phenolic chemicals were analyzed using LC with a MS/MS. Due to methodological complications, phthalates were not possible to analyze.

PBK model development

For the prediction of time-course levels of investigated chemicals in the fish body and modelling of potential toxicokinetic effects, PBK models were developed and applied to the data collected during *in vivo* experiments (**Papers II and III**).

Model structure and parametrization

Overall, PBK models were specific to the test species (zebrafish) and gender (female). All developed PBK models had similar structure inspired from previous work by Grech et al.[105], Chelcea et al.[106] and Wang et al.[107]. Models contained 10 compartments: blood, brain, liver, gastro-intestinal tract, kidney, ovaries/eggs, richly perfused tissues, poorly perfused tissues, skin and fat (Figure 4). Physiological parameters for adult female zebrafish were taken from Grech et al.[105] for models developed both in

Paper II and **Paper III**. For chemical-specific parameters, e.g., $logK_{ow}$, tussue:blood partition coefficients, affinities to blood proteins, various information sources were used including *in vitro*/*in vivo* measured values and parameters predicted by QSAR models.[58, 59, 108-110]

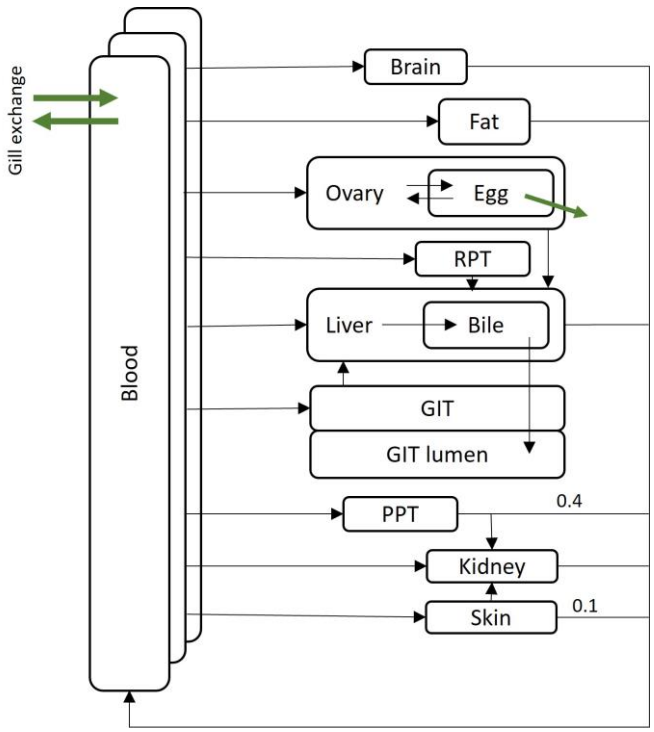

Figure 4. Mixture PBK model scheme. RPT - richly perfused tisues; GIT – gastro-intestinal tract; PPT - poorly perfused tissues

Two different approaches that estimate free fraction of chemical in blood were tested and compared. The first approach is designed for one chemical and requires data on the amount of transport proteins in blood and compound's affinities to the protein. The free fraction is then constant, and compound is assumed to be in equilibrium in blood the moment compound enters this compartment. This approach was used for development of the PFAS single compound model (PBK_{Single}) in **Paper II**. Developed mixture models took into account potential competitive

binding with transport proteins in zebrafish blood. For the model developed in **Paper II**, i.e., the PFAS mixture model (PBK_{Mix}), as well as for the binary mixture model in **Paper III**, a recently proposed algorithm of estimation binding equilibria between many chemicals was incorporated into the models.[111] The algorithm applies protein concentration in blood and compounds' protein affinities, as the first approach, and updates the amount of protein bound and freely available until equilibria is reached (Figure 5). This algorithm was run for each time-step of the PBK model. Both described methods of free fraction estimation assume 1:1 M binding to protein.

PFAS PBK_{Single} and PBK_{Mix} developed in **Paper II** considered ionization of PFAS in the environment by using D_{ow} instead of K_{ow} in estimation of gill exchange coefficients. The models were aimed to be developed by explaining ADME processes mathematically thus no model fitting was performed, and all collected data sets for model evaluation were used as external validation sets. In contrast, the binary mixture PBK model for BPZ and PFOS developed in **Paper III** was partly calibrated to better fit tissue levels of BPZ.

Model performance estimation

In both PBK studies, the developed models were evaluated by comparing their predictions with observed *in vivo* data from this study or from literature. Fold difference (FD) was calculated for each observation as follows:

$$FD = e^{\left|\ln\left(\frac{P_j}{O_j}\right)\right|} \quad (1)$$

where P_j is chemical concentration predicted by a model in a tissue j and O_j is the observed tissue concentration at a certain time point. This

approach allows assessing the quality of model's predictive power and comparison of different models. Model performance was reported in the form of percentages of predictions falling within a 2-, 3-, 5- or 10-fold error interval for each tissue with available *in vivo* data from **Paper II** and **Paper III**, i.e., brain, liver, ovaries, and carcass.

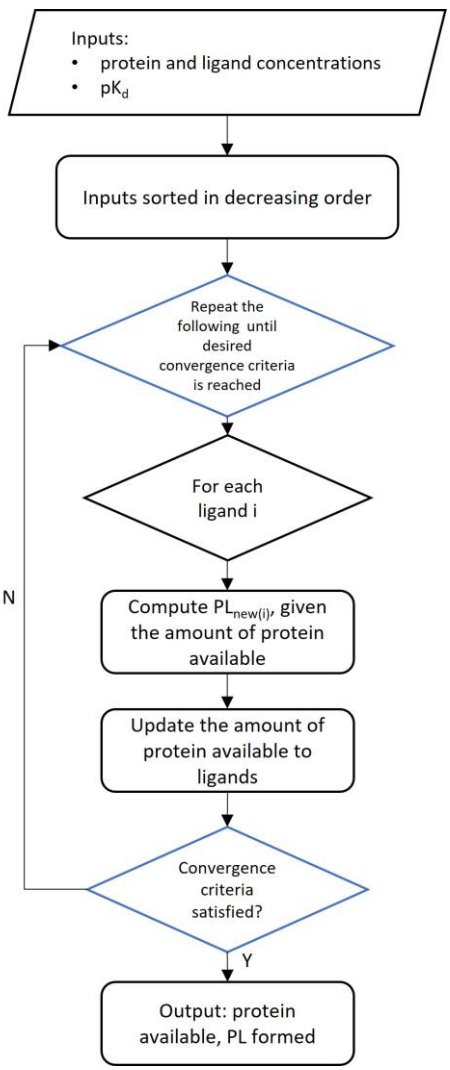

Figure 5. Algorithm used to compute competitive binding equilibria between case chemicals in the blood in PBK models. pKd – dissociation constant; PL – protein-ligand complex. Adapted from ***Paper II****.*

Results and discussion

Identification of thyroid hormone system disrupters (**Paper I**)

In **Paper I**, 19 CP models were developed for 14 biological targets, representing 9 thyroid-specific MIEs (11 models) and 5 xenobiotic transformation MIEs (8 models) to cover TH disruption AOP network at MIE level to the largest degree possible. Models for xenobiotic transformation MIEs are referred to as "general toxicity", as activation of these MIEs has an indirect impact on the regulation of the thyroid hormone system.[112, 113]

QSAR model performance

Performance of developed CP models was investigated at different SL levels. Statistic parameters specific to CP such as validity and efficiency were calculated along with the measures typically used for classification tasks, i.e., TPR, FPR and TDR.

One of the advantages of the CP framework is the ability to control the error rate of predictions when using such models for chemicals screening. The choice of SL is then based on several factors such as the purpose of the study and the desired number of retrieved actives for further testing. In the application one would like to have a high rate of single-label predictions with high TDR for the active class alongside high TPR and low FPR in a valid CP model. When selected SL is high, CP yields more singleton predictions, however, TDR may be lower than desired and more predictions are found outside the model's AD, i.e., in the empty prediction set. In lower SL setting, CPs efficiency tend to be lower, compromised by

higher TDR (Figure 2 in **Paper I**), and more compounds are given uncertain "both" label.

Models were tested at different SL from 0.01 to 0.3 and their performance were evaluated on the test set. All developed CPs were valid at SL of 0.1 or lower, i.e., at a confidence level (1-SL) and above. Thyroid-specific models were generally conservative, i.e., showed validity higher than desired confidence level (thus lower error rate than chosen) especially for the active class. CP models yielding the highest efficiency were developed on more balanced data sets, e.g., DIOs, TTR, and TPO (Figure 2A in **Paper I**). TPR was found to be higher at lower confidence level alongside more chemicals predicted to be active, thus higher FPR and lower TDR. This was noted for both thyroid-specific and general toxicity models. Generally, models built on the Tox21 data set showed lower TDR for the "active" set as compared to those based on ToxCast or literature data sets (Figure 2B in **Paper I**). In case large chemical inventories are to be screened and there is capacity to perform more conformational tests on the predicted chemicals of interests, even predictions from the "both" set can be considered for further testing, if TDR for this set is sufficiently high on the test data. TDR for both "active" and "both" sets varied across models, but this parameter was generally negatively correlated with chosen SL.

Based on the calculated performance metrics, a confidence level of 90% (SL=0.1) was chosen for application to the data sets of interest (HBDB and SE-PR), as higher TDR was preferred over greater efficiency. At the selected SL, TDR for the active class varied from more than 50% for several thyroid-specific models, e.g., DIO2, DIO3, NIS, TTR and TPO, to rates less than 10% for, e.g., TRHR, TRβ agonist and PPAR-δ.

Thyroid hormone system disrupters predicted and prioritized

The developed CP models were then applied to two relevant data bases to identify compounds potentially interfering with the thyroid hormone system. HBDB[74] consists of chemicals identified in human blood, and SE-PR is a list of compounds used and registered in Sweden with predicted high exposure index to consumers based on tonnage and usage patterns. All models were applied with SL of 0.1, and all chemicals were found to be inside the applicability domain of all models under the mentioned SL. Thus, models' output consisted of three possible prediction sets: "active", "inactive" and "both".

In HBDB, 86% of chemicals were either *in vitro* tested positive (appeared in model's training/validation set) or predicted to be active in one or more thyroid-specific models, whereas only 47% of SE-PR compounds satisfied this criterion. A similar trend was noted for the general models' predictions, where 86% of HBDB and 29% of SE-PR compounds were predicted/*in vitro* tested active in at least one model.

Compounds from both data bases tend to share activity labels towards the three DIOs (Figure 6), which was also noted in the models' training sets.84 These results were expected as DIOs are highly homologous isoenzymes sharing inhibition mechanisms.114 The TPO and TTR models predicted remarkably more compounds to be inactive in HBDB and SE-PR than other thyroid-specific models. The NIS inhibition model did not give singleton predictions to the majority of SE-PR chemicals (Figure 6), in contrast with HBDB, where 70% of compounds were predicted as potent NIS inhibitors.

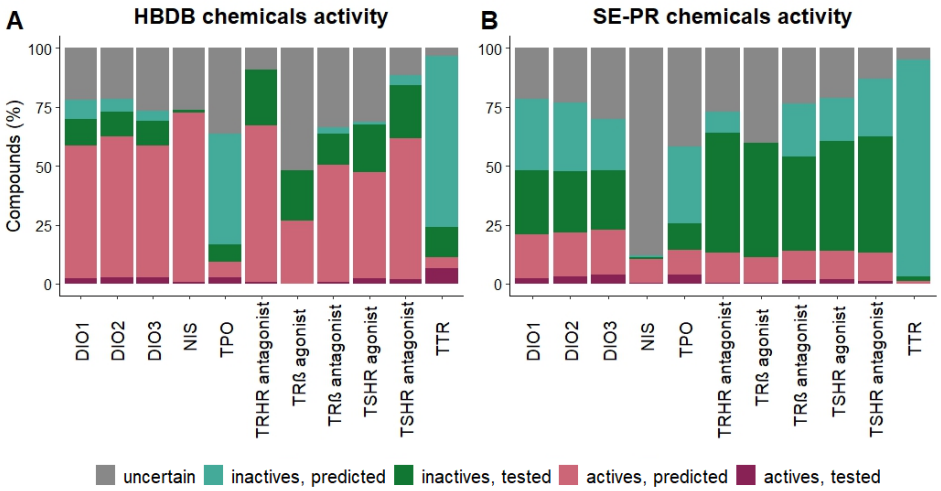

Figure 6. Estimated and experimental activity on thyroid-specific MIEs. A – Human Blood Data Base (HBDB); B – Swedish product register (SE-PR). TRHR — thyrotropin-releasing hormone receptor; TSHR — thyroid-stimulating hormone receptor; TPO — thyroperoxidase; NIS — sodium/iodide symporter; TTR — transthyretin; TRβ — thyroid receptor beta; DIO1, DIO2, DIO3 — iodothyronine deiodinases 1, 2, 3.

The developed models can aid in prioritization of chemicals potentially disrupting the thyroid hormone system. For example, compounds predicted as actives by many models can be prioritized for detailed toxicological investigations. Chemicals in the HBDB predicted as actives by 10 or 11 thyroid-specific models were hydroxylated metabolites of brominated flame retardants and chlorinated biphenyls, which are structurally similar to the thyroid hormones. Most chemicals not predicted to be active by any thyroid-specific model were non-halogenated compounds belonging to different groups, including phthalates, parabens and their metabolites. In the SE-PR data set, pigments were predicted as actives by the majority of models. Toxicity of these chemicals is largely unknown, but metabolites of azo-dyes have been shown to be carcinogens and allergens.[115]

Within the regulatory CRA, there is a need of grouping chemicals using various criteria for several purposes, e.g., filling information gaps, mixture risk assessment and avoiding regrettable substitutions.[116, 117] Identifying groups of chemicals with similar toxicological properties towards a biological system of interest was also discussed in application to MRA. To illustrate and investigate the potential of chemical grouping, model prediction profiles for thyroid related MIEs were illustrated for four chemical classes of emerging concern present in the HBDB, i.e., parabens, phthalates, bisphenols and PFAS.

The models clearly show that parabens and phthalates were mainly inactive in the thyroid hormone system pathways, while bisphenols were predicted to interfere with a range of thyroid system MIEs (Figure 7), and PFAS seem to not have a general pattern in predictions (Figure 8, Appendix A1). The models serve the purpose of filling data gaps with confidence in the presented chemical groups.

Since phthalates and parabens found in HBDB are data-rich groups and were present with a label "inactive" in the training sets of several models, the predictions were not surprising.

Bisphenols, however, is a concerning group, where toxicological information is not available for many BPA substitutes.[118] Many bisphenols from the HBDB were estimated or *in vitro* tested as active towards more thyroid-specific MIEs than BPA. BPAF and TBBPA were *in vitro* tested positive to 6 thyroid-specific MIEs (Figure 7), making them more alarming than BPA in terms of number of thyroid specific MIEs being activated. Especially concerning are predictions for BPM, BPP and BPBP, which were predicted positive towards 10 thyroid-specific MIEs (Figure 7), and at least 7 general toxicity MIEs (Figure S5 in **Paper I**).

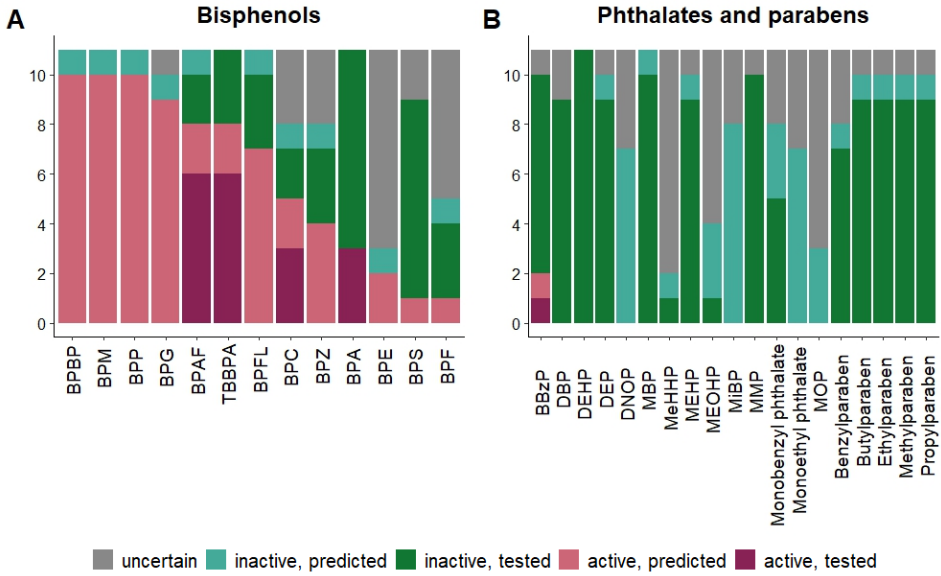

Figure 7. Number of thyroid-specific models giving specific predictions to A) bisphenols and B phthalates and parabens. BPA – bisphenol A; BPF – bisphenol F; BPS – bisphenol S; TBBPA – tetrabromobisphenol A; BPC – bisphenol C; BPE – bisphenol E; BPG – bisphenol G; BPM – bisphenol M; BPP – bisphenol P; BPZ – bisphenol Z; BPFL – bisphenol Fl; BPBP – bisphenol BP; BBzP – Benzyl butyl phthalate; DBP – Dibutyl phthalate; DEHP – Di(2-ethylhexyl) phthalate; DEP – Diethyl phthalate; DNOP – Dioctyl phthalate; MBP – Monobutyl phthalate; MeHHP – Mono-2-ethyl-5-hydroxyhexyl phthalate; MEHP – Mono(2-ethylhexyl) phthalate; MEOHP – Mono(2-ethyl5-oxohexyl)phthalate; MiBP – Isobutyl hydrogen phthalate; MMP – Monomethyl phthalate; MOP – Monooctyl phthalate. Adapted from ***Paper I****.*

41 PFAS compounds were present in HBDB, covering a large and diverse chemical space, including chemicals from short-chain to long-chain PFAS with different functional groups (see Appendix A1 for predictions for all PFAS in HBDB). "Legacy" PFAS, i.e., PFOA and PFOS, were *in vitro* tested against many MIEs in the thyroid hormone system and thus present in the models' training/validation sets (Figure 8). Model predictions reveal that fluorotelomer phosphate diesters (diPAPs), which contain two polyfluoroalkyl groups, were estimated as actives by 9-10 models, while

"legacy" PFAS trigger less TH system specific MIEs. diPAPs have drawn attention in recent years due to their production volumes and unregulated use, since they are known to transform to other PFAS.[119, 120] Recent findings report their potential to disturb the endocrine system.[121, 122] It was shown that diPAPs can interfere with the thyroid hormone system in fish, e.g., zebrafish larvae exposure to 6:2 and 8:2 diPAPs led to a decrease in both T3 and T4 levels.[123] These results suggest that more detailed toxicological investigation of diPAPs are warranted.

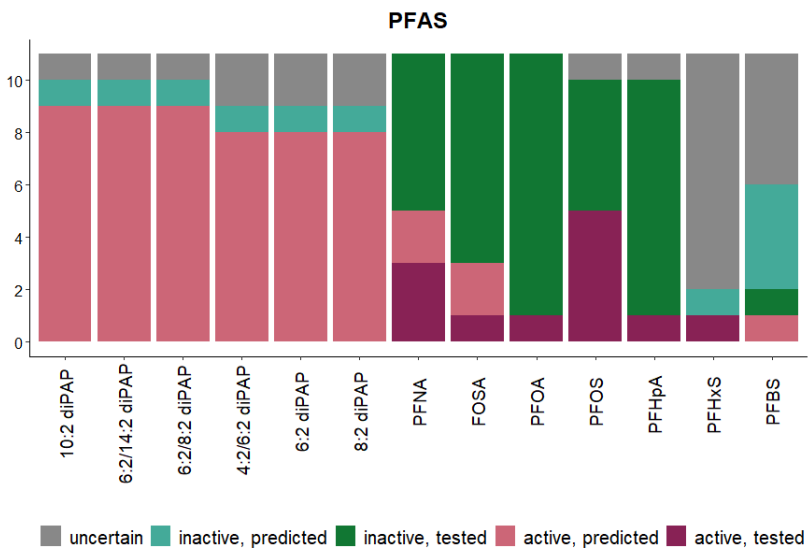

Figure 8. Number of thyroid-specific models giving specific predictions to selected PFAS. diPAP –Fluorotelomer phosphate diester; PFNA - Perfluorononanoic acid; FOSA - Perfluorooctane sulfonamide; PFOA - Perfluorooctanoic acid; PFOS - Perfluorooctane sulfonic acid; PFHpA - Perfluoroheptanoic acid; PFHxS - Perfluorohexane sulfonic acid; PFOA - Perfluorooctanoic acid.

Discussion

In accordance with the 3R (reduction, refinement, and replacement) principles of modern toxicology, the developed models serve as first-tier hazard identification tools in CRA. As reported previously, defined applicability domain is one of the principles for QSAR model development and validation according to the OECD.[50] Several methods have been discussed for estimating applicability domain of QSAR models, and consequently understand confidence of predictions,[124-127] however, there is no general agreement on the selection of applicability domain estimation method.

The idea of AD lies in the estimation of how far a new chemical is from the model's training set in the multidimensional space of chemical descriptors or from the model itself. The AD would then depend on the chosen estimation method, e.g., descriptor-based, leverage-based or distance-to-model based method. The ambiguity of these estimations lies in deciding how close the new chemical should be to consider the prediction accurate enough and how these various methods relate to each other.

The CP framework allows to control model's error rate by varying SL in accordance with the application purpose and thus immediately estimate the applicability domain of a model under applied confidence. Therefore, the CP framework serves as a flexible alternative to AD estimation with a clear concept of confidence that is derived directly from the model.[96] The downside of the CP framework lies in its specific statistical measures, i.e., validity and efficiency, used to estimate performance and error rate, which makes the comparison between it and other methods not transparent.

Selecting a lower SL and thus increasing CP model's confidence would improve the performance statistics, i.e., models will have a higher TPR

and TDR for the active class and lower FPR, but models will be less efficient, as shown in this work (Figure 2 in **Paper I**). Previous work on CP models for endocrine disruption reported negative correlation between the number of false positive and false negative predictions on one side and confidence on the other side.[128] High confidence setting would thus be preferrable in, e.g., hazard identification tasks, when one would want the number of false negatives as low as possible and when efficiency is less critical parameter.

When applying a battery of QSAR models on a data set one has to consider differences in their performance metrics and sizes of the applicability domain. Here we applied and studied models using the same confidence settings to enable comparison of performance on the two external data sets. Chemical prioritization was then based on the number of CP models estimating compound as active. This strategy, however, takes into account neither the severity of an AO nor the quantitative measure of MIE perturbation. The demonstrated prioritization approach serves as first-tier screening tool and aids in selecting chemicals (or chemical groups) of potential concern to the thyroid hormone system for further detailed investigation.

Toxicokinetic studies (**Papers II and III**)

This section discusses results from two toxicokinetic studies included in this thesis, presented in detail in **Paper II** and **Paper III**. Chemicals included in each study are listed in Table 2.

In vivo experiment results

Two *in vivo* mixture experiments were conducted: mixture of 7 PFAS (**Paper II**) and mixture of compounds from diverse groups, including

bisphenols, phenols, phthalates and PFAS (**Paper III**). Both studies were performed on adult female zebrafish exposed to chemicals via the water, and in **Paper II**, maternal transfer of PFAS was investigated. Fish feed analysis showed no significant impact on the tissue concentrations of chemicals from feed.

1. PFAS mixture (**Paper II**)

Zebrafish were exposed to seven PFAS compounds (PFHpA, PFOA, PFNA, PFBS, PFHxS, FOSA and 6:2 FTSA) for 32 days. Nominal water concentration of each PFAS was 10 μg/L, and average measured water concentrations were in the range of 7.8-11.4 μg/L for the studied chemicals. No studied compounds were detected in control water samples, while some PFAS were found in the control fish, suggesting background contamination of fish during the life-span. All PFAS were quantified in carcass and ovaries, while some compounds were not detected in brain, e.g., PFHpA, PFBS, PFHxS and 6:2 FTSA. This might be explained by the small size of brain tissue collected from one single zebrafish which may cause uncertainties in the chemical analysis. Previous studies[97, 129] which successfully measured brain PFAS concentrations of some of the abovementioned chemicals used five pooled fish as a single sample. This approach reduces the measurement error due to low chemical quantities, however, it does not provide information on interindividual variations in tissue levels.

PFOA, PFNA and FOSA were quantified in all four sampled tissues: brain, liver, carcass, and ovaries. While brain was found to accumulate the lowest amount of PFNA and PFOA among analyzed organs, the levels of FOSA in brain were among the highest of the tissues, with the maximum tissue concentration (C_{max}) exceeding 15 μg/g ww. Liver and ovaries were accumulating most of the PFAS, apart from FOSA, which showed the

opposite accumulation pattern, i.e., ovaries<liver<brain. The cause of such high brain levels of FOSA and different accumulation pattern is unclear and might be related to dissimilarity of its chemical structure to the other PFAS compounds in the study leading to different kinetics. An alternative explanation would be active transport processes of the chemicals.

BCFs of several PFAS investigated in **Paper II** were compared to the previously reported for zebrafish. Three literature data sets containing tissue-specific accumulation data were included in this comparison. For four PFAS, similar BCFs were found in the compared studies, showing that mixtures did not have significant influence on chemicals' ADME (Figure 9).

Novel data on maternal transfer of PFAS was reported. Six out of seven studied PFAS (all except PFHpA) were shown to be transferred from mother to embryo with confidence, i.e., quantified in all embryo samples immediately after spawning in clean water. The concentrations of two compounds, 6:2 FTSA and FOSA, significantly decreased in embryos over the 6 days of development, in contrast with other PFAS, which did not show any clear trends. Previous studies observed that changes in tissue composition, e.g., yolk absorption, and volume dilution lead to concentration decrease for some compounds.[130, 131] These processes might also explain drop in embryo levels for FOSA and 6:2 FTSA. Metabolism and shedding of the chorion might be other possible explanations for the phenomenon.

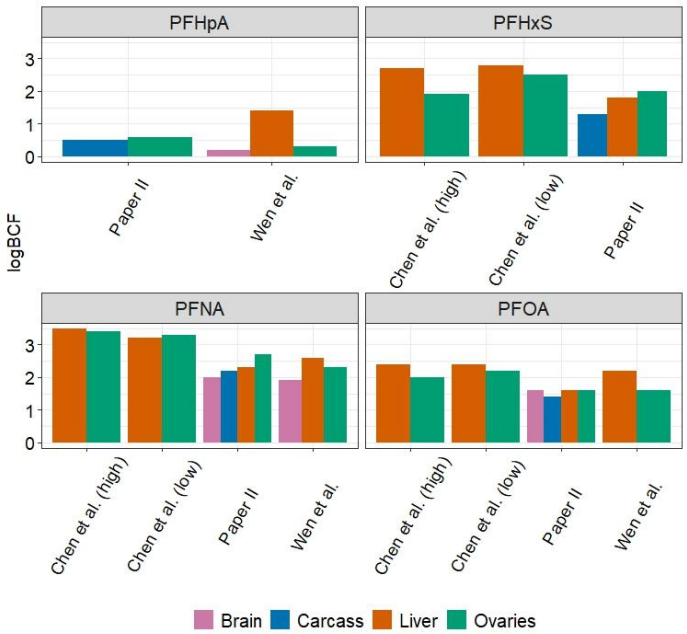

Figure 9. Bioconcentration factors for some of the studied PFAS derived from in vivo experiment in ***Paper II*** *and obtained from scientific literature.*

2. Bisphenol kinetics in a mixture (**Paper III**)

In vivo experiment was conducted with a mixture of BPA, BPAF, BPZ, TCS, TBP, PCP, DEP, DEHP, PFBA, PFBS and PFOS (Table 2). Due to high expected blank levels of the phthalates and difficulties in simultaneous chemical analysis of all chemicals, phthalates were not analyzed.

As expected, case bisphenols and phenols reached steady state in an average of 3-5 days in the analyzed tissues. Almost all studied chemicals showed highest accumulation in the liver, followed by carcass. The brain was the least accumulating organ, apart from PFBS, which showed brain levels exceeding 2000 ng/g at day 1, and TBP, which was accumulated in the brain to a higher degree than in ovaries. BPA, BPZ, BPAF, PCP and TCS eliminated rapidly from all tissues in the depuration phase. PFOS, in contrast with chemicals of other classes, was constantly accumulating

throughout the whole exposure period, and its concentrations only slightly decreased in some tissues during the depuration period (Figure 1 in **Paper III**). Such PFOS pattern was in line with some previously published data on its kinetics,[99] however, other zebrafish studies also suggest rapid elimination of PFOS to a certain degree.[98]

PFOS was the most bioaccumulative compound, with logBCF of more than 3 for all studied tissues except brain, where the accumulation potential was somewhat lower. In contrast, the short-chain PFAS, PFBS showed the highest accumulation in the brain and lowest BCF in ovaries. The three bisphenols were least bioaccumulative. The BPA replacements BPZ and BPAF were previously found to be more bioaccumulative than BPA.[100, 132] Higher bioaccumulation of BPZ was confirmed in the current experiment in carcass, ovaries and the whole body, however, BPAF showed higher BCF than BPA only in the ovaries. Whole body logBCF for TBP, PCP and TCS was in the range of 2.1-2.7, one order of magnitude higher than bisphenols.

When BCFs obtained in **Paper III** were compared to literature data, no statistically significant ($p>0.05$) differences could be seen in the bioaccumulation potential of BPZ between a mixture exposure (this study) and single exposure scenario[100] (Figure 10), which suggests no significant mixture effects on its ADME processes. In addition, two mixture studies with several exposure concentrations showed similar BPA accumulation (**Paper III** and Chen et al.[133]). Same was observed for TCS, supporting the observation of insignificant mixture effects on chemical kinetics.

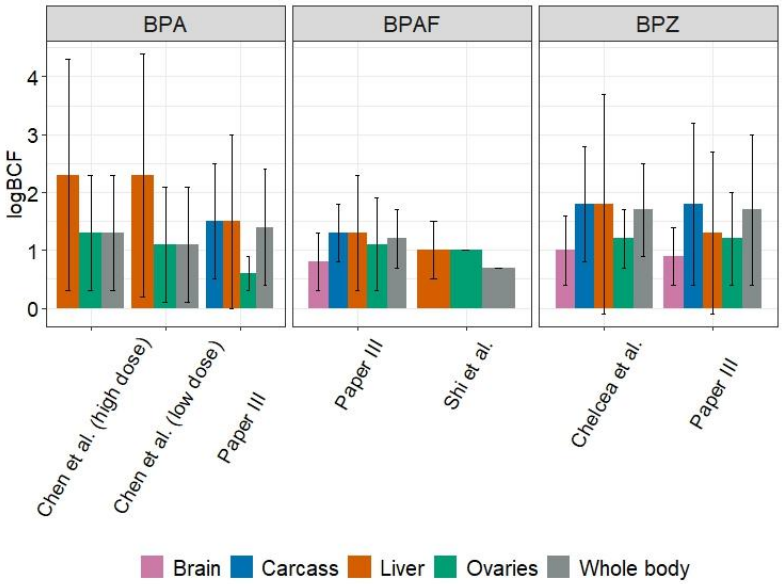

*Figure 10. Comparison of BCF values derived from different in vivo studies: Chen et al.[133] (mixture), **Paper III** (mixture), Shi et al.[101] (single chemical), and Chelcea et al.[100] (single chemical).*

Overall, our two studies on the kinetics of complex mixtures in adult female zebrafish did not reveal statistically significant differences in compounds' accumulation in four sampled tissues between single chemical and mixture exposure. This conclusion was drawn by comparing tissue bioconcentration factors (BCFs) derived from experiments in **Paper II** and **Paper III** and available literature data on some of the studied chemicals, including single chemical and mixture exposure scenarios. However, some discrepancies in derived BCF values were observed and one reason for these may be the differences in experimental approaches and chemical analysis methodologies used. For example, Chen et al.[99, 133] and Wen et al.[97, 129] analyzed 5 pooled fish as one sample, whereas Chelcea et al.[100] as well as **Paper II** and **Paper III** report data on individual fish. While using pooled samples lead to more chemical quantity in a sample and consequently less uncertain measurements, such

practice implies ignoring the individual variation. In addition, available *in vivo* data is often presented for mixtures. Chen et al.[99] reported higher organ-specific bioconcentration factors for some compounds than other studies (Figure 9, **Paper II**). They studied bioconcentration and tissue distribution of a complex PFAS mixture, including several very long-chain compounds as well as PFAS precursors which might transform to other PFAS or influence their ADME.

PBK model performance and predictions

There is currently no agreement on acceptable PBK model error of predicted tissue levels.[134] Predictions that on average fall within a 2-fold error interval are often considered adequate in regulatory context,[135] however, this has been discussed as an unreasonable criterion for, e.g., pharmaceuticals with high pharmacokinetic variability or data-poor compounds.[136] Moreover, 2-fold model errors are difficult to reach due to *in vivo* data variability, which often exceeds this range. For reported generic, compound-unspecific models, deviations up to 10-fold has been recorded.[134, 137]

1. PFAS

In **Paper II**, two PBK models were developed for predicting PFAS kinetics. PBK_{Single} was developed for single PFAS compound and PBK_{Mix}, developed for complex PFAS mixtures, accounted for competitive binding to plasma proteins. Models were tested on experimental data from the study and available literature data. The development of both models aimed at describing ADME processes with mathematical equations and *in vivo* obtained or *in silico* predicted parameters, without fitting them to the data. Thus, all data sets included in the model validation process were external.

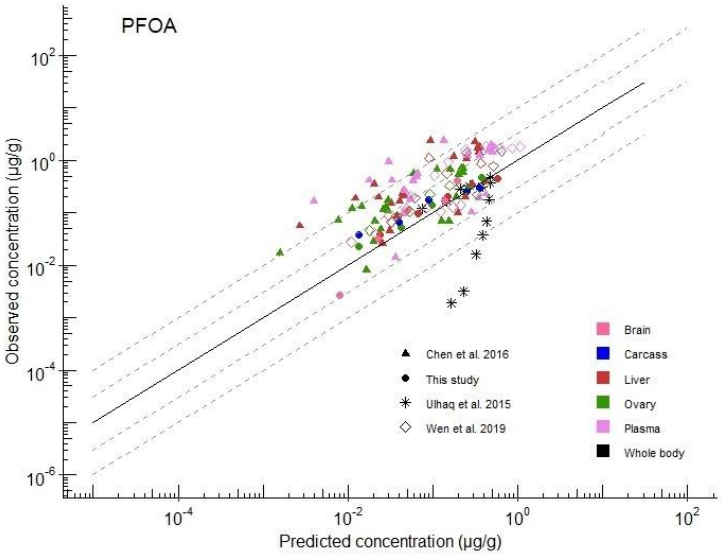

Figure 11. PBK_{Single} model performance on several validation data sets (PFOA given as example). Dotted lines represent 3- and 10-fold errors. Adapted from ***Paper II****.*

Two approaches for calculating the free fraction of chemicals in the blood gave similar results, thus leading to comparable predictive performance (Figure 11, Figure 12). The models demonstrated good performance on two mixture validation data sets on PFAS uptake (**Paper II** and Wen et al.[97]), where most data points were predicted within a 3-fold error. Expectedly less good performance was seen for the complex mixture data set by Chen et al.[99], where the models tended to underpredict the data by more than 3-folds and more than 10-folds in some cases. This is explained by the variation in *in vivo* data obtained in the Chen et al. study [99] as described in the previous section. PBK_{Single} model, applied for the PFOA data set from Ulhaq et al.[98], gave a good approximation of the uptake phase, however, the depuration process was predicted to be slower than observed (Figure 11).

The PBK_{Mix} model was tested on the *in vivo* data set obtained in **Paper II** with all seven mixture chemicals. The model predicted chemical kinetics similar to the PBK_{Single} model (Figure 12), implying no significant impact of competitive binding in blood.

65% of model predictions for the five PFAS for which PCs were obtained from *in vivo* data (PFHpA, PFOA, PFNA, PFBS, PFHxS) were within a 3-fold error. In contrast, the model predicted only 13% of data points of the data-poor chemicals (FOSA and 6:2 FTSA) within a 3-fold deviation from the observed values (Figure 12). The uncertainty of QSAR predicted PCs used for FOSA and 6:2 FTSA might explain the lower predictivity for these compounds. In addition, FOSA and 6:2 FTSA are structurally different from perfluoroalkyl carboxylates and perfluoroalkyl sulfonates, which might lead to different kinetic processes not captured by PBK_{Mix}.

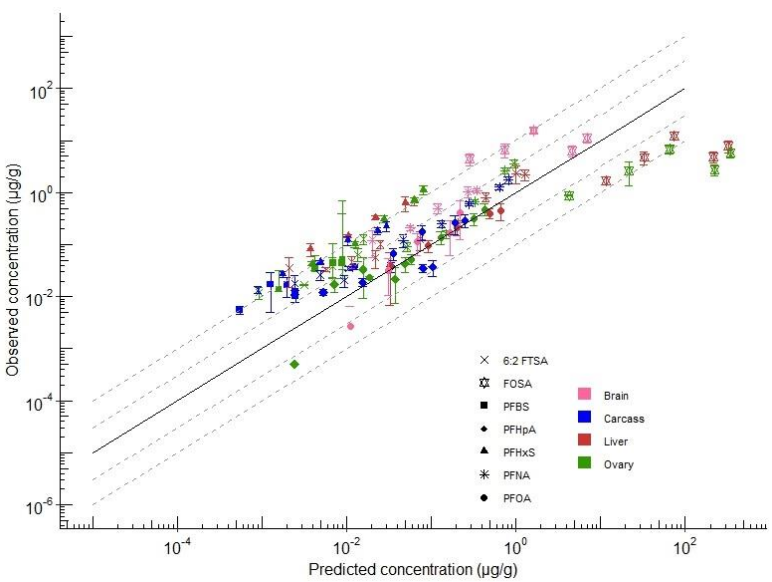

*Figure 12. Observed vs predicted plot for PBK_{Mix} model tested on in vivo data from **Paper II**. Dotted lines represent 3- and 10-fold errors. Adapted from **Paper II**.*

2. Bisphenols in complex mixture

In **Paper III**, two existing models were tested on the data set from our zebrafish experiment. One of the models was previously developed and validated for single bisphenols[100] and applied in this study to predict kinetics of the three bisphenols in the mixture. The second model was PBK_{Single} model from **Paper II** which was applied to predict PFOS kinetics. From these tested models, a new binary mixture model was developed to enable predicting kinetics of one bisphenol and PFOS simultaneously, accounting for competitive plasma protein binding. This competitive binding module was similar to what was added in the PBK_{Mix} model presented in **Paper II**.

The zebrafish PBK model for bisphenols from Chelcea et al.[100] predicted the majority of experimental data from **Paper III** (more than 70% for each bisphenol) within a 5-fold error. Overall, the model's performance statistics agreed well with those reported in the original study.

The PBK_{Single} model developed in **Paper II** showed reasonable performance in predicting kinetics of PFOS exposed to zebrafish in a mixture, with most of the liver, ovaries and carcass data within a 3-fold error. The model however showed systematic underprediction of concentrations in ovaries with 71% of values within a 10-fold error. Such systematic error implies the presence of processes in the tissue not captured by the model, e.g., active transport of PFOS into the eggs by fatty acid binding proteins alongside with fatty acids, as they are structurally similar.[138, 139]

The new binary PBK model was applied to *in vivo* data from **Paper III**, predicting kinetics of two pairs of chemicals: i) BPA and PFOS; and ii) BPZ and PFOS. The binary model predictions of bisphenol kinetics did not differ significantly from the ones given by the single chemical model

developed previously[100] (Figure 13). As expected, PFOS did not affect tissue distribution of bisphenols at applied doses representing *in vivo* experiment in **Paper III**, with exposure levels of PFOS at 12 ng/L.

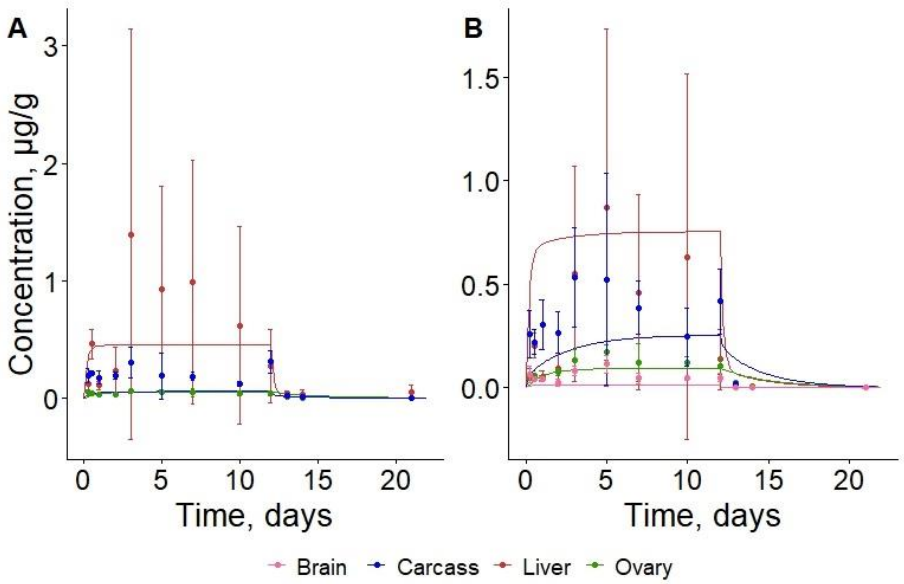

Figure 13. BPA (A) and BPZ (B) kinetics in adult female zebrafish predicted by the binary Bisphenol-PFOS model. BPA and BPZ exposure concentrations were 9 and 6.5 µg/L, respectively. PFOS exposure concentration: 12 ng/L. Adapted from ***Paper III****.*

Estimating exposure threshold for toxicokinetic interactions

A PBK simulation experiment was performed using the binary PBK model developed in **Paper III** to explore if and at which concentrations PFOS can theoretically alter the kinetics of co-exposed bisphenols. Binary mixtures of bisphenol–PFOS (BPA–PFOS and BPZ–PFOS) were simulated with varying PFOS exposure concentrations from 12 ng/L to 100 µg/L, while the bisphenol external dose was kept at the same level

(BPA – 9.0 μg/L, BPZ – 6.5 μg/L, as in zebrafish experiment in **Paper III**).

Simulations of bisphenols with 12 ng/L PFOS did not show any significant differences in bisphenol concentrations, as described above. Slightly decreased concentrations after reaching C_{max} in the liver were noted for both BPA and BPZ when co-exposure with PFOS was simulated at 1 μg/L. This decrease became larger as the PFOS exposure dose was increased to 10 μg/L, while C_{max} remained at the same level. When bisphenol kinetics were simulated with a PFOS dose at 100 μg/L, the liver C_{max} was lower than that predicted by single chemical model and by the binary PBK model in a co-exposure scenario with 12 ng/L PFOS. The other tissues demonstrated similar pattern in tissue levels, however, it was less distinct as compared with liver possibly due to generally lower accumulation of bisphenols in these tissues (Figure 14).

Observed changes in internal levels of bisphenols due to the PFOS co-exposure simulation scenarios can be explained by the mechanism implemented in the model. The free fractions of chemicals in the blood, calculated with the competitive binding equilibria algorithm, were estimated to be constant over time in simulation of bisphenols co-exposure with PFOS at ng/L level. However, when the PFOS exposure concentration was at μg/L levels, the free fractions of bisphenols were steadily increasing until nearly all molecules were unbound in the blood at around day five in the simulation. This indicates that at high exposure levels of PFOS, these molecules outcompete less strong plasma protein binders, such as bisphenols, leading to faster elimination of the latter.

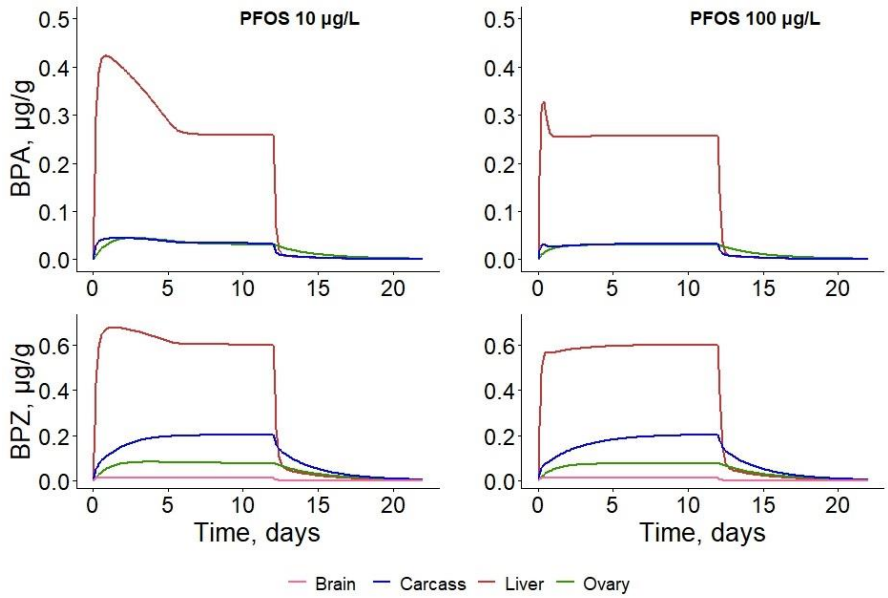

Figure 14. Predicted kinetics of BPA and BPZ with different PFOS exposure concentrations. Left – PFOS at 10 µg/L, right – PFOS at 100 µg/L. Adapted from ***Paper III****.*

Discussion

The aim of the present toxicokinetic studies was to investigate mixture effects on chemicals' ADME processes using *in vivo* and *in silico* approaches. It is generally assumed that ADME is not affected by co-exposed chemicals under environmentally relevant concentrations. Studies on possible perturbations of ADME are important for CRA as organisms are exposed to hundreds of chemicals which might act in concert, and low exposure concentrations of many chemicals could lead to altered ADME processes. In addition, laboratory studies are often performed with doses exceeding environmental levels and thus it is crucial to account for possible mixture kinetic interactions under higher exposure levels leading to over- or underestimated BCFs. In line with the higher BCFs of PFAS reported by Chen et al.,[99] PBK models developed in **Paper**

II tended to underestimate tissue levels of compounds in this data set. This is attributed to inclusion of several PFAS precursors which may transform to other PFAS in Chen et al.[99] study.

Results of applying the binary PBK model of **Paper III** to predict bisphenols kinetics with elevated PFAS levels confirmed the findings of Wen et al.[129] who hypothesized that observed *in vivo* influence of long-chain PFAS on the tissue distribution of short-chain PFAS may be due to competitive protein binding in the blood. In contrast with observation in the Wen et al.[129] study, the *in vivo* PFAS experiment and the PBK_{Mix} model (**Paper II**) suggested no significant influence of mixture on the kinetics of its constituents. The reasons for these disagreements between observations are unclear, but may be attributed to exposure concentration differences. Wen et al.[129] co-exposed several short-chain PFAS with a total concentration of six long-chain PFAS of 60 µg/L. That suggests that higher exposure concentration than 10 µg/L and chemicals acting together could lead to the observed effect.

When applying PBK models for dose-at-target predictions, their applicability domain needs to be considered, as suggested in the recent OECD guidance for PBK model development.[140] PBK models are of complex nature, and there is currently no established method to assess their AD. However, the OECD[140] suggests several considerations, including model structure and parametrization, uncertainty and sensitivity analysis, and models predictive capacity (for the last aspect, *in vivo* validation data is required). Several recent publications discuss the need of better defining the AD of PBK models.[137, 141-143] PBK models developed in this thesis represent narrow AD when it comes to physiology as models were parametrized for adult female zebrafish. The chemical domain of the models is wider and covers chemical groups, i.e., PFAS and

bisphenols. When applying a model to new chemicals (even from the same chemical class) one, however, has to consider that prediction performance might deviate from that reported dependent on variations in chemical structure and properties.

In the PBK models for PFAS developed in **Paper II**, chemicals were assumed to not undergo metabolism in the fish body. This assumption is based on the persistency of PFAS chemicals[144] and lack of evidence of their transformations *in vivo*.[59, 145] There is a growing interest in research of transformation reactions and accumulation of PFAS precursors since they appear at low levels in the environment but still have a high bioaccumulation potential.[145, 146] As shown in this thesis, PBK_{Mix} model demonstrated larger deviations in tissue level predictions for 6:2 FTSA and FOSA, which could be attributed to several reasons, including the possibility that these chemicals may behave differently than other studied PFAS and for example undergo metabolism.[145, 147, 148] This means that the proposed model might need to be updated to account for metabolism for better predictions for FOSA and 6:2 FTSA. This might also be true for other PFAS chemicals for which assumption of no metabolism may not be valid. In addition, the route of administration has to be considered. In **Paper II** and **Paper III**, only water exposure was considered in the models, i.e., chemicals entering fish via the gills. The models can be parametrized for other exposure routes, e.g., oral or intravenous, like some previously developed generic fish models.[105] However, to tailor PBK models from **Paper II** and **Paper III** for other exposure routes than the present they need to be validated on available *in vivo* data measured after exposure via the route of interest.

Uncertainties in model parametrization have been described in detail in **Paper II** and **Paper III** and are revisited here shortly. One of the

essential parameters for PBK model development is the octanol-water partition coefficient (K_{ow}), however, in case of ionizable chemicals such as several PFAS, this parameter is less relevant. PFAS from **Paper II** and PFAS sub-model from **Paper III** accounted for ionization by introducing the pH adjusted octanol-water distribution ratio (D_{ow}) in the gill exchange equations, however, this parameter still uses K_{ow} as an input. K_{ow} is very difficult to measure for PFAS, and QSAR predictions for it show notable discrepancies,[149] adding uncertainty to the model parametrization, especially given that K_{ow} was shown to be one of the most sensitive parameters in **Paper II** models (Figure S17 in **Paper II**).

Another source of uncertainty lies in the parametrization of blood protein binding process in the models (**Paper II**, **Paper III**). In the **Paper II** models, PFAS binding affinities to human serum albumin were used as an approximation to parametrize the plasma protein binding[110] and in **Paper III**, the binary PBK model was parametrized with affinities of bisphenols[108] and PFOS[109] to bovine serum albumin. Unlike human plasma, where albumin makes up more than half of serum proteins concentration, zebrafish lack albumin genes. This makes it unclear which protein(s) are main carriers of chemicals in blood. Moreover, concentrations of plasma constituents in zebrafish blood depend on feeding and gender.[150-152] In both **Paper II** and **Paper III**, total protein content was used for estimating the unbound fraction of chemicals, though it is likely that only a certain fraction of plasma proteins is able to transport studied chemicals (Table 2). Detailed characterization of zebrafish plasma proteins and measurements of chemicals' affinities to them would provide a basis of improving the parametrization of current models and reduce uncertainty.

According to OECD guidance,[140] *in vivo* data is used in **Paper II** and **Paper III** for the validation of PBK models. Further application of these models aids in significant reduction of animal testing, when various aspects of models' applicability domain are carefully considered. Development of PBK models, which do not require *in vivo* data for validation (New Generation PBK models), have been recently discussed in human risk assessment.[66] Alternative PBK model performance evaluations such as conservative scenario approach can be applicable for data-poor chemicals in environmental risk assessment.

Summary and future perspectives

The research presented in this thesis introduces novel developments of *in silico* NAMs for hazard identification and exposure assessment of chemicals including impact of mixtures.

In **Paper I**, a panel of 19 SAR models was developed using the CP framework method for a fast first-tier identification of potential thyroid hormone system disrupting chemicals. Here we propose a chemical prioritization strategy where the number of models predicting a compound as active plays a role, assuming that a larger number of models indicating activity, the higher the potential concern is. Application of these models with suggested prioritization strategy to HBDB and SE-PR inventories revealed that chemicals found in human blood pose greater risks of being thyroid hormone system disrupters. These potentially hazardous chemicals merit further toxicological investigation.

In **Papers II and III**, we investigated how chemicals' ADME processes and bioaccumulation may be affected in complex mixtures. Both studies used a combination of *in vivo* and *in silico* methods to elucidate possible mixture effects on chemical kinetics. **Paper II** focused on a PFAS mixture and in **Paper III,** we investigated a mixture of chemicals from different classes, including bisphenols and PFAS. Mixture chemicals in **Paper III** were selected to represent those can perturb various ADME processes, e.g., metabolism and plasma protein binding.

Main results of these ADME studies can be summarized as follows:

1. *In vivo* studies.

Novel *in vivo* data showed that FOSA was highly bioaccumulative in the brain, in comparison with other studied PFAS. The study highlights concerns regarding maternal transfer of PFAS due to ovaries were among the most accumulative organs of the majority of investigated PFAS. Furthermore, all compounds were detected in eggs and larvae after spawning.

The zebrafish mixture experiment conducted in **Paper II** showed no significant mixture effects on ADME of the studied PFAS. To conclude this, previously reported BCF values of the chemicals were compared with values obtained from this experiment.

In **Paper III**, we compared the BCF values for bisphenols derived from mixture exposure experiment with those reported previously for single chemical exposure. This comparison neither revealed statistically significant differences in chemicals bioaccumulation. This suggests that the investigated mixture did not impact the ADME of the chemicals at environmentally relevant concentrations.

2. PBK modelling

In **Paper II**, a PBK model for a complex mixture of PFAS (PBK_{Mix}) was developed. One potential mixture ADME effect was implemented in the model: competitive plasma protein binding. The PBK_{Mix} model demonstrated good predictive capacity on mixture data sets and suggested no significant influence of competitive plasma protein binding on bioconcentration of studied PFAS. In **Paper III**, the focus shifted towards a complex mixture of chemicals belonging to different chemical classes that may interfere with ADME processes causing impact on kinetics of individual chemicals. Here, effects of chemicals at environmentally relevant concentrations on kinetics of three bisphenols

were investigated. A binary bisphenol–PFOS PBK model was developed and validated on *in vivo* data presented in **Paper III**. Application of this model in a simulation experiment showed that competitive binding to plasma proteins between PFOS and bisphenols can lead to decrease in tissue concentrations of the latter chemicals at higher exposure level of PFOS.

Future perspectives. Further development of SAR models presented in this thesis has two possible directions: 1) existing models can be improved to show better performance metrics, e.g., using additional data sets; 2) models can be complemented with new ones for thyroid hormone system MIEs not covered in **Paper I** when *in vitro* data becomes available. The CP framework with its advantages of inbuilt error rate control and applicability domain estimation could be used for development of first-tier screening models for other endocrine systems.

Several developments can be foreseen with mixture PBK models presented in this thesis. First, the model's uncertainty can be reduced by using zebrafish-specific plasma protein parameters instead of mammalian when such data is available. Second, PBK_{Mix} model can be updated to incorporate other potential ADME interactions between chemicals, e.g., inhibition or induction of metabolism. Such developments would require additional parametrization, including V_{max} and K_m, as well as inhibitory constants for each pair of chemicals in a mixture. The competitive binding equilibria algorithm implemented in **Paper II** and **Paper III** can also be applied to metabolic interactions for compounds metabolized by the same protein. Third, in this work, zebrafish were used as a model organism, which limits the models' applicability in environmental risk assessment, where many species are considered. However, given the availability of physiological parameters, the model can

be parametrized for other fish species. Finally, integrating a toxicodynamic part to established PBK models (PBK-TD model) can be used to link external exposure dose, dose-at-target, and adverse endpoint. A TD part would aid in estimating the magnitude of response, e.g., half maximal effect concentration (EC50) in connection to the predicted target tissue levels. This would widen the model's applicability in MRA, accounting for both toxicokinetic and toxicodynamic effects of a complex mixture.

Overall, the present research is relevant in the chemical risk assessment field. Developed QSAR models showed good predictive power and the ability to identify and prioritize chemicals of concern with confidence. Mixture PBK models demonstrated the ability to assess internal doses for mixture constituents simultaneously. Additionally, PBK models aid in hypotheses testing and predicting exposure concentrations under which co-exposed chemicals could potentially influence each other's ADME properties.

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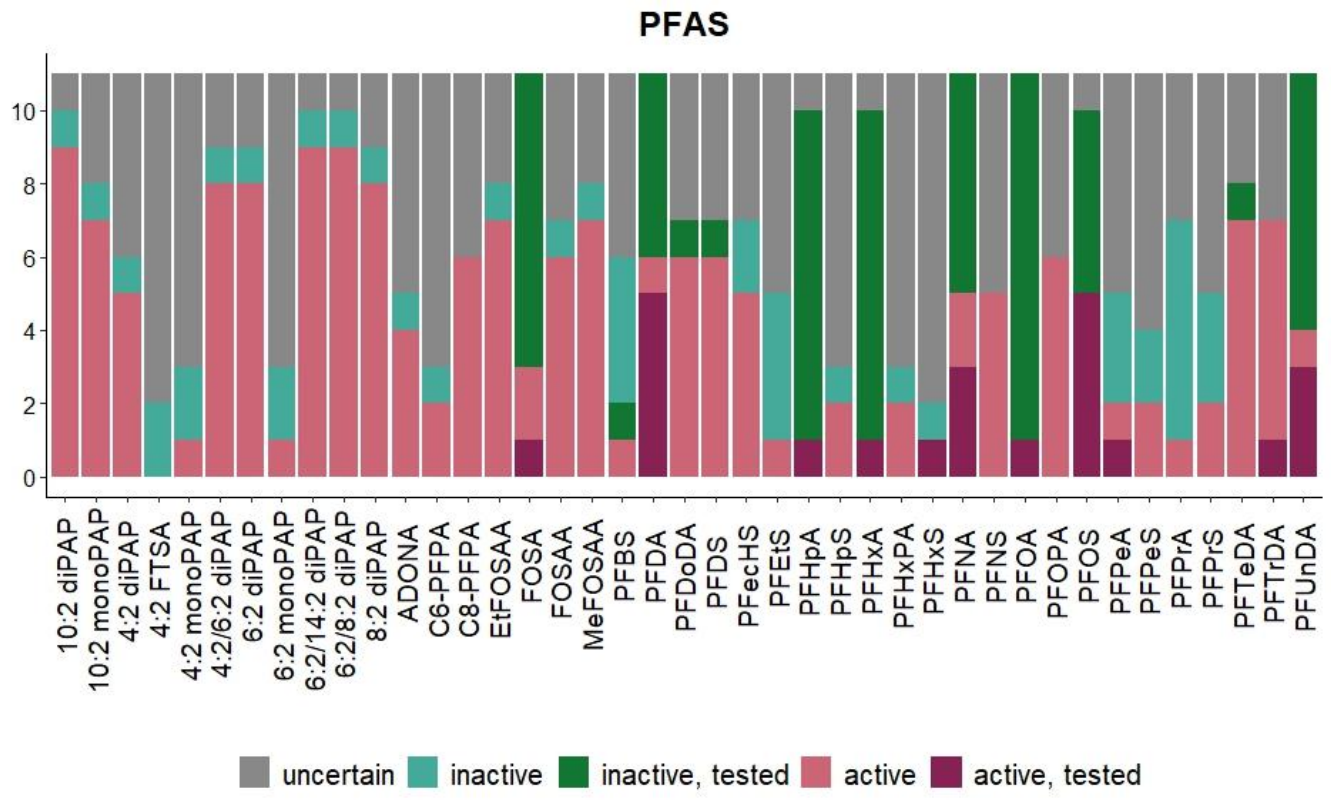

Appendix A1. All PFAS compounds in HBDB predicted with thyroid-specific models.