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Studying interaction effects on toxicokinetics in zebrafish combining experimental and modelling approaches

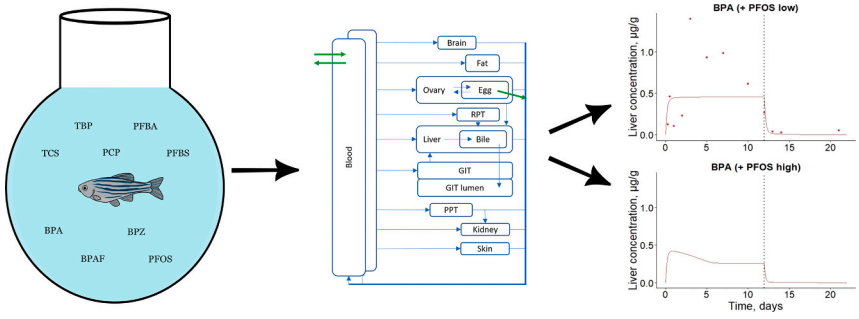
Elena Golosovskaia [a,*], Stefan Örn [b], Pim Leonards [c], Jacco Koekkoek [c], Patrik L. Andersson [a,*]

[a] Department of Chemistry, Umeå University, Umeå, Sweden
[b] Department of Animal Biosciences, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden
[c] Faculty of Science, Chemistry for Environment & Health, Vrije Universiteit Amsterdam, Amsterdam, the Netherlands

HIGHLIGHTS

- BCFs for zebrafish tissues determined for BPA, BPAF, BPZ, TCS, TBP, PCP, PFBA, PFBS, and PFOS.
- PFOS, TCS and PCP showed largest BCFs in female zebrafish tissues.
- Experiments indicated that kinetics of individual chemicals were not affected in a mixture.
- Single PBK model predicted BCFs of individual chemicals in a mixture.
- Binary PBK model simulations indicated that PFOS influence tissue concentrations of BPA and BPZ.

GRAPHICAL ABSTRACT



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ABSTRACT

Humans and wildlife are exposed to a complex mixture of anthropogenic chemicals of which only a few have been subjected to regulations. Chemical risk assessment is currently based on evaluating single chemicals, which is costly, time-consuming, and neglect toxicokinetic and toxicodynamic mixture effects. This study focused on interaction effects on the absorption, distribution, metabolism and excretion (ADME) processes of selected chemicals representing potential modulators of these processes. Adult female zebrafish (*Danio rerio*) were exposed to selected mixture of 11 chemicals and bioconcentration factors (BCFs) on tissue level were determined for 9 of them: bisphenol A (BPA), bisphenol AF (BPAF), bisphenol Z (BPZ), triclosan, tribromophenol, pentachlorophenol, heptafluorobutyric acid (PFBA), perfluorobutanesulfonic acid (PFBS), and perfluorooctanesulfonic acid (PFOS). Comparison of BCFs of bisphenols obtained from single chemical exposure experiments versus the current study revealed no statistically significant differences ($p > 0.05$), implying no mixture effects on kinetics of bisphenols at investigated concentrations. The same conclusion was reached using two physiologically based kinetic (PBK) models, developed for individual bisphenols and *per-* and polyfluoroalkyl substances (PFAS), showing good model fit for BPA, BPZ, BPAF, and PFOS. To simulate exposure scenarios where kinetic interaction effects may occur through competitive protein binding in blood, a new PBK model was developed. Simulations where zebrafish were dosed with BPA and BPZ, individually, and combined with varying levels of PFOS, showed that competitive binding to serum proteins alter tissue levels of bisphenols when levels of PFOS exceeded 1 µg/L.

* Corresponding authors.
E-mail addresses: elena.dracheva@umu.se (E. Golosovskaia), patrik.andersson@umu.se (P.L. Andersson).

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This indicates that chemicals acting in concert could perturb ADME but only at higher levels or in complex mixtures.

1. Introduction

Humans and wildlife are exposed to a complex mixture of various kinds of anthropogenic chemicals from small organic compounds to large and complex polymers including microplastics. These chemicals are present in e.g., building materials, electronics, textiles, food, drinking water, cosmetics, and other materials and products. Over the lifetime of these materials, recycling or end-of-life processing, chemicals may emit to the surrounding environment and reach compartments, such as soil, water or air, that may mediate risks of exposure to chemicals for humans and animals. Exposure is never to single chemicals but occurs to a multitude of chemicals from various sources simultaneously. Yet, regulatory chemical risk assessment is largely based on individual chemicals (Kienzler et al., 2016), although proposals on group assessment of chemicals is being discussed in e.g., the European Union for *per*- and polyfluoroalkyl substances (PFAS) (Anon, 2023a) and bisphenols (Anon, 2023b).

It is known that chemicals can act in concert or even potentiating or inhibiting toxicological effects in organisms. Risk assessment methods have been developed to include mixture effects using concentration addition and independent action approaches. These models cover toxicodynamics but not effects on toxicokinetics. It is currently being discussed how these approaches can be implemented in legislation using e.g., mixture assessment factors (Kortenkamp and Faust, 2018; Backhaus, 2023). Much less is known on effects on toxicokinetics in mixture exposure scenarios. Generally, it is assumed that no interactions occur between compounds that affect kinetics at environmentally relevant concentrations (European Commission, 2012). However, organisms are exposed to a large range of chemicals simultaneously and certain environments, such as urban surface waters, may contain high levels of contaminants and thus pose a risk for both toxicokinetic and toxicodynamic effects. These exposure scenarios may impact various processes related to the absorption, distribution, metabolism and excretion (ADME) of chemicals (Bart et al., 2022). For example, the distribution of an individual chemical in blood mediated through transport proteins could be disturbed by another chemical competing for the same protein. It has been reported that long-chain perfluoroalkyl acids (PFAAs) affect the bioconcentration and tissue levels of short-chain PFAAs in zebrafish (Wen et al., 2017). These effects were however only detected at higher concentrations (10 μg/L). Another ADME process which can be perturbed in a mixture is metabolism, caused by chemicals interacting with e.g., cytochrome P450 enzymes (Hanioka et al., 1996). It is also known that BPA inhibits glucuronidation of zidovudine (Li et al., 2014), and triclosan inhibits cytosolic sulfonation of 3-hydroxybenzo(*a*)pyrene, BPA, p-nitrophenol, and acetaminophen (Wang et al., 2004).

Physiologically based (toxico)kinetic (PB(*T*)K) models are widely used in pharmacology and toxicology to translate external exposure dose to internal exposure, and thus estimate the "dose-at-target", which is required to trigger a toxicological effect. These models can include various number compartments, i.e., tissues, such as, brain, gonads, and muscle, in addition to typical organs including liver, kidney, and blood. Such models require both physiological parameters, e.g., tissue volumes and blood flows, and chemical-specific parameters, e.g., blood to tissues partition coefficients and metabolic constants. PBK models can be generic (validated for a number of chemicals and using quantitative structure-property relationship (QSPR) models for parametrization) or local (applicable for a particular chemical only). Very few PBK models for fish consider kinetic interaction effects with a few exceptions mainly covering binary mixtures or volatile organics (Desalegn et al., 2019; Liu et al., 2022; Golosovskaia et al., 2024; Mit et al., 2021; Tebby et al., 2019).

Understanding kinetics of chemicals is critical including mixture effects to yield solid data for assessing dose at target. Most experimental studies on uptake and depuration of chemicals including data on bioconcentration factors are undertaken in complex mixtures. That is beneficial for several reasons including yielding data for several chemicals in the same study and thus reducing animal testing. However, if kinetic processes are perturbed by the mixture, the data will be skewed. Chemicals may not only directly disturb ADME processes of other compounds, but also physiological parameters like cardiac output (Haddad and Krishnan, 1998). Thus, this study aimed at investigating kinetic interactions between chemicals of various classes with representatives that potentially could induce interaction effects on ADME processes, i.e., kinetic interaction effects. The selected mixture contained phenols, phthalates, and *per*- and polyfluoroalkyl substances (PFAS), and the three bisphenols: bisphenol A (BPA), bisphenol AF (BPAF), and bisphenol Z (BPZ). The mixture cover chemicals known to induce phase I and II metabolism and perturb protein mediated distribution in blood.

The particular focus was on investigating whether kinetics of bisphenols is affected by co-exposed chemicals. First, female zebrafish were exposed to the selected chemical mixture, and uptake and depuration were studied. Derived data were compared with tissue distributions from BPZ single-chemical exposure experiments performed previously by our group using a similar experimental protocol (Chelcea et al., 2022), as well as PBK model data. In addition, a new PBK model tailored for binary mixtures was developed to simulate plausible effects induced by PFOS on kinetics of BPA and BPZ, simulating a range of PFOS exposure concentrations. The focus was to study interaction effects on protein mediated distribution in blood serum potentially causing variation in the free fraction and thus modulating tissue concentrations.

2. Materials and methods

2.1. Chemicals

Chemicals for the zebrafish study were purchased from Sigma-Aldrich including in crystal form; BPA (CAS 80-05-7), BPZ (CAS 843-55-0), BPAF (1478-61-1), triclosan (TCS) (CAS 3380-34-5), tribromophenol (TBP) (CAS 118-79-6), bis(2-ethylhexyl) phthalate (DEHP) (CAS 117-81-7), diethyl phthalate (DEP) (CAS 84-66-2), PFBA (CAS 375-22-4), PFBS (CAS 375-73-5) and PFOS (CAS 1763-23-1); and 500 μg/mL solution of pentachlorophenol (PCP) (CAS 87-86-5). Chemicals and their properties are listed in Table 1.

2.2. Adult zebrafish experiment

Adult female zebrafish were kept at the aquatic laboratory at the Department of Animal Biosciences. 50 fish were held in 40-L glass tank with a flow-through system using charcoal-filtered tap water at a constant temperature of 26 °C, 12:12 light:dark cycle and fed daily with commercial food (Sera Vipan Nature). Animal experiments were approved by the regional ethical committee on animal experiments (Dnr 5.8.18-02613/2022).

The female zebrafish were exposed to a mixture of selected emerging chemicals (Table 1) for 12 days followed by 23 days depuration phase. Nominal exposure concentration of bisphenols in the mixture was set to 10 μg/L in accordance with a previous study on single chemical exposure of BPZ (Chelcea et al., 2022). For remaining chemicals, nominal exposure concentration was set to reflect environmentally relevant levels in surface water. PFOS has been recorded in levels up to 286 ng/L, with a median at 2 ng/L. (Li et al., 2021) PCP has been quantified in

Table 1
Studied chemicals with selected physicochemical properties.

Abbreviation	Name	CAS	MW	logKow[a]	pKa[a]
BPA	Bisphenol A	80-05-7	228.3	3.42[b]	9.46
PBZ	Bisphenol Z	843-55-0	268.3	4.34	10.2
BPAF	Bisphenol AF	1478-61-1	336.2	3.75	8.22
PCP	Pentachlorophenol	87-86-5	266.3	4.91	6.95
TCS	Triclosan	3380-34-5	289.5	4.97	8.66
TBP	Tribromophenol	118-79-6	330.8	4.15	7.55
PFBA	Heptafluorobutyric acid	375-22-4	214.0	2.82[c]	−0.20
PFBS	Perfluorobutanesulfonic acid	375-73-5	300.1	3.90[c]	−3.57
PFOS	Perfluorooctanesulfonic acid	1763-23-1	500.1	6.43[c]	−1.64
DEHP[d]	Bis (2-ethylhexyl) phthalate	117-81-7	390.6	7.53	NA[e]
DEP[d]	Diethyl phthalate	84-66-2	222.2	2.45	NA[e]

[a] Predicted form ComptoxDashboard (CompTox Chemicals Dashboard, 2024).
[b] Staples et al. (Staples et al., 1998)
[c] Predicted with COSMOtherm 2011 (C2.1 release 01.10) (Wang et al., 2011).
[d] Chemical not detectable by applied analytical method.
[e] Not applicable.

levels from 30 ng/L to μg/L (Chen et al., 2021), and TCS at 10–74 ng/L (Okumura and Nishikawa, 1996; Bester, 2005; Dhillon et al., 2015) in European surface water. Based on these data, a nominal exposure concentration was set to 20 ng/L for all chemicals except bisphenols, to reflect low but environmentally relevant levels in surface waters and to be feasible for chemical analysis. The stock solution with the mixture was pumped into the tank with a peristaltic pump, and water flow was set to 3 water renewals daily. After the exposure phase, fish were transferred to another 40-L tank with the same conditions but without chemical exposure.

Fish were sampled before the start of exposure (controls) and at 6 h, 12 h and at days 1, 2, 3, 5, 7, 10, and 12 for the exposure phase, and at days 13, 14, 21, 28, and 35 for the depuration phase. At each timepoint, 3 fish (e.g., pseudo replicates) were sacrificed and analyzed for chemical content. Fish were sedated with 150 mg/L MS-222 water solution and decapitated prior to sampling. Weights of whole fish and collected organs (ovaries, liver, brain and carcass) were determined. Samples were frozen immediately (−20 °C) until further chemical analysis at VU Amsterdam, the Netherlands. Chemical concentration in the tank was monitored throughout the sampling period. Water samples were taken on day 1, 3, 6, and 10, during the exposure phase and 1 day after fish were transferred to clean water. Control water samples from the fish tank were taken prior to chemical exposure. In addition, fish feed was analyzed to account for possible unintentional exposure. The estimated daily oral intake of studied chemicals was calculated, assuming that fish consume approximately 4 % of their total weight (approximately 500 mg) per day.

2.3. Chemical analysis

Prior to the extraction isotopic labeled internal standards (Wellington Laboratories, Toronto Research Chemicals and Cambridge Isotope laboratories) were added to the sample material (Section S1). In a two-step extraction with 1 mL acetonitrile – methanol (3/1) mixture (Biosolve) the target compounds were extracted from the organ tissues by 30 min sonication. The carcasses were first manually cut and subsequently extracted with two times 5 mL acetonitrile – methanol (3/1) mixture. The volume of the combined fractions was reduced to ca 0.3 mL followed by an addition of 1 mL acetonitrile. To freeze out the interfering matrix the extracts were stored for one night at −20 °C.

The next day the extracts were centrifuged for 10 min at 14000 rpm, subsequently loaded on an acetonitrile conditioned C18 cartridge (SEP-PAK 200 mg, Waters) and eluted with two-times 0.8 mL acetonitrile. The eluates were evaporated until dryness, and the remaining residues were reconstituted in 50 μL 0.5 M CO_3 buffer (Na_2CO_3 – $NaHCO_3$, both Sigma – Aldrich) and 50 μL methanol. The extracts were stored at 4 °C until measurement. The water samples were diluted with the isotopic labeled internal standard and directly injected.

The PFASs were measured on a LC (ExionLC, Sciex) coupled with a Turbo V Ion source (ESI) operating in the negative ion mode prior to triple quadrupole mass selective detection (6500+, Sciex). Separation of the PFASs was done on a C18 XP column (X-bridge, 100 × 2.1 mm, 2.5 μm, Waters) set at 50 °C using a gradient of 20 mM NH4Ace (Merck) and methanol. The applied MRM transitions and MS/MS settings are described in Tables S1-S3.

The extract was first analyzed for PFAS followed by a derivatization for the phenolic compounds. The PFAS extracts were diluted in 0.5 mL 0.5 M CO_3 buffer and 0.5 mL acetone (Biosolve). Prior to the analysis of the phenolic compounds, the compounds were derivatized with dansyl chloride (Sigma-Aldrich). Derivatization was carried out at 60 °C over 20 min. After cooling down to room temperature 4 mL hexane (Biosolve) was added. The hexane phase was evaporated till dryness and the residues were reconstituted in 0.1 mL acetonitrile. The extracts were stored at 4 °C until analysis. For the analysis of phenolic compounds in water, samples were diluted with the isotopic labeled internal standard and 0.5 mL CO_3 buffer, dansylated according to protocol and injected.

The phenolic compounds were analyzed using LC with a tandem mass spectrometry, MS/MS – ESI positive mode (XEVO TQ-XS, Waters). The chromatographic separation was achieved on a C18+ column (Cortecs, 150 mm × 2.1 mm × 1.6 μm, Waters) using a gradient of 0.05 % formic acid (Biosolve) in type 1 water and methanol. The applied MRM transitions and MS/MS settings are described in Tables S4-S6.

2.4. Application of existing PBK models

Literature search was performed in Web of Science and PubMed (completed August 16, 2023) to identify existing PBK models suitable for predicting the tissue levels of the target chemicals in this study. The models should fulfill the following criteria: 1) be parametrized for zebrafish; 2) external validation on in vivo data should be performed; 3) be applicable to the studied bisphenols; and 4) be readily available for application, e.g., model codes should be provided. Two recently published models were found satisfying the abovementioned criteria, i.e., Grech et al. (Grech et al., 2019) and Chelcea et al. (Chelcea et al., 2022) Since the latter model is based on the model developed by Grech et al. (Grech et al., 2019) and have shown improved performance, the Chelcea model (Chelcea et al., 2022) was tested on our dataset with all parameter values presented in the original study.

In addition, a recently developed PBK model by Golosovskaia et al. (Golosovskaia et al., 2024) (PBK_{Single}) validated for several individual PFAS was used to predict PFOS concentrations. This model was parametrized for PFOS using $logK_{ow}$ predicted by COSMOtherm (Table 1), binding affinity to bovine serum albumin (BSA) to account for PFOS binding to the plasma proteins, and exposure conditions corresponding to the experimental settings of this study.

2.5. Development of the binary PBK model

Two previously developed models, i.e., the Chelcea et al. (Chelcea et al., 2022) model developed for bisphenols and the PBK_{Mix} model by Golosovskaia et al. (Golosovskaia et al., 2024) developed for PFAS were merged to account for different kinetics of bisphenols and PFAS, and plausible plasma protein interactions. We refer to the original studies (Golosovskaia et al., 2024; Chelcea et al., 2022) for detailed descriptions

and assumptions of the models. The competitive binding algorithm implemented into the PBK_{Mix} model (Golosovskaia et al., 2024) requires data on the amount of protein in the blood and binding affinities of each chemical in a mixture. In vitro data on serum albumin binding constants for PFOS vary from 10^4 to 10^6 M^{-1} (Chen and Guo, 2009; Chen et al., 2015; Beesoon and Martin, 2015; Bischel et al., 2010) measured with different methods, while values of 10^2–10^4 M^{-1} were reported for BPA and BPZ (Gu et al., 2022; Xie et al., 2010; Ikhlas et al., 2019). Binding affinities to bovine serum albumin (BSA) of BPA (pKd = 3.2) (Gu et al., 2022), BPZ (pKd = 2.5) (Gu et al., 2022) and PFOS (pKd = 5.7) (Chen et al., 2015), estimated at temperatures around 300 K were used as an approximation to represent binding to plasma proteins. Zebrafish is known to lack albumin gene (Noel et al., 2010; Nolte et al., n.d.) and it is currently uncertain which protein is responsible for small molecule transport in blood, thus, we used the total protein content in zebrafish blood as reported by Li et al. (Li et al., 2016) The developed binary model was applied using levels of the bisphenols in line with the experimental studies and varying PFOS levels; 12 ng/L (in line with the experimental study), 1 μg/L, 10 μg/L, and 100 μg/L.

The two previously developed PBK models represent different approaches to account for various aspects of compound's kinetics which were preserved in the binary PBK model. For example, the gill exchange rate constant *kx* (mL/day) was modelled for bisphenol model (Chelcea et al., 2022) as follows:

$$kx = min(F_{water}, Y_{blood}) \tag{1}$$

while for PFOS model (Golosovskaia et al., 2024) it was calculated according to the equation below:

$$kx = \frac{\left(\frac{BW}{1000}\right)^{0.75}}{\rho_{water} + \frac{\rho_{lip}}{Dow} + \frac{1}{Y_{water}} + \frac{1}{Y_{blood}}} * 1000 \tag{2}$$

where F_{water} is effective respiratory volume; BW – body weight (g); Y_{water} – gill ventilation coefficient; ρ_{water} – resistance of water; ρ_{lip} – resistance of lipid layers; and Y_{blood} – mass-normalized blood perfusion coefficient, calculated as a product of cardiac output and blood:water partition coefficient.

Subsequently, excretion via the gills was modelled differently. For bisphenols, gill exchange rate constant (*kx*) was used for both intake and excretion via the gills (Chelcea et al., 2022), i.e.:

$$\frac{dA_{admin_gill}}{dt} = kx * \frac{A_{water}}{V_{water}} \tag{3}$$

$$\frac{dA_{excret_gill}}{dt} = kx * \left(\frac{A_{blood}}{V_{blood}} * f_u \Big/ PC_{blood:water} \right) \tag{4}$$

where A is amount of chemical; f_u is the fraction unbound; $PC_{blood:water}$ is the blood:water partition coefficient.

For PFOS, in contrast, gill excretion was modelled in accordance with the model proposed by Hendriks et al. (Hendriks et al., 2001):

$$\frac{dA_{excret_gill}}{dt} = \left(\frac{1}{\rho_{lip} * (Dow - 1) + 1} + 1 \right) * kx * \left(\frac{A_{blood}}{V_{blood}} * f_u \right) \tag{5}$$

The binary PBK model, incorporating the two previously developed models, contains 10 compartments: blood, brain, ovaries/eggs, adipose tissue (fat), liver, gastrointestinal tract (GIT), kidney, skin, poorly perfused tissues (PPT), and richly perfused tissues (RPT). The bisphenol and PFOS parts of the binary model were combined in the blood compartment, with competitive protein binding algorithm adapted from Blay et al (Blay et al., 2020) and described in more detail in Golosovskaia et al. (Golosovskaia et al., 2024)

The mass balance for non-eliminating tissues was modelled for the bisphenols according to the following equation:

$$\frac{dA_t}{dt} = F_t * \left(\frac{A_{blood}}{V_{blood}} - \frac{A_t}{V_t} \Big/ PC_{t:blood} \right) \tag{6}$$

For PFOS, chemical amount for non-eliminating tissues was modelled as follows:

$$\frac{dA_t}{dt} = f_u * F_t * \left(\frac{A_{blood}}{V_{blood}} - \frac{A_t}{V_t} \Big/ PC_{t:blood} \right) \tag{7}$$

Two binary mixture simulations were run with the binary model: BPA + PFOS and BPZ + PFOS. Liver:blood and skin:blood partition coefficients in the binary BPZ + PFOS model were fitted to the data to adjust for the high observed liver concentrations and where the single chemical PBK model showed large prediction error. The modFit function in the FME R package with the Nelder-Mead algorithm (Soetaert and Petzoldt, 2010) was used for fitting. Parameters were chosen based on the smallest residuals for the four organs, i.e., brain, ovary, liver, and carcass.

2.6. Model validation

The models were evaluated using fold difference (FD) metric, comparing the predictions in four fish tissues (brain, ovaries, liver and carcass) to the in vivo observed chemical concentrations. Note that we were only able to evaluate scenarios with PFOS exposure being at 2 ng/L, in accordance with our zebrafish experiment. FD was calculated as follows:

$$FD = e^{\left| ln\left(\frac{P_{ij}}{O_{ij}}\right) \right|} \tag{8}$$

where P_{ij} is a predicted concentration of a chemical i in tissue j, and O_{ij} is the observed concentration of the same chemical in the same tissue.

3. Results and discussion

3.1. In vivo zebrafish experiment

Uptake of the studied chemicals were analyzed in four different fish tissues, i.e., brain, liver, ovaries, and carcass (Fig. 1). Fish feed was analyzed where trace levels of the bisphenols and PFAS were quantified and estimated to contribute during the exposure period with 3.42 ng per fish of BPA, 0.04 ng BPZ, 0.15 ng BPAF, and 0.71 ng PFOS (minor amounts of PFBS and PFBA), suggesting no significant impact on the internal concentrations (Fig. 1, Section S2, Table S9). The three studied bisphenols reached the maximum tissue concentration (C_{max}) after 3 to 5 days of exposure followed by dropping levels. This phenomenon has been noticed previously (Chelcea et al., 2022) and may be due to metabolic processes. PFBA and PFBS showed limited potential to accumulate, whereas the levels of PFOS continuously increased in all tissues throughout the exposure period and remained in the tissues over the depuration phase. PCP, TCS, and TBP reached rather stable levels after 5 to 10 days of which highest steady-state levels were noted for TCS (Fig. 1, Fig. S1, Tables S7, S8). The three bisphenols, PCP, and TCS showed rapid elimination and nearly disappeared from the fish already after 48 h of depuration (Fig. 1), whereas TBP showed unclear trends in the depuration phase, probably due to background contamination. This chemical was detected in both control water and water samples taken during the depuration phase indicating background contamination (Tables S7-S9).

PFOS was the most bioaccumulative compound in the mixture (whole body BCF of over 1000 L/kg) followed by TCS (455 ± 152 L/kg),

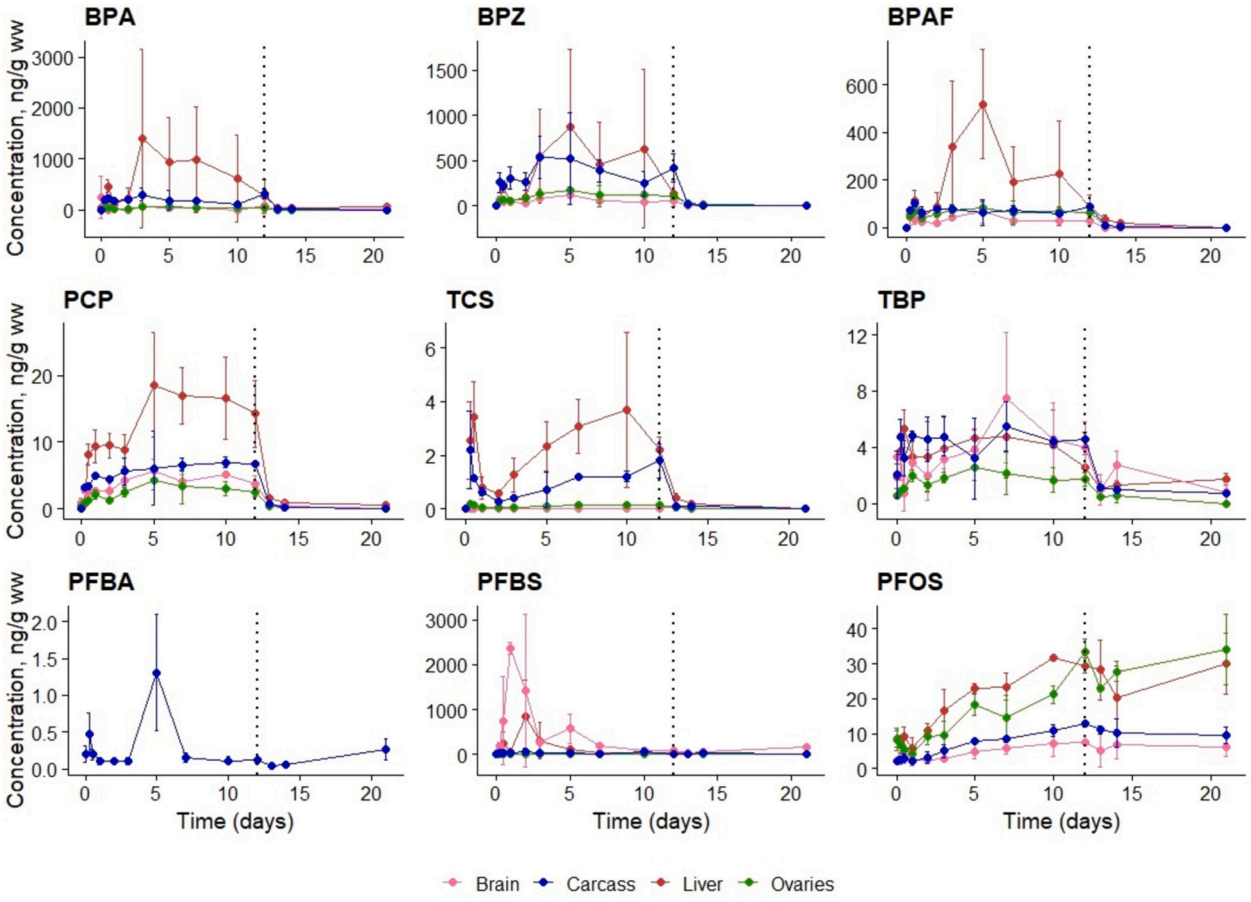

Fig. 1. Adult zebrafish tissue levels over time obtained from the mixture exposure experiment, with uptake phase from day 0 to 12 and depuration from day 12 to 21. Note that the last two time points, days 28 and 35, are not presented due to very low levels for the majority of chemicals. These data can be seen in Table S8.

PCP (270 ± 6 L/kg), and TBP (139 ± 14 L/kg), respectively. The highest BCF for PFOS was observed in the ovaries, however, levels in the liver were the highest during the uptake phase. In contrast, PCP, TCS, and TBP showed the lowest bioaccumulation potential in the ovaries. All studied chemicals, except for PFBA, were quantified in the liver, with that being one of the main accumulating tissues. That can be explained

Table 2
Calculated bioconcentration factors (BCFs) (L/kg) in zebrafish for studied chemicals from this study and reported in literature for bisphenols (data from female fish, otherwise stated). BCFs are calculated using tissue concentration at last exposure timepoint and average water concentration.

Compound	Average measured water concentration (μg/L)	Carcass	Liver	Brain	Ovaries	Whole body[a]	Reference
BPA	9	35 ± 11	30 ± 35	ND	4 ± 2	28 ± 9	This study
	97.5					6	Lindholst et al. (Lindholst et al., 2003)[b]
	1.94	NA	183 ± 134	NA	12 ± 9	14 ± 10	Chen et al. (Chen et al., 2017)
	5.72	NA	183 ± 91	NA	22 ± 11	18 ± 9	Chen et al. (Chen et al., 2017)
BPZ	6.5	65 ± 24	21 ± 23	8 ± 3	16 ± 6	52 ± 18	This study
	17	66 ± 11	59 ± 74	10 ± 4	16 ± 3	53 ± 7	Chelcea et al. (Chelcea et al., 2022)
BPAF	4.6	19 ± 3	20 ± 11	6 ± 3	14 ± 6	17 ± 3	This study
	20	NA	10 ± 3	NA	10 ± 1	5 ± 1	Shi et al. (Shi et al., 2016)
TBP	0.027	167 ± 20	95 ± 34	147 ± 67	64 ± 18	139 ± 14	This study
TCS	0.003	566 ± 200	701 ± 153	ND	48 ± 8	455 ± 152	This study
	0.04	NA	6460 ± 1560	NA	2261 ± 127	465 ± 113	Chen et al. (Chen et al., 2017)
	0.23	NA	4730 ± 1500	NA	104 ± 38	332 ± 191	Chen et al. (Chen et al., 2017)
PCP	0.021	314 ± 17	674 ± 237	173 ± 58	116 ± 35	270 ± 6	This study
PFBA	0.02	6 ± 2	ND	ND	ND	ND	This study
PFBS	0.055	252 ± 352	659 ± 87	1512 ± 916	9 ± 8	220 ± 277	This study
PFOS	0.009	1063 ± 71	2455 ± 179	621 ± 193	2799 ± 288	1200 ± 81	This study

[a] Whole-body concentration was calculated as weighted average of the tissue concentrations.
[b] Gender not specified.

by the liver being a responsible organ for metabolism and excretion of chemicals. In the case of PFOS, fatty acid binding proteins (FABPs) are highly expressed in the liver and PFOS are known to bind to these proteins which potentially also contribute to the high levels (Zhao et al., 2023). The PFOS levels in ovaries raise concern for maternal transfer of PFOS as it may be transported to eggs by FABPs alongside with fatty acids which are stored in yolk (Monroig et al., 2009; Liu et al., 2003).

The estimated BCFs of BPA, BPZ, and BPAF can be ordered as carcass ≈ liver > whole body > ovaries > brain. BPZ showed higher bioaccumulation potential than BPA and BPAF in carcass, ovaries and the whole body (Table 2), which was also noted in Chelcea et al. (Chelcea et al., 2022) BPA was least accumulated in the ovaries among the three bisphenols in the mixture with BCF of 4 ± 2 L/kg as compared to 16 ± 6 L/kg and 14 ± 6 L/kg for BPZ and BPAF, respectively. Higher levels of BPZ and BPAF in ovaries raise concerns about maternal transfer of BPA alternatives and potential offspring developmental toxicity (Gao et al., 2022; Mu et al., 2018). The bioaccumulation potential of BPA in the whole body of zebrafish was almost 5 times higher than reported by Lindholst et al. (Lindholst et al., 2003), where the exposure concentration was 10 times higher (Table 2). The whole body BCFs of both BPA and TCS were, however, in the same range as reported by Chen et al. (Chen et al., 2017) However, measured BCFs of these chemicals in the ovaries was somewhat lower (Chen et al., 2017).

Overall, the BCFs of BPZ estimated in this study was in accordance with the single chemical exposure study by Chelcea et al. (Chelcea et al., 2022) (Table 2). Some discrepancies were observed in the mean BCF of the liver, i.e., 21.2 ± 22.5 L/kg reported in this study versus 59.2 ± 74 L/kg observed by Chelcea et al. (Chelcea et al., 2022) Given the high variation in liver data of both studies, we cannot conclude that these discrepancies are of statistical significance. In the study by Chelcea et al. (Chelcea et al., 2022), a worst-case scenario was considered when C_{max} was used to derive BCFs. We compared our data with data of Chelcea et al. (Chelcea et al., 2022) using the worse-case scenario and the last exposure time point tissue concentration as the basis for calculating BCFs (Fig. S2). No significant differences ($p > 0.05$ using two-sample *t*-test with unequal variance) were found between the two approaches and studies, indicating that the mixture had no significant effect on ADME properties of BPZ under the studied concentrations.

Whole body BCF of BPA was three times higher than that obtained in a single chemical exposure study by Lindholst et al. (Lindholst et al., 2003), but in line with BCFs derived in a mixture exposure study by Chen et al. (Chen et al., 2017) Liver and ovaries BCFs were somewhat lower than those found in Chen et al. (Chen et al., 2017) As for BPAF, BCFs observed in the liver and ovaries in the current study align with the single chemical study by Shi et al. (Shi et al., 2016), with however whole body BCF being lower in the latter study. TCS kinetics, investigated in the complex mixture by Chen et al. (Chen et al., 2017) showed the same bioaccumulation potential in the whole body, with differences observed in tissues between the studies (Table 2). Overall, BCFs vary slightly between studies with lower and higher values that may be due to exposure levels or other experimental variations.

3.2. Studying interaction effects using single chemical PBK models

A zebrafish PBK model by Chelcea et al. (Chelcea et al., 2022) was applied to predict the kinetics of the three bisphenols in the current study (Fig. 2). The majority of experimental data of BPZ was predicted within a 5-fold error, with 43 % of carcass predictions and 36 % of liver and ovaries predictions within a 2-fold error (Fig. 2). The data showed that liver BCF was among the highest for BPZ (Table 2), however, the PBK model systematically underestimated liver concentrations. The estimated concentrations for the other bisphenols agreed well with the experimental data of this study with 36 % (BPA) and 57 % (BPAF) of ovary levels and 50 % of liver concentrations predicted within a 2-fold error. Slight underprediction of brain levels for BPAF (29 % of predictions in the 2-fold error interval) may be explained by uncertainties of

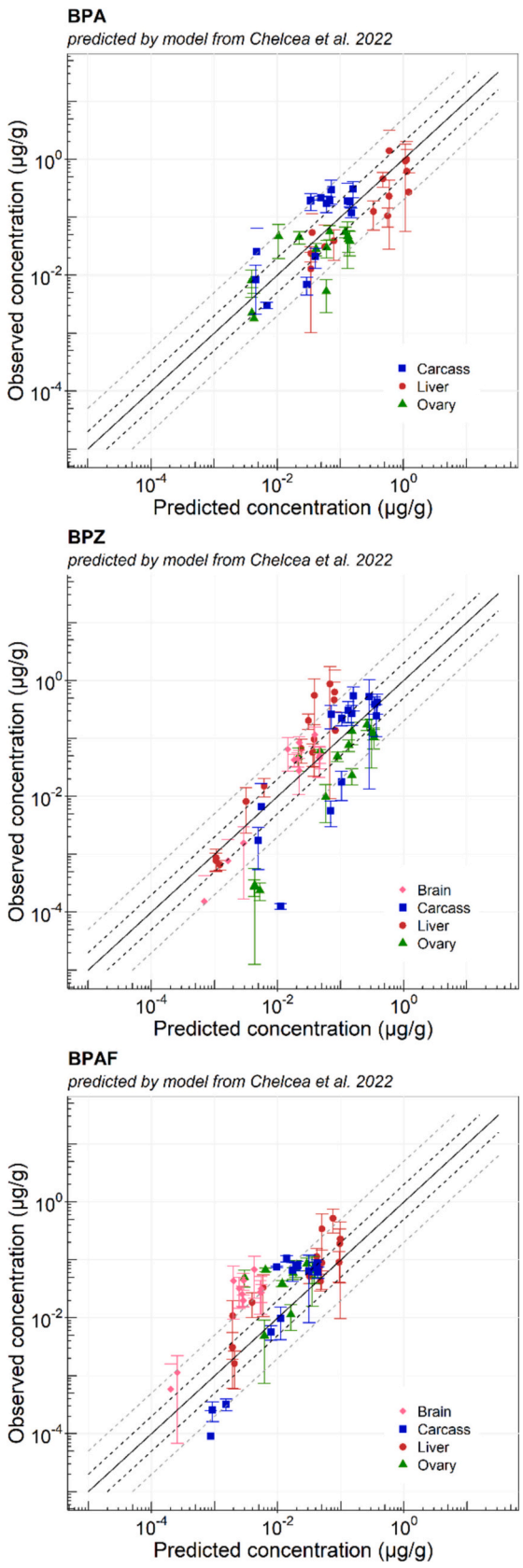

Fig. 2. Single chemical PBK model from Chelcea et al. (Chelcea et al., 2022) predicting BPA, BPZ, and BPAF levels of the current mixture experiment. Water dose was adjusted to the measured levels in this study. The dotted lines represent 2 and 5-fold errors.

the QSAR model used to calculate the brain:blood partition coefficient. The vast majority of all tissue data points (82 % for BPA, 71 % for BPZ, 70 % for BPAF) fall within a 5-fold error (Fig. 2) indicating good model performance that also fits well with reported performance in the original publication (Chelcea et al., 2022).

The PFOS levels in exposed zebrafish was predicted with the PBK_{Single} model developed by Golosovskaia et al. (Golosovskaia et al., 2024) resulting in the vast majority of predictions within a 3-fold error for brain, carcass, and liver (Fig. 3). Levels in ovaries were less well predicted with 71 % of predicted concentrations within a 10-fold error with systematic underprediction. Overall, the two previously developed single chemical PBK models applied to the mixture data of the current study, showed errors similar to those reported in the original studies. Thus, these models can be applied in situations where fish are being exposed to mixtures of chemicals at low concentrations, i.e., levels found in surface waters, as considered in the current study.

3.3. Simulation of interaction effects on ADME using a binary PBK model

The kinetics of BPA and BPZ were simulated using a novel binary PBK model where we varied levels of PFOS from 12 ng/L to 100 μg/L. The modelling focused on the effect of protein binding in the blood and PFOS was selected as a known strong plasma protein binder that could have an effect on the bioavailability of the bisphenols (Forsthuber et al., 2020; Jones et al., 2003). BPA and BPZ were selected as representatives of emerging bisphenols with available experimental kinetic data from the same laboratory. The binary model was first evaluated using the experimental data of this study, simulating a mixture of bisphenol (BPA 9 μg/L or BPZ 6 μg/L) and PFOS at 12 ng/L water exposure dose. The predicted tissue levels were compared to observed data and fold differences were estimated (Fig. 4). Overall the model showed good predictivity for simulating exposure to BPA+ PFOS (Fig. 4A) and BPZ + PFOS (Fig. 4B).

For the BPA + PFOS simulation, 56 % of the predictions were within a 3-fold error rate and 87 % within a 10-fold error (Fig. 4A). While the most accurate PFOS predictions were obtained for carcass (86 % within a 3-fold error), this tissue was predicted least accurate for BPA (only 21 % within a 3-fold error). For ovaries, PFOS predictions showed large variation in errors, with more than a 10-fold error during depuration phase, whereas BPA predictions were more accurate (>70 % of data points in the 3-fold error range). For the BPZ + PFOS simulation, 60 % of predictions fall within a 3-fold error, and 92 % within a 10-fold error interval (Fig. 4B). The model predicted PFOS internal concentrations overall with 75 % predictions at a 3-fold error interval. However, only 43 % of PFOS prediction in ovaries fall within a 3-fold error, while this organ was predicted the best for BPZ (71 % predictions within 3-fold error).

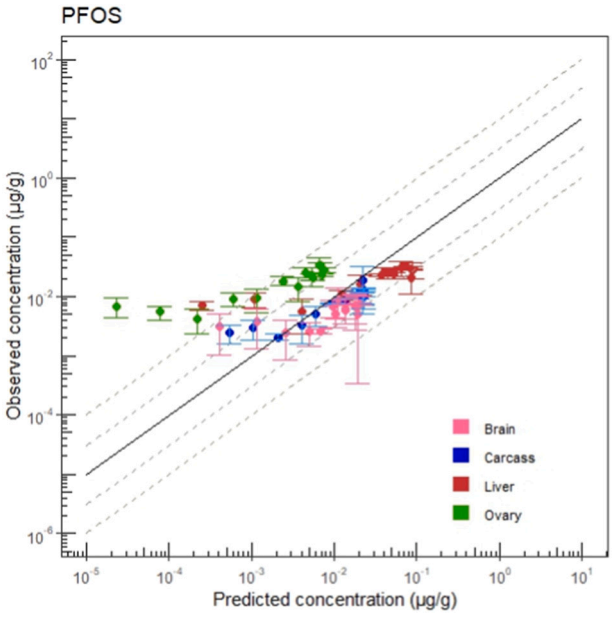

Fig. 3. Observed (this study) versus predicted PFOS tissue concentrations obtained with PBK model from Golosovskaia et al. (Golosovskaia et al., 2024)

In both simulations, the model showed reasonable, however somewhat varied predictions. For example, the model gave good predictions for carcass and brain for PFOS in simulation with both bisphenols, and ovaries and carcass for BPZ and BPA. Predictions for brain for BPZ and liver for both bisphenols were less accurate. This can be due to several factors, including metabolization processes in the liver not covered by the model, as well as larger uncertainty in experimental data.

In a next step, PFOS concentrations were increased stepwise aiming at increasing our understanding on potential effects on mixture kinetics caused by chemicals competing for blood protein carriers. That mechanism could potentially influence the bioavailable fraction of studied bisphenols and thus their concentration in tissues. A simulated level of PFOS at 1 μg/L slightly changed liver levels of BPA, with decreasing levels after reaching C_{max} as compared to the 12 ng/L dose of PFOS (Fig. 5A and B). No changes in ovaries, carcass and brain levels were noted at this exposure concentration (Table S10), however, these tissues reached slightly lower C_{max} at the two highest concentrations of PFOS, i.e., 10 μg/L and 100 μg/L (Fig. 5C, D, Table S10). The decrease in BPA tissue levels after the peak, as discussed for the 1 μg/L PFOS concentration, was also observed at the two highest concentrations, i.e., 10 and 100 μg/L PFOS (Table S10). However, at the concentration of 100 μg/L PFOS, only liver levels showed this behavior (Fig. 5D), while other tissues peaked at lower concentrations (Table S10).

Simulated BPZ tissue concentrations were not perturbed by co-exposures of PFOS at ng/L levels, and only a minor decrease in liver concentrations after C_{max} could be noted at 1 μg/L PFOS (Fig. 6B). At PFOS levels of 10 μg/L (Fig. 6C), BPZ levels in liver started to decrease after reaching maximum concentration at day 1 to 2 while carcass levels peaked at lower C_{max}, as compared with lower concentrations of PFOS, and did not show any concentration decrease by time (Table S10). For BPZ, the kinetic interaction effects at 100 μg/L PFOS slightly differed from that of BPA (Figs. 5D, 6D) in that both compounds reached lower steady-state concentrations, than in the exposure scenario with 12 ng/L PFOS, but that BPA showed a more clear drop in steady-state concentration after reaching C_{max}.

Usually in PBK modelling, the free fraction of chemicals in blood is considered to be constant. However, efforts have been made to account for possible temporal changes of free fraction of PFOS and PFOA in blood, where it was allowed to decrease over time until saturation of binding to proteins was reached (Tan et al., 2008; Loccisano et al., 2012). This has shown to better represent mammalian intravenous data of these PFAS chemicals. In this study, free fractions of BPA and BPZ in blood were compared when fish were exposed to different PFOS concentrations (Fig. S3). Free fractions of each chemical were calculated iteratively at each time point until equilibrium was reached. Notably, under the lowest exposure of PFOS, free fractions of BPA and BPZ in blood were estimated to be constant, however, at PFOS levels of 10 μg/L, free fractions increased over time until all bisphenol molecules were unbound. This can be explained by a stronger binding affinity to albumin of PFOS that outcompeted the bisphenols resulting in increased bioavailability of bisphenols leading to higher excretion rates (Fig. S3).

It has been shown that long-chain PFAS at μg/L levels impact tissue accumulation of short-chain PFAS (Wen et al., 2017). In that study the levels of the short-chain PFAS reached lower C_{max} and/or decreased after peak concentrations. It was suggested that these kinetic interaction effects could occur due to competitive binding to organic anion transporting polypeptides, liver fatty acid-binding proteins or serum proteins (Wen et al., 2017). The simulation presented here with BPA + PFOS and BPZ + PFOS with varying PFOS concentrations showed similar trends in

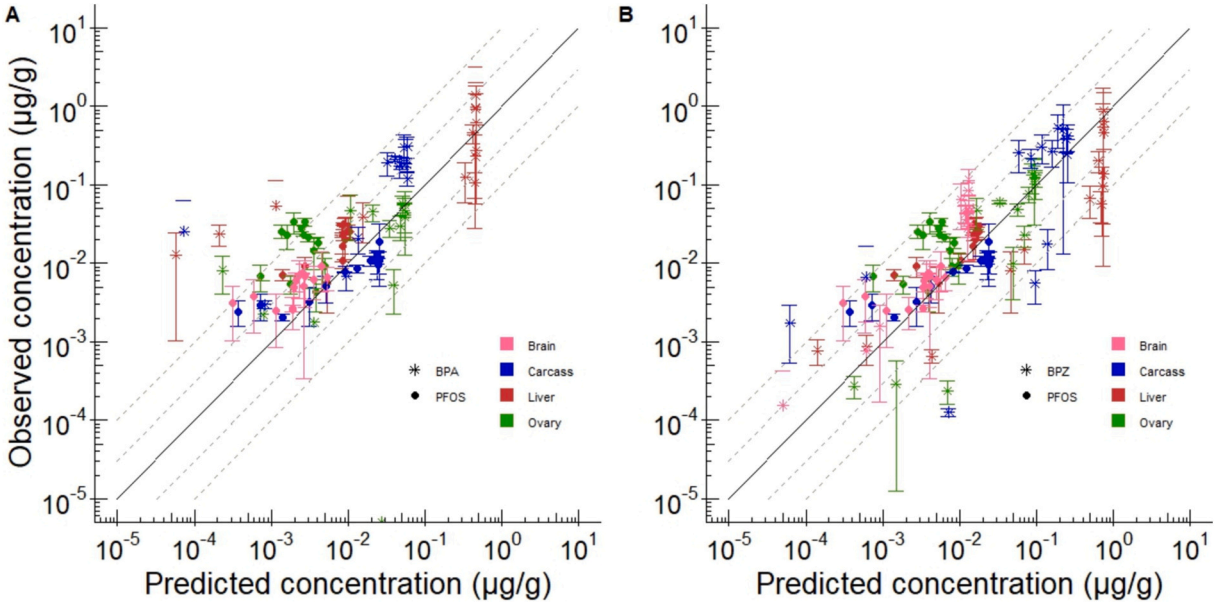

Fig. 4. Predicted versus observed concentrations for binary BPA + PFOS (A) and BPZ + PFOS (B) models where exposure concentration of bisphenol was 10 µg/L, and 12 ng/L for PFOS. Dotted lines represent 3- and 10-fold errors.

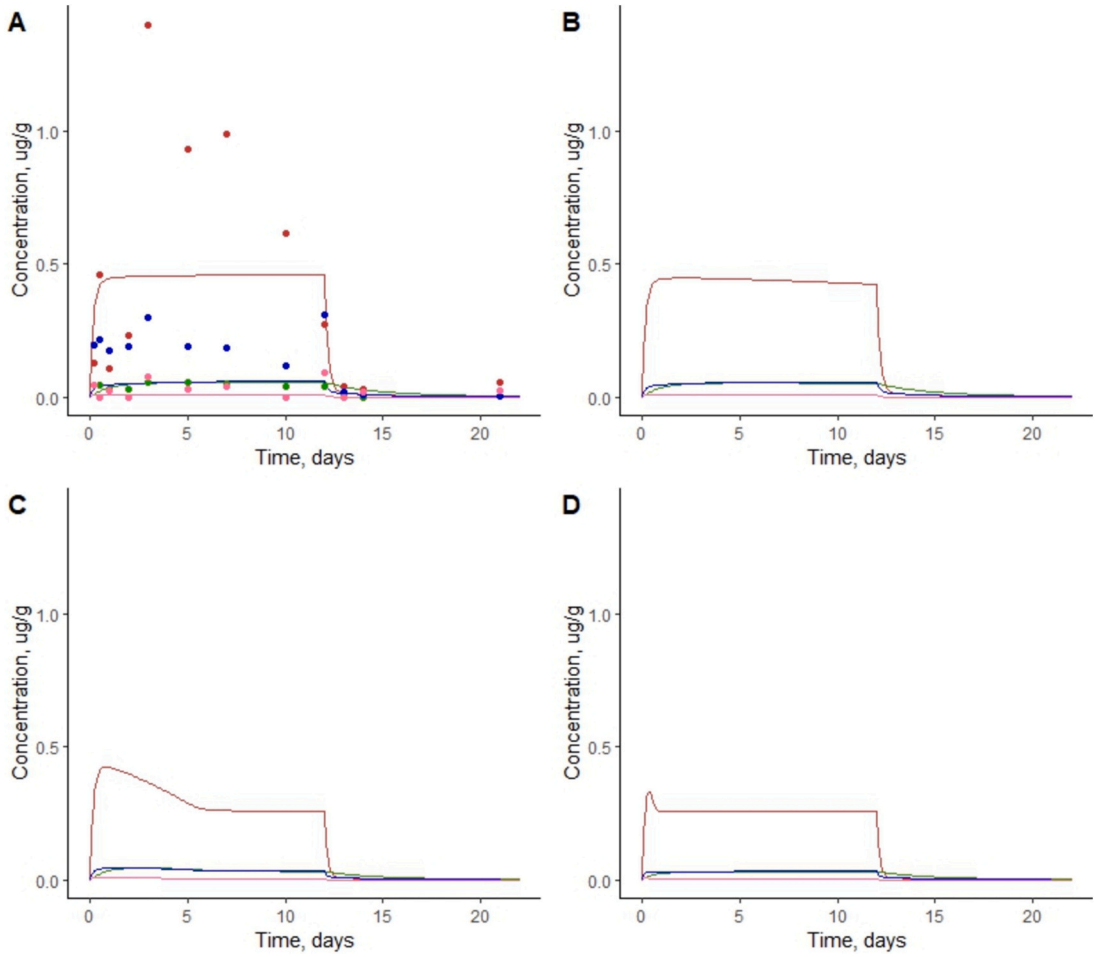

Fig. 5. Predicted tissue concentrations of BPA after combined exposure for 12 days with different PFOS concentrations; A - 12 ng/L PFOS, B – 1 µg/L PFOS, C – 10 µg/L PFOS, and D – 100 µg/L PFOS. Following the exposure phase, depuration was simulated for another 16 days (depuration period of 10 days is shown). The red line indicates predicted levels in liver, blue carcass, green ovaries, and rose brain. Dots in A represent the observed tissue levels in this study.

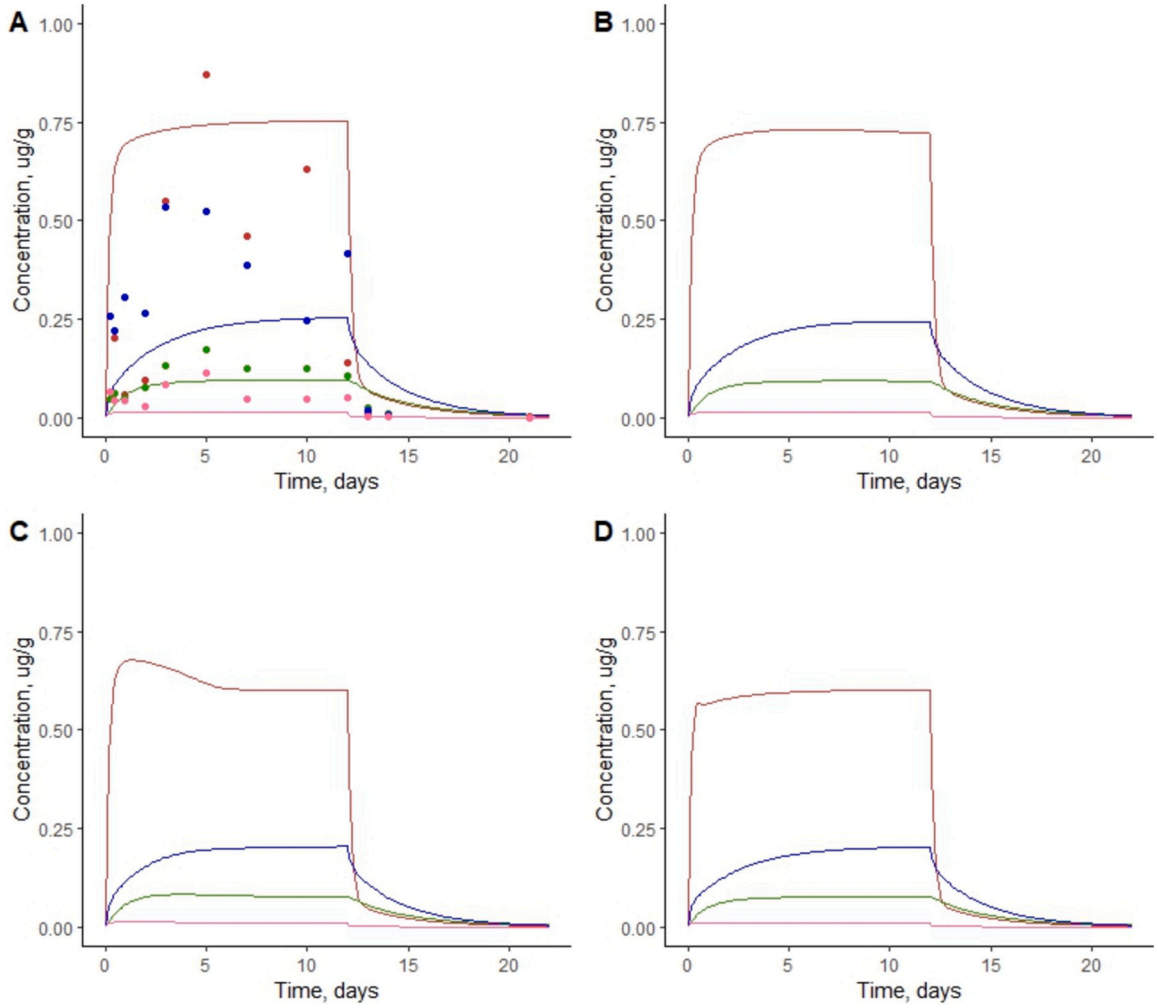

Fig. 6. Predicted tissue concentrations of BPZ after combined exposure for 12 days with different PFOS concentrations; A – 12 ng/L PFOS, B – 1 μg/L PFOS, C – 10 μg/L PFOS, and D – 100 μg/L PFOS. Following the exposure phase, depuration was simulated for another 16 days (depuration period of 10 days is shown). The red line indicates predicted levels in liver, blue carcass, green ovaries, and rose brain. Dots in A represent the observed tissue levels in this study.

tissue levels of bisphenols. Competitive binding to serum proteins was modelled and the simulation indicated that this process might be responsible for changes in tissue distribution of the studied bisphenols.

In the binary PBK model a competitive protein binding algorithm was implemented where an assumption on 1:1 binding of target chemical and protein. This may be correct for bisphenols where the number of binding sites for BSA has been estimated to be around 1 (Gu et al., 2022; Ikhlas et al., 2019). For PFOS, however, several binding constants have been reported, for multiple binding sites (Chen and Guo, 2009). That may mean that higher levels are required for PFOS to outcompete bisphenols, if binding of PFOS is less specific. Another uncertainty is the lack of information on protein characteristics of zebrafish specific serum transporters including their binding affinities. As a result, mammalian albumin data was used. Note also that we applied the total level of proteins in zebrafish blood, and likely far from all proteins are able to transport studied chemicals and thus competitive binding may occur at lower concentrations of PFOS. Another potential source of error of the binary model is the simulated exposure concentrations, which were above the applicability domain as the studied concentrations of PFOS were three orders of magnitude higher than those experimentally tested and used for model development.

3.4. Environmental implications

In this study, we investigated the effect on ADME of individual compounds in co-exposure scenarios. The in vivo experiment did not reveal any statistically significant differences in tissue distribution of the studied bisphenols between single chemical exposure and co-exposures with different classes of contaminants at concentrations close to environmentally relevant. This was also indicated by applying two existing PBK models on experimental data from this study, where models tailored for single chemicals were able to predict kinetics of chemicals in the mixture. Overall, these observations imply that interaction effects on ADME processes are not relevant at environmentally relevant concentrations.

To understand and reveal if and at which concentrations interaction effects on ADME may occur, a binary PBK model was developed, and simulations were conducted with varying levels of PFOS mixed with set levels of each BPZ and BPA. At μg/L PFOS levels, the bisphenols started to show different distribution pattern and lower C_{max} in tissues. The simulations indicated that the free fraction in blood of bisphenols decreased due to weaker plasma protein binding as compared with PFOS leading to faster elimination via the gills and lower tissue concentrations. The simulation is, however, only an indication for potential effects and has to be interpreted with consideration of its limitations. It should be noted that the binary PBK model has not been validated and that simulated PFOS levels were above those used to develop the single PFOS model used as a basis. However, modelled changes in tissue levels align well with recent in vivo observations with short- and long-chain PFAS kinetics in zebrafish (Wen et al., 2017). This phenomenon could be

relevant in laboratory settings, in highly polluted waters or if an organism is exposed to a complex mixture of chemicals acting in concert. Overall, these modelling results highlight the importance of further investigation on plausible kinetic interaction effects in mixture exposure scenarios as well as development of PBK models capable of accounting for interaction effects on ADME processes including metabolism and uptake processes; and physiological parameters.

CRediT authorship contribution statement

Elena Golosovskaia: Writing – review & editing, Writing – original draft, Visualization, Software, Investigation, Formal analysis, Data curation, Conceptualization. **Stefan Örn:** Writing – review & editing, Resources, Methodology, Formal analysis. **Pim Leonards:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis. **Jacco Koekkoek:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis. **Patrik L. Andersson:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

Patrik L. Andersson reports financial support was provided by Swedish Research Council Formas. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2025.178663.

Data availability

Data will be made available on request.

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