



Full length article

Investigating the bioaccumulation potential of anionic organic compounds using a permanent rainbow trout liver cell line

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ABSTRACT

Permanent rainbow trout (*Oncorhynchus mykiss*) cell lines represent potential *in vitro* alternatives to experiments with fish. We here developed a method to assess the bioaccumulation potential of anionic organic compounds in fish, using the rainbow trout liver-derived RTL-W1 cell line. Based on the availability of high quality *in vivo* bioconcentration (BCF) and biomagnification (BMF) data and the substances' charge state at physiological pH, four anionic compounds were selected: pentachlorophenol (PCP), diclofenac (DCF), tecloftalam (TT) and benzotriazol-*tert*-butyl-hydroxyl-phenyl propanoic acid (BHPP). The fish cell line acute toxicity assay (OECD TG249) was used to derive effective concentrations 50 % and non-toxic exposure concentrations to determine exposure concentrations for bioaccumulation experiments. Bioaccumulation experiments were performed over 48 h with a total of six time points, at which cell, medium and plastic fractions were sampled and measured using high resolution tandem mass spectrometry after online solid phase extraction. Observed cell internal concentrations were over-predicted by K_{OW} -derived predictions while pH-dependent octanol–water partitioning (D_{OW}) and membrane lipid-water partitioning (D_{MLW}) gave better predictions of cell internal concentrations. Measured medium and cell internal concentrations at steady state were used to calculate RTL-W1-based BCF, which were compared to D_{OW} - or D_{MLW} -based model approaches and *in vivo* data. With the exception of PCP, the cell-derived BCF best compared to D_{OW} -based model predictions, which were higher than predictions based on D_{MLW} . All methods predicted the *in vivo* BCF for diclofenac well. For PCP, the cell-derived BCF was lowest although all BCF predictions underestimated the *in vivo* BCF by ≥ 1 order of magnitude. The RTL-W1 cells, and all other prediction methods, largely overestimated *in vivo* BMF, which were available for PCP, TT and BHPP. We conclude that the RTL-W1 cell line can supplement BCF predictions for anionic compounds. For BMF estimations, however, *in vitro*-*in vivo* extrapolations need adaptation or a multiple cell line approach.

1. Introduction

Bioaccumulation is an important parameter for environmental risk assessment of organic compounds as the accumulated compounds potentially endanger environmental and human health. Bioaccumulation occurs as bioconcentration from the surrounding environment, expressed as bioconcentration factor (BCF), or as biomagnification from dietary uptake, expressed as biomagnification factor (BMF). Most commonly fish, and more specifically, rainbow trout (*Oncorhynchus mykiss*), are used for bioaccumulation assessments concerned with the aquatic environment (OECD, 2012). Such assessments are standardized according to OECD Test Guideline (TG) 305, where enrichment and depuration of a compound are

measured in week-long exposures of over 100 fish in a resource-intensive and ethically questionable manner (OECD, 2012).

One alternative method to assess bioaccumulation in fish are modelling approaches, which predict bioaccumulation with varying degrees of complexity and, most commonly, on the bases of the compound's octanol–water partition coefficient, K_{OW} (Nichols et al., 2013). The octanol phase is used as surrogate of the organisms' lipid phase, which is assumed to drive the bioaccumulation of neutral compounds. However, such K_{OW} – based models often overestimate bioaccumulation relative to the *in vivo* reference, since depuration processes, such as biotransformation, are not considered (Laue et al., 2020; Fu et al., 2009).

In vitro methods have been developed to obtain information about

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the capacity of fish liver to biotransform chemicals. OECD TG319a/b detail the use of freshly isolated and cryopreserved hepatocytes or of S9 fractions from fish to determine biotransformation rates (OECD, 2018a; OECD, 2018b). Via *In Vitro* - *In Vivo* Extrapolations (IVIVE), these biotransformation rates aid in the refinement of bioaccumulation models (Nichols et al., 2013; OECD, 2018a; OECD, 2018b). Yet, these *in vitro* methods still require the sacrifice of fish; as well, the activity of the hepatocytes and S9 fractions may vary depending on the health status and strain of the fish, the season of isolation and the isolation procedure itself.

Another *in vitro* alternative, which avoids experimentation with fish altogether, are permanent fish cell lines (Stadnicka-Michalak et al., 2014; Stadnicka-Michalak et al., 2018b). Two approaches for the prediction of bioaccumulation in fish were tested with permanent fish cell lines to date using cell lines from rainbow trout, which stem from gill (RTgill-W1, (Bols et al., 1994), liver (RTL-W1, (Lee et al., 1993) and intestinal tissue (RTgutGC, (Kawano et al., 2011)). The simpler of the two approaches derives a BCF directly from measured cellular concentrations in an IVIVE procedure: the cellular concentration of a compound at steady state, expressed as per cell mass, divided by the exposure medium concentration (Stadnicka-Michalak et al., 2014). This was done using the RTgill-W1 cell line in Stadnicka-Michalak et al. (Stadnicka-Michalak et al., 2014), though the focus of that study was on extrapolation of internal effect concentrations and not a comparison to *in vivo* bioaccumulation. In the second approach, the measured *in vitro* biotransformation rate of the test compound serves as input parameter for physiology-based toxicokinetic (PBTK) models, enabling IVIVE and BCF prediction (Stadnicka-Michalak et al., 2022). This approach was successfully demonstrated for all three above mentioned rainbow trout cell lines for the polycyclic aromatic hydrocarbon, benzo(a)pyrene (Stadnicka-Michalak et al., 2018b).

Cell line-based approaches operate on a small scale, which has advantages in terms of test material needs and waste produced. Yet, the small scale makes quantifying test compounds in cell samples challenging due to the minute amounts added to these test systems. Stadnicka-Michalak et al. (Stadnicka-Michalak et al., 2014; Stadnicka-Michalak et al., 2018b) addressed this challenge by using radiolabeled test compounds (Stadnicka-Michalak et al., 2014, 2018b). More recently, fish cell-internal concentrations have also been determined in a mass balance-type approach for non-radiolabeled compounds (Chen et al., 2014; Pietsch et al., 2014; Schug et al., 2018; Schug et al., 2019; Mahoney et al., 2022). One of these studies examined the formation of biotransformation products (BTP) in the RTgill-W1 and RTL-W1 cell lines upon test compound exposure and found a putative BTP, a hydroxy-metabolite in RTL-W1 cells, which implies the activity of phase I biotransformation enzymes (Mahoney et al., 2022). Other studies, which examined the transfer of volatile and hydrophobic organic compounds across an RTgutGC epithelium, demonstrated biotransformation activity and suggested to use the test system for different cell lines and for the identification of BTPs of test compounds (Schug et al., 2018; Schug et al., 2019). These advances in applications of fish cell lines and analytical methods provide impetus to expand these types of investigations to a wider chemical space.

Ionizable organic compounds (IOC) are a group of chemicals for which the mechanisms and extent of bioaccumulation is little understood. IOC are distinct from neutral compounds by having a charged fraction, either permanently or depending on the pH of the surrounding milieu, which results in organic anions, cations or zwitterions. IOC comprise a great number of chemical classes (Arp et al., 2017; Franco et al., 2010) and are used as, for example, surfactants (Fredell et al., 1994; Cowan-Ellsberry et al., 2014; Kahrilas et al., 2015), pharmaceuticals (Manallack, 2007) and pesticides (Fawcett et al., 1994), with the concurrent release into the aquatic environment (UNESCO, 2009; Sánchez-Bayo and Wyckhuys, 2019; Trombini et al., 2020). The bioaccumulation of IOC strongly depends on their molecular structure. For example, pharmaceuticals, such as the anionic diclofenac, express a low

bioaccumulation in fish compared to neutral compounds despite a log K_{OW} of > 4 (Schwaiger et al., 2004; Memmert et al., 2013). However, certain anionic surfactants, such as perfluoroalkyl substances and long chained alkyl sulfonates (alkyl chain ≥ 14) (Martin et al., 2003; Inoue et al., 2012; Ribbenstedt et al., 2022), exert a high bioaccumulation in fish relative to other anionic compounds (Könnecker et al., 2011; Tolls et al., 2000; Mueller et al., 2020). Anionic compounds that lack the typical surfactant-like structure but have a largely hydrophobic surface, such as pentachlorophenol, also show high bioaccumulation in fish (McKim et al., 1986; ECHA, 2017). Thus, it seems that the neutral and charged fraction of an IOC determine bioaccumulation (Armitage et al., 2017). In accordance with this observation, the K_{OW} was found to be an inappropriate descriptor for the bioaccumulation of IOC that are mainly or permanently charged (Fu et al., 2009). The pH-dependent octanol–water distribution ratio, D_{OW} (K_{OW} corrected for the neutral and charged fraction at a specific pH), appeared more suitable for bioaccumulation prediction (Arnot and Gobas, 2003; Fu et al., 2009). Recent investigations found that the compound's pH-dependent membrane lipid–water distribution ratio, D_{MLW} , describes the accumulation in fish for surfactants well, since the cell membranes appear to be the main sink for surfactants in fish (Avdeef et al., 1998; Armitage et al., 2013; Bittermann et al., 2014; Goss et al., 2018; Droge et al., 2021; Ribbenstedt et al., 2022). Beside the cell membranes' phospholipid, the interaction with proteins can pose another significant matrix for bioaccumulation (Rodgers et al., 2005; Poulin and Theil, 2000; Rodgers and Rowland, 2006; Trainor et al., 2007; Schmitt et al., 2008) as shown for perfluorooctanoic acid (Han et al., 2003). Further, membrane-integrated proteins can play an important role in the uptake and efflux of IOC, which would otherwise permeate the cell membrane in negligible amounts (Armitage et al., 2017). Comprising functional entities with phospholipids and proteins in place, it is reasonable to assume that fish cell lines possess all the relevant matrices to assess the bioaccumulation potential of IOC in fish.

We therefore set out in this study to assess the potential of the RTL-W1 fish cell line to predict the bioaccumulation of four purposefully selected anionic organic compounds in fish. The RTL-W1 cell line was selected for testing because it represents the liver as primary organ for biotransformation and is among the best studied fish cell lines with regard to its biotransformation capabilities (Lee et al., 1993; Nehls and Segner, 2001; Thibaut et al., 2009; Stadnicka-Michalak et al., 2018b). Indeed, the expression of cytochrome CYP1A, glutathione-S-transferase, sulfotransferase and UDP-glucuronosyltransferase have been confirmed in this cell line (Lee et al., 1993; Nehls and Segner, 2001; Thibaut et al., 2009; Stadnicka-Michalak et al., 2018b). We hypothesized that the RTL-W1 cell line possesses the principal ability to accumulate and biotransform IOC and that derived *in vitro*-based BCF are comparable to *in vivo* bioaccumulation. To test this hypothesis, we 1) determined non-toxic exposure concentrations for bioaccumulation experiments based on the established RTgill-W1 cell viability assay following OECD TG249 (Stadnicka-Michalak et al., 2018a; OECD, 2021); 2) established a method to measure cell-internal concentrations of anionic organic compounds in RTL-W1 cell cultures over time for mass balance analysis; and 3) derived *in vitro* BCF from measured concentrations in cells and exposure medium and compared these with *in vivo* data and common BCF prediction methods.

2. Materials and methods

2.1. Test compound selection

Detailed information on the test compounds is documented in the supporting information (SI): CAS registry number, structural formula, vendor and purity in SI Table S1, and influence of pH on degree of ionization in SI Figure S1. Three criteria were applied to select the four test compounds. The first criterion was that high quality, in line with OECD TG305 (OECD, 2012), bioaccumulation data in rainbow trout are

available for reference. The second criterion was that the compounds are largely negatively charged at physiologically relevant pH (7–8) while the third criterion was environmental relevance. Based on these criteria, the selected anionic compounds were: benzotriazol-*tert*-butyl-hydroxyl-phenyl propanoic acid (BHPP), diclofenac (DCF), pentachlorophenol (PCP) and tecloftalam (TT). BHPP belongs to a group of ultraviolet stabilizers, which are most prominently used as additive in plastic polymers and car paints (Crawford, 1999; Himmelsbach et al., 2009). DCF is a well-known pharmaceutical used for its pain relieving and anti-inflammatory properties (Maddrey et al., 2013; NHS, 2022). PCP is a pesticide disinfectant that was used in the past as a preservative in wood, leather, agricultural seeds and in paper mill systems (Kobayashi, 1979), but has been listed in Annex A of the Stockholm Convention due to its high toxicity and environmental persistence since 2015 (Stockholm Convention, 2022). TT is a pesticide used to control bacterial leaf blight (*Xanthomonas oryzae*) in rice (Kirkpatrick et al., 1981; Ngo et al., 2016). It is expected to end up in the aquatic environment, although this has not been addressed in the accessible scientific literature. In contrast, BHPP, DCF and PCP have been ubiquitously found in surface waters and in aquatic organisms (Holmbom, 1980; Oikari and Kunnamo-Ojala, 1987; Loos et al., 2009; Lu et al., 2019; Trombini et al., 2020).

2.2. Preparation of stock solutions of test compounds

The test compounds (Table 1) were delivered via dimethyl sulfoxide (DMSO, CAS 67–68-5, Sigma Aldrich) stock solutions in concentrations that resulted in a DMSO concentration of 0.5 % (v/v) in the final exposure medium of the fish cell line acute cytotoxicity assays (OECD TG249 (OECD, 2021), while it was 0.1 % (v/v) for the bioaccumulation experiments (same as in OECD TG 319b (OECD, 2018)). The same DMSO stock solution per test compound, stored at -20 °C in between experiments, was used for all biological replicates for each cytotoxicity or bioaccumulation experiment. The computation of the compound's K_{OW} values (of the neutral species) has been attempted with the software COSMOtherm but was not successful for some of the substances. Therefore, the K_{OW} and D_{OW} were taken from other estimation programs as detailed in Table 1.

2.3. Cell culture

RTL-W1 (Lee et al., 1993) and RTgill-W1 cell lines (Bols et al., 1994) were routinely cultured in cell culture flasks with 150 cm² growth area (Techno Plastic Product AG) at 19 ± 1 °C in the dark at normal atmosphere in 20 mL routine cell culture medium, i.e. Leibovitz's medium (L-15, Invitrogen), supplemented with 5 % (v/v) fetal bovine serum (FBS, Eurobio Scientific). When a cell culture reached approximately 95 % confluency, the cells were diluted in a 1:1 (v/v) ratio to obtain two new cell culture flasks or seeded for an experiment. To detach the cells, the medium was removed, the cell layer rinsed twice with 1.4 mL Versene solution (Gibco™ Versene Solution, Thermo Fisher Scientific Inc.) and 0.7 mL trypsin (Pan Biotech) added. When the cell layer visibly detached

from the plastic bottle, the trypsin reaction was stopped by the addition of routine cell culture medium, after which cells were brought into suspension. For the acute cytotoxicity assays with RTgill-W1 cells, cell passages 62 to 74 were used; for confirmation of non-toxic concentrations and the bioaccumulation experiments with RTL-W1 cells, cell passages were 80 to 94.

2.4. Determination of non-toxic exposure concentrations

It was crucial to use non-toxic exposure concentrations of the test compounds in the bioaccumulation experiments to avoid that toxic effects mask the accumulation behavior of the exposed cells. RTL-W1 cells cannot easily be transferred to the test conditions of the standardized acute cytotoxicity assay, due to the absence of FBS in the exposure medium in this test (OECD, 2021). Therefore, acute cytotoxicity assays were conducted with RTgill-W1 cells according to OECD TG249, assuming an overall comparability in the acute cytotoxicity between rainbow trout cell lines, as it was demonstrated for RTgill-W1 vs RTgutGC cells by Schug et al. (2020). In brief, RTgill-W1 cells were exposed in a 24 well plate format to a range of six exposure concentrations of a single test compound dissolved in DMSO (OECD, 2021) with a 48 h exposure duration. For the exposures, the protein-free type of Leibovitz's medium, L-15/ex, was applied (OECD, 2021). At the start and termination of exposure (C_{0h} and C_{48h}), the exposure medium, was sampled for later chemical analysis (SI section 2, Table S2). After exposure, cell viability was quantified based on a set of three fluorescent indicator dyes. These were alamarBlue™, 5-carboxyfluorescein diacetate acetoxy methyl ester (CFDA-AM) and Neutral Red, indicating cell metabolic activity, cell membrane integrity and lysosomal membrane integrity, respectively (OECD, 2021). The raw fluorescent data were expressed as % of control and corrected for the actual measured exposure concentrations based on the geometric mean, which was calculated with C_{0h} and C_{48h} of the respective exposure concentrations of the biological replicates. These data were used to produce sigmoidal concentration–response curves of cell viability (OECD, 2021) and calculate the effective concentrations causing 50 % decline of cell viability (EC_{50}). Moreover, the non-toxic concentrations were calculated in an online application (Stadnicka-Michalak et al., 2018c), according to Stadnicka-Michalak et al. (2018a), which applies an algorithm on the toxicity data to determine a reproducible and conservative estimate.

The exposure concentrations for the bioaccumulation experiments were chosen based on three criteria: 1) the exposure concentration should be as low as possible and not exceed the non-toxic concentrations (see above); 2) to avoid enzyme inhibition in the exposed cells, the exposure concentration should be ≤ 1 μM (OECD, 2018), and 3) the chosen concentration should be at least 10 times above the method limit of quantification (LOQ). Final exposure concentrations for the bioaccumulation experiments were confirmed to be non-toxic upon exposure of RTL-W1 cells over 72 h (SI section 2, Table S3, Figure S2), i.e. the longest time span foreseen for the bioaccumulation studies (see below). For this purpose, the same fluorescence-based cytotoxicity assay was

Table 1

Overview of test compounds with usage and key physico-chemical properties. Partition coefficient estimations were taken from the same source to keep uncertainties comparable.

Test compound	Abbreviation	CAS	Category/Usage	pK_a^5	$\log K_{OW}^6$	$\log D_{OW}$ (pH 7.4) ⁷	$\log D_{MLW}^8$
Benzotriazol- <i>tert</i> -butyl-hydroxyl-phenyl propanoic acid	BHPP	84268–36-0	UV stabilizer in plastics and paints ¹	4.65	4.23	1.75	2.2
Diclofenac	DCF	15307–79-6	Pharmaceutical ²	4.18	4.04	1.37	1.4
Pentachlorophenol	PCP	87–86-5	Pesticide Disinfectant ³	4.68	4.76	2.45	2.9
Tecloftalam	TT	76280–91-6	Pesticide ⁴	1.07	5.48	3.13	2

¹(Crawford, 1999; Himmelsbach et al., 2009); ²(Maddrey et al., 2013; NHS., 2022); ³(Kobayashi, 1979; Stockholm Convention, 2022); ⁴(Kirkpatrick et al., 1981; Ngo et al., 2016); ⁵ACD/Labs prediction; ⁶Mean of EPI Suite and ACD/Labs predictions; ⁷ACD/Labs; ⁸Prediction method in Armitage et al. (2013).

applied as outlined above, but with 5 % FBS supplementation of the exposure medium to sustain the viability and metabolic activity of the RTL-W1 cells.

2.5. Experimental procedure for bioaccumulation assessment

The presented experimental procedure was inspired by previous studies that focused on IVIVE using rainbow trout cell lines to predict bioaccumulation in fish (Stadnicka-Michalak et al., 2014). Initially, two formats were considered, all using RTL-W1 cells: 24 well plates (1.9 cm² growth area/well, Greiner Bio-One) holding 2.5×10^5 cells/well in a volume of 1 mL and cell culture flasks (25 cm² growth area/flask, Techno Plastic Product AG) holding 3.3×10^6 cells/flask in a volume of 6 mL. Cell internal concentrations were better measurable in the flask format (SI section 3); it hence was decided to conduct the bioaccumulation experiments in the 25 cm² cell culture flasks.

2.6. Cell seeding and exposure start

For each experimental run, ten 25 cm² cell culture flasks were seeded with 3.3×10^6 RTL-W1 cells per flask in a volume of 6 mL routine cell culture medium. This cell density ensured a confluent monolayer with minimal fluctuation of cell number during the experimental duration (SI section 4, Figure S3). Cells from routine culture at about 95 % confluency were detached and suspended as described above for cell counting, using the electronic cell counting system CASY TCC (BIO-VENDIS Products GmbH). A 10 µL sample of the cell suspension was diluted in 10 mL CASY solution and the cell number, viability and cell diameter measured in two technical replicates, aiming for no more than ± 10 % variability to be valid. The seeding density was calculated and the cell suspension appropriately prepared in routine cell culture medium. Seeded cells were incubated for 48 h to 72 h under routine cell

culture conditions (see above) to obtain the confluent monolayers for experimentation. The experiments were started by the removal of the cell culture medium and the addition of 3 mL of L-15 medium with test compound solved in DMSO and 5 % FBS (v/v) to sustain the cells (Lee et al., 1993).

2.7. Bioaccumulation experiments

Fig. 1 depicts the experimental set up and all subsequent sampling and extraction steps. Two flasks were sampled at each sampling time point, which were 0 h, 4 h, 8 h, 16 h, 24 h and 48 h of exposure: the flask with exposed cells (A, Fig. 1) and the cell-free negative control (B). At experimental onset and termination, the cell count control (C) and the process control (D) were sampled additionally. The process control contained cells but no test compound and was used to account for potential chemical background contamination, while the cell count control contained cells and test compound and was used to monitor changes in cell number. For the latter, cells were sampled and numbers determined with the CASY TCC as described above. Cell numbers of all bioaccumulation experiments are reported in the SI (section 5, Figure S4). Finally, the prepared exposure medium was stored at 19 °C during the experiment and sampled at the onset and termination of each experiment to monitor the test compound's stability. These samples served as reference to account for potential abiotic degradation of the test compounds. Each bioaccumulation experiment was done in two independent experiments per test compound with one sample per treatment and sampling time point (i.e. one cell culture flask). We considered two independent experiments sufficient, because neither opposing trends nor unreasonable variations in cell internal concentrations were observed in the two replicates (SI, section 5, Table S5 and S6).

Three fractions of the test system were considered relevant for sampling: the exposure medium, the cell monolayer and the plastic, i.e.

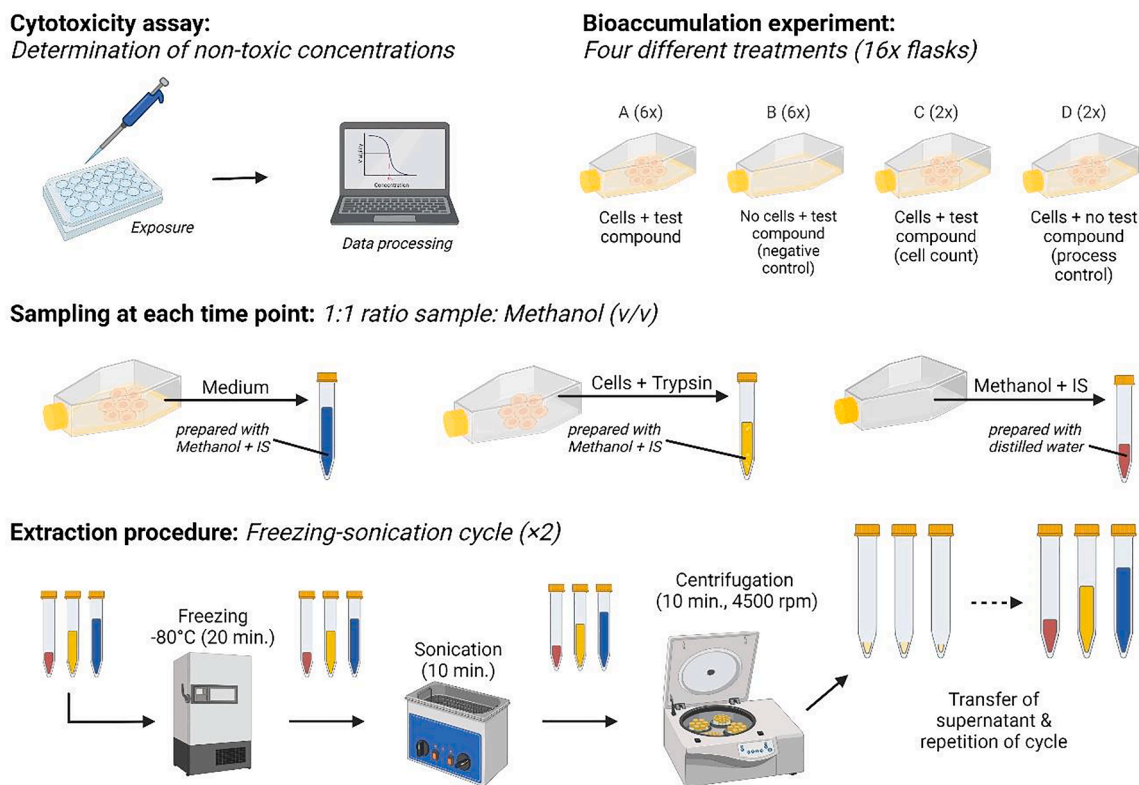


Fig. 1. Work flow from exposure concentration determination to bioaccumulation experiments. In a first step, a suitable non-toxic exposure concentration was determined for each compound. Each bioaccumulation experiment had four different treatment types to (A) measure test compound accumulation in cells, (B) to monitor abiotic loss, such as sorption to plastic, (C) count the cell number over the experimental duration, and (D) monitor background contaminations of test compound during exposure and sampling. IS = Internal Standard.

test compound adsorbed to it (Fig. 1). In contrast, since IOC are not volatile in their charged state, the air-filled headspace was not considered. All samples were collected in 15 mL centrifuge tubes (91015, TPP Techno Plastic Products AG). To obtain the three fractions, the medium was sampled first. A volume of 1 mL was sampled for pH measurement by means of a small pH probe (microFET, Welling) to account for potential pH differences, which might affect the IOC ionization state. The remaining 2 mL were then sampled for chemical quantification in the medium fraction. Next, the cell surface was rinsed for 30 s with 3 mL of test compound-free cell culture medium. 2 mL of this rinse solution were pooled with the initial 2 mL of exposure medium and the remaining rinse medium was discarded. Cells were harvested by the addition of 1 mL trypsin solution followed by incubation until the cell monolayer visibly detached and further dislodged by use of a cell scraper (Techno Plastic Products AG) to ensure complete capture of cells. The trypsin solution was sampled and an additional 1 mL of trypsin solution added to the flask to collect all remaining cells. This second trypsin application was combined with the first trypsin sample. The rinsing steps for exposure medium and cells were necessary to clearly distinguish the test compounds associated with each sample fraction and reduce carry over across the different sample matrices (SI section 5.2, Figure S5). At last, the test compound sorbed to plastic was sampled by the addition of 2 mL methanol, containing internal standard. The flask, now only containing methanol, was shaken on a plate shaker for 5 min at 200 rpm, after which the methanol was transferred to a 15 mL centrifuge tube and diluted with distilled water (CAS 7732-18-5) in a 1:1 (v/v) ratio. The medium and cell samples were each diluted with methanol in a 1:1 ratio (v/v), to assure sufficient extraction of test compounds and aid protein precipitation. The applied trypsin and methanol solutions were sampled and extracted in the same manner and measured to account for background contaminations.

For sample extraction and matrix removal, all samples were frozen for 20 min at -80°C , and sonicated for 15 min at room temperature thereafter (Fig. 1). Then, the samples were centrifuged for 10 min at 4347 m/s^2 to precipitate the protein and cell debris. The supernatant was transferred into a new 15 mL centrifuge tube. The samples went through the extraction process twice to remove the matrix and were stored at -20°C until chemical analysis. Mass balances were derived at each sample time point according to Equation (1):

$$\% \text{ of total amount} = \frac{Y_t}{\sum Y_t} \quad (1)$$

where the compound amount in fraction Y_t (ng) was either taken from the exposure medium, the cells or the plastic and sum of total compound amount in test system, $\sum Y_t$ (ng), both present at a sample time point t . Further, the total summed up amounts at each time point, $\sum Y_t$, were compared to the initially added amount at the experimental start $\sum Y_{0h}$, to detect potentially occurring biotransformation activity or uncontrolled losses:

$$\% \text{ of total initial amount} = \frac{\sum Y_t}{\sum Y_{0h}} \quad (2)$$

2.8. Derivation of in vitro-based bioconcentration factors

Using the mean cell number (C , Fig. 1), the mean cell diameter, d ($16.6\text{ }\mu\text{m}$), and the calculated absolute amount of test compound in the cell samples, $cell_t$, (SI, section 5.1, Table S5), the internal cellular concentration, C_{cell} , at steady state was determined, assuming that the cell volumes can be approximated as being spherical (Stadnicka-Michalak et al., 2018b):

$$C_{cell} \left[\frac{\text{ng}}{\text{L}} \right] = \frac{cell_t [\text{ng}]}{\text{mean cell number of experiment} \times \left(\frac{1}{6} \times \pi \times d^3 \right) [\text{L}]} \quad (3)$$

The *in vitro* BCF (RTL-W1 BCF, Equation (4)) was calculated as the ratio of C_{cell} over the measured exposure medium concentration, C_{medium} ,

per biological replicate and the mean of those was used for presentation (SI, section 5.3, Table S8):

$$\text{RTL} - \text{W1 BCF} = \frac{C_{cell}}{C_{medium}} \quad (4)$$

The obtained RTL-W1 BCF values were compared to other common prediction methods, including *in vivo* data (SI, section 5.3, Table S9), to assess the RTL-W1 cell's suitability for bioaccumulation prediction. The prediction methods covered empirical regression-based models that use the compound's D_{OW} , (Bruijn et al., 2022); BCF_{DOW} or the compound's D_{MLW} (Droge et al., 2021), BCF_{DMLW} . Further, a more refined prediction tool that applies the compound's D_{OW} in a one-compartment PBTK model was applied (Nichols et al., 2013, PBTK with K_{OW}/D_{OW}). The BCF_{DOW} and the BCF_{DMLW} , together with the PBTK model predictions, are referred to as "numerical predictions" in the following.

2.9. Chemical analysis

For quantification, 1 mL of sample extract was added to 19 mL distilled water and enriched via online solid phase extraction prior to measurement using a high performance liquid chromatography - electrospray ionization - tandem high resolution mass spectrometry system (HPLC-ESI-HRMS/MS, QExactive or QExactive Plus, Thermo Fisher Scientific) (Lauper et al., 2022). The chromatographic separation on the column (XBridge C18, $3.5\text{ }\mu\text{m}$, $2.1 \times 50\text{ mm}$) was achieved by a methanol/water gradient (SI section 6.1), both containing formic acid (0.5 % (v/v)). In addition to the target screening of the test compounds, a suspect screening for known and suspected biotransformation products of the test compounds was performed and evaluated using Compound Discoverer 3.3 (Thermo Fisher Scientific). For further details on the analysis set-up, see SI section 6.

Target screening for all test compounds was performed by taking full scan MS (resolution of 70 000 at m/z 200) with subsequent data-dependent MS2 acquisition (resolution of 17 500, isolation window of 1 m/z) in positive mode for BHPP and DCF, while TT and PCP were measured in negative mode. Quantification was done by standard calibration in ultrapure water (with equivalent percentage of methanol as in sample) using internal standards. The Software Tracefinder 4.1 (Thermo Fisher Scientific) was used to analyze the obtained MS data. The limits of quantification were determined by the peak shape with at least five mass scans forming the peak and a signal to noise ratio greater than ten. Isotope labeled homologs were only available for DCF (DCF- D_4) and PCP (PCP- $^{13}\text{C}_6$) and used as internal standards. For BHPP and TT, the best fitting internal standard was mefenamic acid- D_4 , as it showed closest retention time and structural similarity to the test compounds. For samples in which a detection of test compound was possible but below LOQ, half of the LOQ ($0.5 \times \text{LOQ}$ (ng/L)) was used to approximate the test compound amount. To correct for potential matrix effects and compound losses during the extraction process (Fig. 1), a known amount of each test compound was spiked into exposure medium, harvested cell solution and pure methanol from the plastic fraction (relative recovery, SI, section 6.2, Table S11). These samples went through the same extraction process as outlined above (section 2.7) and the recovery was determined (SI, section 6.2, Table S12). Table S11 in the SI (section 6.1) contains the method LOQ for the different matrices as well as the final exposure concentrations per compound, the matrix-dependent relative recoveries and the comparison of measured and nominal exposure concentrations in the bioaccumulation experiments.

2.10. Data analysis and visualization

Obtained sample concentrations were further analyzed and visualized using the programming language R (Core Team, 2020) and the packages openxlsx, tidy, dplyr, ggplot and patchwork (Schauberger and Walker, 2022; Pedersen, 2022; Wickham, 2022; Wickham et al., 2022; Wickham et al., 2016). Software Graphpad Prism 9.4.0 (GraphPad

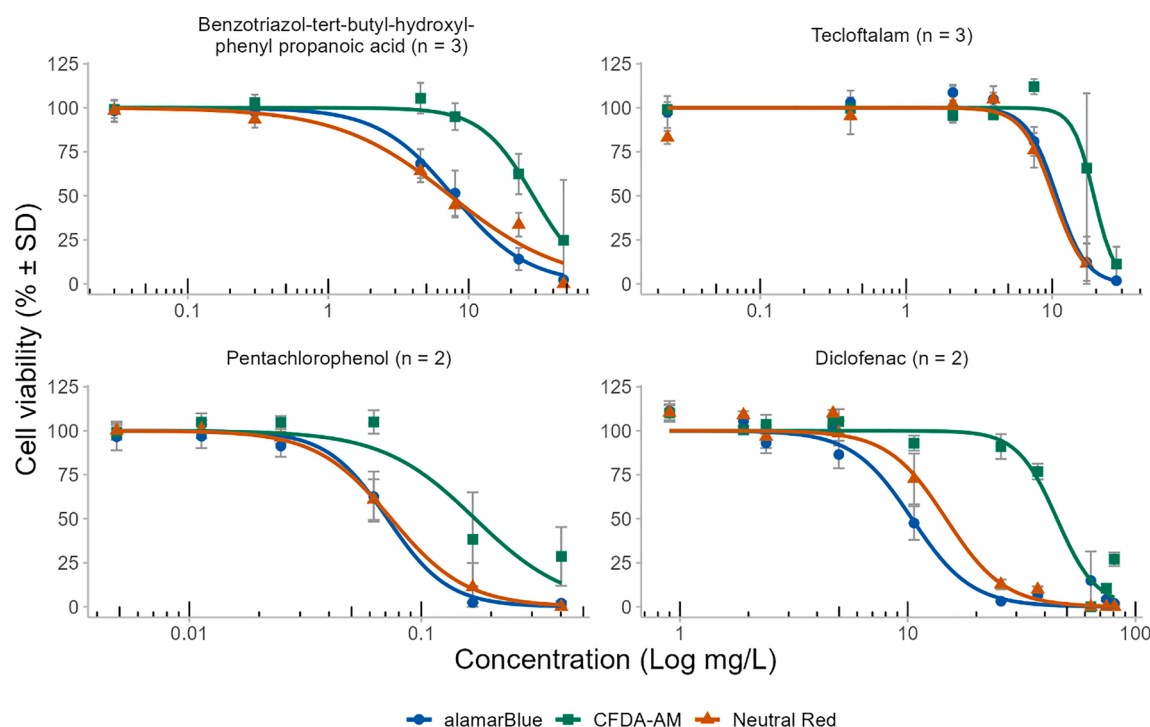


Fig. 2. Acute cytotoxicity of the test compounds to RTgill-W1 cell cultures over 48 h exposure. alamarBlue indicates for cell metabolic activity, CFDA-AM for cell membrane integrity and Neutral Red for lysosomal membrane integrity. Errors bars = standard deviation. For more information, consult Table S2 and SI section 2. n = number of independent replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Software, US) and Biorender (BioRender.com, Toronto) were also used for visualization.

3. Results and discussion

3.1. General observations

The goal of this study was to assess the potential of a fish liver cell line, RTL-W1, to predict the bioaccumulation potential of anionic organic compounds in rainbow trout. This required careful method set-up, including determination of non-toxic exposure concentrations and chemical quantification for mass balance analysis. The results were finally put into the context of bioaccumulation predictions in fish.

3.2. Impact of chemicals on cell viability

All test compounds were toxic to RTgill-W1 cells, following the OECD TG249 acute toxicity assay procedure, with the only variation being a 48 h rather than 24 h exposure duration to account for the

prolonged exposures for bioaccumulation assessment. Cell toxicity data were corrected for the geometric mean (C_{0h}/C_{48h}) of measured compound concentrations (Fig. 2, SI, section 2, Table S2). PCP was the most toxic test compound with its EC_{50} of 72 $\mu\text{g/L}$ (60 – 90 $\mu\text{g/L}$, 95 % confidence interval) being about 100-fold lower relative to the other test compound's EC_{50} values (Table 2). Cell metabolic activity, as measured by alamarBlue, and lysosomal membrane integrity, assessed by Neutral Red, responded more sensitively to compound exposure than cell membrane integrity based on CFDA-AM. This order in sensitivity is commonly observed (Fischer et al., 2019).

The EC_{50} values from the most sensitive RTgill-W1 acute cytotoxicity assay, i.e. cell metabolic activity in all cases, were compared with available *in vivo* LC_{50} values and found to be in good agreement (Table 2) (Tanneberger et al., 2013; Natsch et al., 2018; Fischer et al., 2019; Schug et al., 2020; OECD, 2021). The difference for DCF is largest if the EC_{50} is compared to the LC_{50} in juvenile zebrafish, being 166 mg/L (Praskova et al., 2011) compared to 10.4 mg/L in RTgill-W1, indicating that the cell line is more sensitive to DCF exposure than the juvenile stage in fish, which was observed elsewhere for non-polar chemicals (Tanneberger

Table 2

Results of acute cytotoxicity assay with RTgill-W1 cells. EC_{50} = Concentration causing 50 % reduction in cell viability, CI = confidence interval, LC_{50} = Concentration causing 50 % mortality in tested fish batch, SD = standard deviation, BHPP = Benzotriazol-tert-butyl-hydroxyl-phenyl propanoic acid, DCF = Diclofenac, PCP = Pentachlorophenol, TT = Tecloftalam.

Test Compound	EC_{50} of cell metabolic activity (mg/L [95 % CI])	LC_{50} (mg/L, <i>in vivo</i>)	Reference
BHPP	7.4 [2.9–14.5]	not available	–
DCF	10.4 [8.4–13.4]	$6.9 \pm 1.2^*$, 166^{**}	*mean of 72 h (van den Brandhof and Montforts, 2010) and 144 h exposure (Praskova et al., 2011) in zebra fish embryo, **in juvenile zebra fish (96 h exposure) (Praskova et al., 2011)
PCP	0.072 [0.06–0.09]	0.14 ± 0.03 , 0.19 ± 0.05 , 0.28 ± 0.12 , 0.87 ± 0.45	mean (\pm SD) in various stages of rainbow trout, bluegill, fat head minnow and guppy respectively at 96 h exposure (SI, section 7, Table S13 for references)
TT	10.7 [8.9–13.0]	30 ***	in common carp (Lewis et al., 2016)

et al., 2013; Fischer et al., 2019). A similar observation can be made for the PCP EC₅₀ relative to LC₅₀ from species other than rainbow trout: on average, LC₅₀ were 0.19 mg/L in bluegill (*Lepomis macrochirus*) to 0.87 mg/L in guppy (*Poecilia reticulata*) compared to EC₅₀ of 0.072 mg/L in RTgill-W1. The LC₅₀ for rainbow trout with 0.14 mg/L, however, was only 2-fold higher than the RTgill-W1-based EC₅₀. Species differences in sensitivity may be the reason for the observed discrepancies between *in vivo* and RTgill-W1-based values (Fischer et al., 2019; Laue et al., 2020; Wassenaar et al., 2020).

For PCP, literature EC₅₀ values were available for comparison from identical RTgill-W1 acute cytotoxicity assays. The studies reported EC₅₀ values of 10 µg/L (10 – 20 µg/L 95 % confidence interval) (Tanneberger et al., 2013) and 163 µg/L ± 46 µg/L (mean of interlaboratory study ± standard deviation) (Fischer et al., 2019) after 24 h exposure. Our EC₅₀, with 72 µg/L (61 – 92 µg/L 95 % confidence interval) after 48 h exposure, lies in between this range despite the extended exposure duration. This fits the observation that toxicity generally develops well within 24 h (OECD, 2021) and therefore supports the notion that exposure durations > 24 h appear to often have a negligible influence on cytotoxicity in the RTgill-W1 cell line. This is the first report on the acute cytotoxicity of test compounds in RTgill-W1 with a deliberate focus on the compound's charge. Although only four anionic compounds were tested here, the results add to the growing evidence that the acute cytotoxicity assay with RTgill-W1 cells also predicts acute fish toxicity of negatively charged compounds (Tanneberger et al., 2013; Natsch et al., 2018; Fischer et al., 2019; Schug et al., 2020).

The concentration–response curves were used to derive the non-toxic

exposure concentrations (Stadnicka-Michalak et al., 2018a), which served as one base to set the exposure concentrations for bioaccumulation assessment (section 2.4 and SI section 2). The such chosen exposure concentrations (Fig. 3) were confirmed to be non-toxic in the RTL-W1 cell line under the exposure conditions applied for bioaccumulation assessment, i.e. monolayer exposure in the presence of 5 % FBS, over a period of ≤ 72 h (SI, section 2). These final exposure concentrations were therefore then used to run the bioaccumulation experiments.

3.3. Bioaccumulation experiments

3.3.1. Mass balances and biotransformation activity

Based on the optimization of cell sampling and analytical procedures, all test compounds were recoverable from all sampled test compartments, i.e. medium, cells and plastic (Fig. 3). Comparison between cell-containing flasks and cell-free flasks (negative control; B, Fig. 1) allowed to differentiate compound amounts truly taken up by the cells (SI, section 5, Table S5 and S6). The calculated steady state of accumulated compound in the cells was reached within 14 h (BHPP, PCP and TT) to 24 h (DCF) of experimental duration (for calculation see SI section 8). Up to 4 ± 0.7 % of BHPP, 1.2 ± 0.9 % of TT, 0.34 ± 0.03 % of PCP and 0.24 ± 0.15 % of DCF of the initially added compound mass accumulated in the RTL-W1 cells at steady state (Fig. 3). The by far largest amounts of test compound, ≥ 93 %, were found in the medium, while amounts sorbed to plastic were only minor and well below 1 % or < LOQ (Fig. 3). The mass balances indicate little influence of compound loss

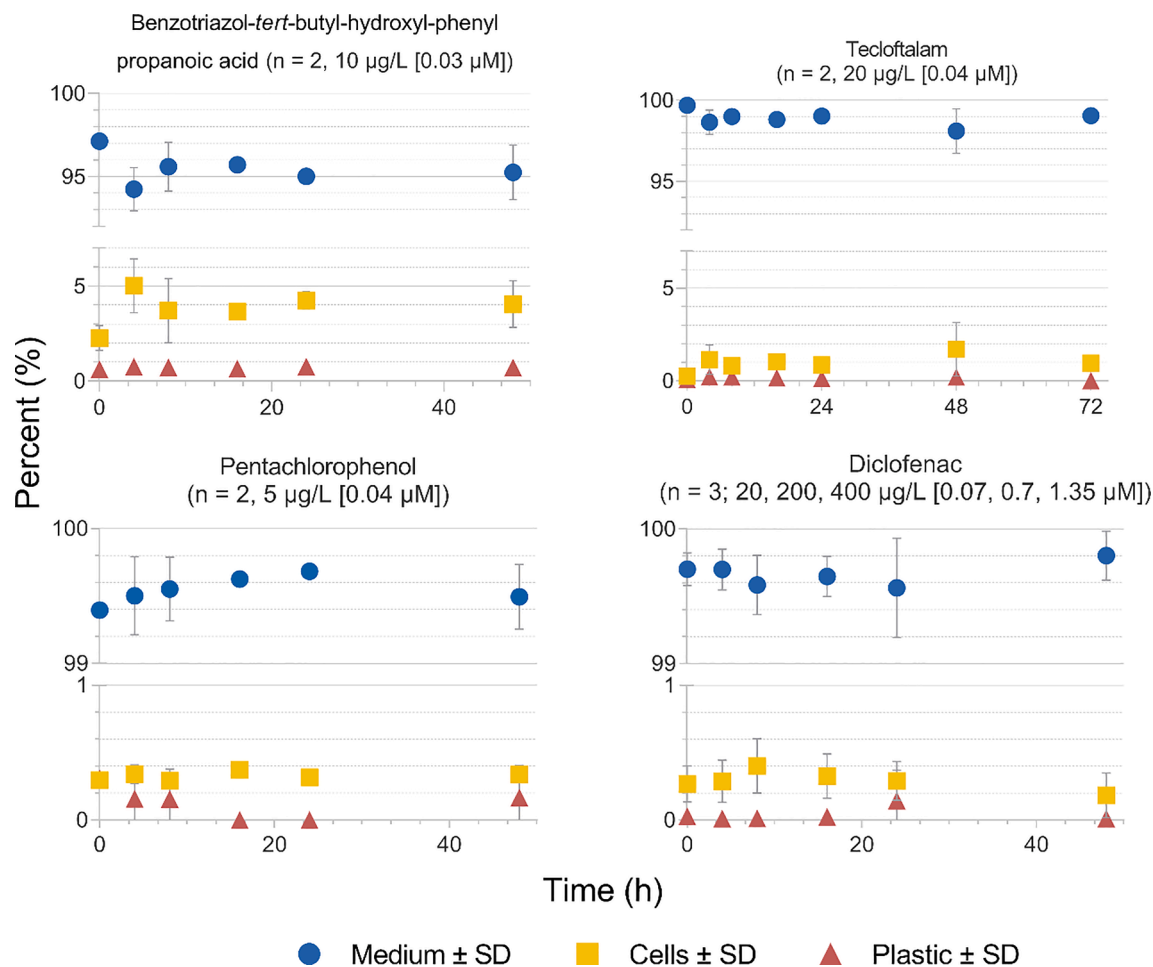


Fig. 3. Distribution of the compounds in the test system during bioaccumulation experiments. Note that for Diclofenac the average of three biological replicates with three different exposure concentrations is depicted. In the second replicate of tecloftalam, the time point 72 h was sampled rather than the 16 h time point. Exposure concentrations given as µg/L and as µM in brackets. SD = Standard Deviation, n = number of biological replicates.

due to biotransformation in the cells: total amounts were on average over all time points around 94 ± 15 % for BHPP (mean \pm standard deviation), 72 ± 29 % for TT, 106 ± 11 % for DCF and 99 ± 2 % for PCP (SI, section 5, Table S5, S6 and section 9, Table S16). In an attempt to improve quantification of chemical distribution and test concentration dependency of bioaccumulation, DCF exposure was explored at different concentrations tested in the same way. These experiments demonstrated an independence of bioaccumulation from the initial exposure concentration and showed that exposure concentrations of 200 $\mu\text{g/L}$ and 400 $\mu\text{g/L}$ were better quantifiable in the cell samples than the initially chosen exposure concentration of 20 $\mu\text{g/L}$ (Fig. 3 and SI, section 10, Figure S6). The pH decreased from 7.4 to 7.0 over the experimental duration irrespective of the compound exposure (SI, section 11, Figure S7), i.e. solely due to the presence of cells. This change in pH only marginally changed the large charged fraction of the test compounds (SI, section 1, Figure S1) but changed the neutral fraction of BHPP, DCF and PCP by approximately 2.5-fold. Under the assumption that the neutral fraction at least partly drives the bioconcentration, an influence of the observed pH changes on test compound accumulation cannot entirely be ruled out.

Stadnicka-Michalak et al. (2014) conducted similar experiments in 24 well plates with RTgill-W1 cells and observed comparable accumulation, i.e. from 0.5 to 2.5 % of added mass, of 8 neutral compounds (3 partly charged) with low to moderate K_{OW} from 0.57 to 4.05 (Stadnicka-Michalak et al., 2014). One exception was PCP, for which an apparent higher accumulation was found in the RTgill-W1 cells, about 6 % of added mass (Stadnicka-Michalak et al., 2014), compared to 0.4 % in the present study. This difference might stem from the different analytical methods. Stadnicka-Michalak et al. (2014) used radiolabeled compounds and liquid scintillation counting without HPLC for sample measurement, while here unlabeled compounds were used in an HPLC-HRMS/MS method. The liquid scintillation counting method without HPLC separation does not differentiate between parent compound and its biotransformation products so that the fraction measured in the cells reflects the sum of those. Interestingly, tetrachlorohydroquinone (THQ), a PCP biotransformation product, was found in low amounts in the exposure medium of PCP-exposed RTL-W1 cells (Pietsch et al., 2014), which has been confirmed in only one *in vivo* study with striped bass to date (Gates and Tjeerdema, 1993). In our measurements, this biotransformation product could not be detected. A possible reason is that Pietsch et al. (2014) applied high (and toxic) PCP-exposure concentrations, which may have facilitated a sufficiently high and detectable production of THQ.

Another reason for the different amounts of accumulated PCP in Stadnicka-Michalak et al.'s work (2014) and the present study may be differing biotransformation capabilities of RTgill-W1 and RTL-W1 cells. For example, biotransformation of benzo(a)pyrene was faster in the RTL-W1 compared to the RTgill-W1 cell line (Stadnicka-Michalak et al., 2018b). Yet, the presented mass balances in the current study neither indicate a measurable loss attributable to biotransformation activity (SI, section 9, Table S16), nor were biotransformation products detected (SI, section 12). Further, the criteria for biotransformation activity of the test guidelines for *in vitro* biotransformation were not met with the exception of a significant slope in the case of PCP (OECD, 2018), SI, section 13, Table S18). This latter finding can be seen as an indication that RTL-W1 perform PCP biotransformation, albeit at a low extent, in line with the finding by Pietsch et al. (2014) and the proposal by Stadnicka-Michalak et al. (2014) for RTgill-W1. Amounts of formed PCP biotransformation products in the RTL-W1 cells may have been too low to be detectable by the applied analytical method. Another reason for the failure to observe biotransformation activity and formed biotransformation products in our experiments may be the uncertainty of the quantification of test compound and the resulting variation in the mass balance. This could mask small biotransformation activity, as discussed above for PCP (SI, section 9, Table S16).

While the removal of xenobiotics in fish via biotransformation, or

rather general elimination, has been extensively studied, knowledge on the responsible biotransformation enzymes in fish and their cell lines is limited (Armitage et al., 2017). The phase I enzymes CYP1A and CYP3A (Schlenk et al., 2008; Mehinto et al., 2010) were shown to be expressed in fish and DCF exposure in rainbow trout was demonstrated to induce cytochrome CYP1A1 gene expression (Mehinto et al., 2010). On a genetic bases, several subfamilies of the cytochrome 450 family were found in rainbow trout (1A, 3A, 2 K and 2 N), along with Flavin containing monooxygenases, nitroreductase, alcohol and aldehyde dehydrogenases, peroxidases and uridine diphosphate glucuronosyltransferase (Schlenk et al., 2008). RTL-W1 cells are known to have an inducible activity of CYP1A (Lee et al., 1993; Stadnicka-Michalak et al., 2018b) and basal activities of 17 β -HSD (dehydrogenation), 5 α -reductase, UDP-glucuronosyltransferase isoforms, phenol sulfotransferase isoforms (Thibaut et al., 2009) and glutathione-S-transferase (Nehls and Segner, 2001). Thus, RTL-W1 cells express enzymes that are involved in phase I (addition of functional group) and II (conjugation) of biotransformation.

DCF and PCP are known to biotransform in rainbow trout (Kobayashi et al., 1976; Kobayashi, 1979; McKim et al., 1986; Renner and Mücke, 1986; Stehly and Hayton, 1989; Oikari and Kunnamo-Ojala, 1987; Frankovic et al., 1995; Cravedi et al., 1999; Mehinto et al., 2010; Kallio et al., 2010; Lahti et al., 2011), while there is no information available on the biotransformation of BHPP and TT in fish. PCP was found to be biotransformed in rainbow trout to dechlorinated congeners (Frankovic et al., 1995) and its glucuronide and sulfate conjugates (Kobayashi et al., 1976; Kobayashi, 1979; Renner and Mücke, 1986; Stehly and Hayton, 1989; Cravedi et al., 1999), while DCF was biotransformed to hydroxylated DCF isomers and a variety of their conjugate isoforms, such as glucuronide, sulfate and glutathione conjugates (Kallio et al., 2010; Lahti et al., 2011; Fu et al., 2020; Kosfeld et al., 2020). However, *in vitro* clearance of DCF in different assays, which used rainbow trout liver tissue, was relatively low (but significantly different from controls) (Connors et al., 2013; Baron et al., 2017; Kosfeld et al., 2020) and we could not detect the formation of DCF BTPs in RTL-W1 cells. Both, the low DCF clearance previously documented *in vitro* and our observations, confirm the general difficulty to detect BTPs in *in vitro* systems. However, it appears that the formation of BTPs by the RTL-W1 cells might not be relevant to predict *in vivo* bioaccumulation as discussed below (section 3.4).

3.3.2. Prediction of cell internal concentrations

When considering the differences of the test compounds' K_{OW} and D_{OW} , the compounds' pH-dependent speciation probably played a role in the observed accumulation in RTL-W1 cells (Timmer and Droge, 2017; Ribbenstedt et al., 2022; Kierkegaard et al., 2020). A simple partitioning exercise, which assumes an accumulation into RTL-W1 cells according to the compounds' K_{OW} , D_{OW} or D_{MLW} , and the cells' approximated fractional volume of lipid and membrane lipid, was conducted to gain an insight into the partitioning of the test compounds (Fig. 4, SI section 14).

This exercise revealed that the K_{OW} consistently overestimates the accumulation in RTL-W1 cells by about two orders of magnitudes. In contrast, the D_{OW} and D_{MLW} -based predictions are mostly within one order of magnitude of the observed accumulation in RTL-W1 cells (SI, section 14, Table S20). It stands out that the D_{OW} and D_{MLW} predictions lie close to each other for all compounds, between 0.2 and 0.7 log units, except for TT. For PCP and DCF, the partition-based prediction appears to suffice to predict the accumulation in RTL-W1 cells, since observed and predicted values were within one order of magnitude. For BHPP, however, the predicted values were about one order of magnitude lower than what was observed in the cells while the observed value lay between the D_{OW} and D_{MLW} predictions for TT. The discrepancies between observed and predicted accumulation may be caused by two reasons. First, the applied input values — partition coefficients and volumetric fractions in cells — are approximations, which could be refined if measured values or refined estimation methods became available. For

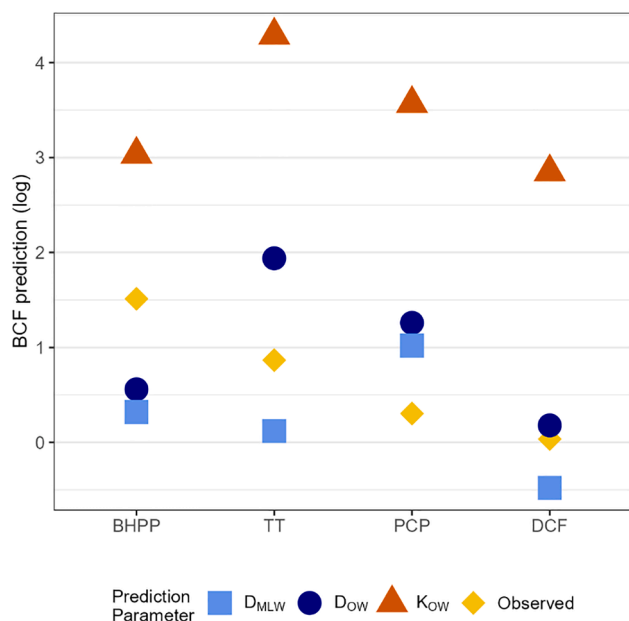


Fig. 4. Comparison of observed and partition coefficient-based accumulation in RTL-W1 cells. Under the assumption of 4 % (v/v) lipid and 1 % membrane lipid content in RTL-W1 cells, the test compound's K_{OW} , D_{OW} or D_{MLW} were applied to predict test compound accumulation in RTL-W1 cells. The RTL-W1 BCF values showed little variation among all replicates per test compound (SI, section 5.3, Table S8). For more information see SI section 14. BHPP = Benzotriazol-*tert*-butyl-hydroxyl-phenyl propanoic acid, TT = Tecloftalam, PCP = Pentachlorophenol, DCF = Diclofenac.

TT for example, D_{OW} values range between -0.76 to 3.13 depending on which estimation software is applied, which results in very different cellular TT accumulations. However, an extensive discussion on the uncertainties of parameter estimations is beyond the focus of the present study. The volumetric cell fraction is calculated from experimental cell counts (section 2.7 and SI section 14) and reflects variations in handling that may influence the partition predictions in terms of cell mass. Second, facilitated transmembrane flux of charged compounds via transport proteins in membranes could modulate the observed accumulation in RTL-W1 cells relative to the partition-based predictions (Leslie et al., 2005; Dobson and Kell, 2008; Sugano et al., 2010; Hagenbuch and Stieger, 2013; Armitage et al., 2017). There is evidence, that RTL-W1 cells do possess such transporter proteins, either because they were studied in the cell line or in rainbow trout and other fish (Fischer et al., 2011; Steiner et al., 2014; Luckenbach et al., 2014; Kropf et al., 2016). It can be assumed that some of the test compounds are substrates of such transporters in the RTL-W1 cells, contributing to the accumulation behavior. For example, organic anion transporting polypeptides may be responsible for xenobiotic uptake (Steiner et al., 2014; Kropf et al., 2016), while some members of the ATP binding cassette transporter family may contribute to xenobiotic efflux (Fischer et al., 2011; Luckenbach et al., 2014).

Another influencing factor, applying to all compounds studied here, is their charge. The neutral as much as the ionized fraction of an IOC may permeate the phospholipid bilayer of the cell membrane although the charged species diffuses considerably slower through the cell membrane compared to the neutral species (Fu et al., 2009; Ebert et al., 2020). The permeation of the ionized species depends on favorable interactions between the test compound's charge and electrostatic charges of the phospholipid bilayer, steric effects, as well as pH gradients across membranes (Fu et al., 2009; Watson, 2015; Ribbenstedt et al., 2022). As summarized in Fu et al. (Fu et al., 2009), acidic organic compounds, which are largely charged at pH 7, show a low BCF, due to their repulsion caused by the negative electrical potential of a cell (Fu et al.,

2009; Watson, 2015). On the opposite, strong basic compounds (positively charged, $pK_a > 7$) accumulate in cells, because of the attraction from the negative cell potential (Fu et al., 2009). Further, Fu et al. (2009) report that a higher BCF for anionic organic compounds along increasing pK_a values from 3 to 6 (Fu et al., 2009) is caused by the ion trap effect. The ion trap mechanism was shown to be present in rainbow trout cell lines (Dayeh et al., 2009; Yue et al., 2015) and is relevant for bioconcentration in fish (Erickson et al., 2006). On a cellular level, it occurs especially when the neutral species permeate the lysosomal membrane and, due to pH differences between cytoplasm (pH ~ 7) and inside the intralysosomal space (pH ~ 5), dissociates inside the lysosome. However, a correlation of pK_a and BCF was not found in the present study, although the pK_a values fall within the range where ion trapping may be observed (Table 1). Testing of compounds with a broader range of pK_a values in RTL-W1 cell exposures would give more insight into the relevance of this effect for IOC uptake into RTL-W1 cells.

It is possible that the charged fraction of test compound sorbed to the cell surface, specifically to positively charged head groups of some phospholipid species (Kierkegaard et al., 2020; Ribbenstedt et al., 2022) or proteins. However, under the consideration that the overall cell surface charge is negative, the sorption of anionic compounds to the external cell surface is likely small. Rather, we predict that the association of cationic compounds with the cell membrane would be more relevant, due to opposing charges of the cell membrane and the compound (Timmer and Droge, 2017). A validated mechanism, which describes the compound-dependent sorption to cell surfaces, has not been developed to date. Therefore, cell surface sorption could not be considered in the presented partition exercise. Dedicated experiments and model approaches are needed to scrutinize the effect and contribution of each phenomenon to the overall observed accumulation in RTL-W1 cells (Schug et al., 2018; Stott et al., 2015; Minghetti et al., 2017; Chang et al., 2021; Fuchylo et al., 2022). In the larger context of IOC, it would be interesting to test a set of anionic organic compounds with similar molecular structures that resemble the structure of the cell membranes' phospholipids, such as surfactants (Ribbenstedt et al., 2022), or cationic organic compounds, which bear positive charges (Kierkegaard et al., 2021).

3.4. Comparison of *in vitro* and *in vivo* bioaccumulation

Fig. 5 compares the RTL-W1 BCF to the available *in vivo* bioaccumulation data and several common numerical predictions that focus on the chemical accumulation predictions in fish. Overall, the use of RTL-W1 cells to directly predict the bioaccumulation in fish performs in a similar manner as the numerical BCF predictions. With the exception of PCP, this is particularly true for D_{OW} -based BCFs (PBTK D_{OW} and BCF_{OW}), which are higher and more consistent with RTL-W1-derived BCF than BCF predicted based on D_{MLW} . This finding again supports the notion that uptake of chemicals into living cells involves a multitude of interactions, as discussed above. It moreover is apparent that the RTL-W1 BCF, along with the numerical BCF predictions, cannot account for *in vivo* BMFs. Indeed, the comparison of the RTL-W1 BCFs with *in vivo* BCF or BMF deviates considerably and needs to be discussed as per test compound with the caveat that no BCF information exists for TT and BHPP.

DCF is the only test compound where all accumulation predictions and the *in vivo* BCF studies lie within an order of magnitude from each other (Fig. 5). It is notable that the RTL-W1 BCF agrees well with the *in vivo* BCF values in whole fish and liver. This indicates that the RTL-W1 cells contain the relevant accumulation mechanisms that govern the DCF accumulation in the whole fish.

For PCP, the RTL-W1 BCF is at least half an order of magnitude lower than the numerical predictions of BCFs and more than two orders of magnitude lower than the *in vivo* BCF. The difference to the numerical predictions may be caused by the suggested, although albeit small, biotransformation of PCP in the RTL-W1 cells, which reduces the RTL-

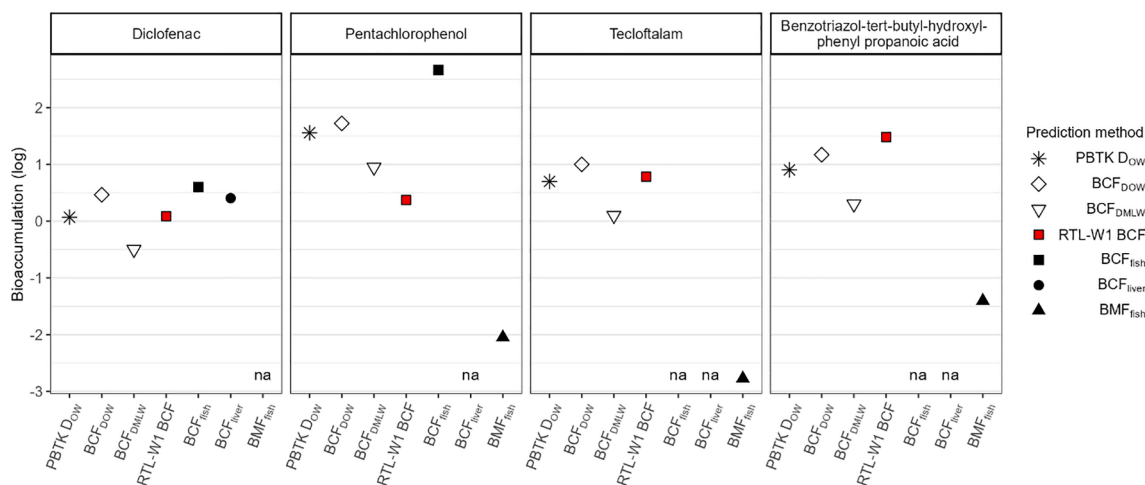


Fig. 5. Comparison of different prediction methods for bioaccumulation in fish. The RTL-W1 BCF values showed little variation among all replicates per test compound (SI, section 5.3, Table S8) and thus are not shown. All *in vivo* values (Fish/Liver $BCF_{fish/liver}/BMF_{fish}$) were taken from rainbow trout (SI, section 5.3, Table S9). $\log BCF_{DMLW} = \log D_{MLW} - 1.9$, $\log BCF_{DOW} = 0.85 \times \log D_{OW} - 0.7$, $\log \text{RTL-W1 BCF} = \text{derived cell-based } in vitro \text{ BCF}$, PBTK model D_{OW} : (Nichols et al., 2013) using D_{OW} instead of K_{OW} and neglectation of biotransformation, BCF/BMF_{fish} = Bioconcentration or Biomagnification factor in whole fish derived from feeding study, BCF_{liver} = BCF based on sampled liver tissue (Mommert et al., 2013) SD = Standard deviation, na = not available, *Droge et al. (2021) **EU Commission Technical Guidance Document (2004) (Bruijn et al., 2022).

W1 BCF relative to the numerical predictions that do not consider biotransformation or elimination in general. Apparently, neither RTL-W1 bioaccumulation nor numerical predictions reflect well what was measured in the one *in vivo* study using rainbow trout. BCFs of PCP in fish species other than rainbow trout indicate varying accumulations with values ranging from $\log BCF$ of 0.7 to 3.7, with a geometric mean of $\log BCF$ of 2.1 (median at 2.3) (SI, section 15, Table S21).

For both, DCF and PCP, the lack of BTP identification appears to be irrelevant for the *in vivo* bioaccumulation prediction. Our RTL-W1 BCF for DCF compares well with the *in vivo* BCF despite the absence of detecting BTPs, while for PCP, the *in vivo* BCF was much higher than the RTL-W1 BCF, which appears to be independent of potential BTP formation. Biotransformation activities vary in *in vitro* as well as in *in vivo* experiments (Connors et al., 2013; Stadnicka-Michalak et al., 2018b; Kosfeld et al., 2020) and depend on tissue type as well as prior and ongoing exposure of test animals. These aspects require consideration when discussing the role of BTPs in BCF determinations.

All *in vivo* BMF values were considerably lower than any numerical prediction method, the RTL-W1 BCFs and the *in vivo* BCFs (Fig. 5). This is not surprising because biomagnification is controlled by different processes than bioconcentration. Biomagnification strongly depends on the xenobiotic entry via the intestines and associated residence times and depuration mechanisms, which contrast the entry via the gill (Erickson et al., 2006). A combination of cell lines may instead aid in BMF predictions in the future, such as a first exposure of the rainbow trout intestinal cell line, RTgutGC, followed by exposure of the RTL-W1 liver cell line.

4. Conclusions and outlook

We here developed a procedure using a permanent fish cell line, RTL-W1, to enable the measurement of intracellular amounts of IOC for bioaccumulation assessment in fish. This complements the set of assays that use fish cell lines for diverse endpoints in risk assessment, such as the measurement of biotransformation rate constants for IVIVE, prediction of acute toxicity in fish and the study of *trans*-epithelial transport. The results imply that our developed method is suitable to test diverse groups of chemicals, including anionic compounds. In the future, it would be relevant to study other structures of anionic organic compounds as well as cationic organic compounds to further evaluate the role of chemical structure and charge on bioaccumulation mechanisms.

The measured cell internal concentrations could also be used in a PBTK-based model approach to back-calculate to the exposure concentration and derive a BCF.

From a regulatory perspective, both the RTL-W1-based as well as the numerical bioaccumulation predictions indicate that the bioaccumulation of the test compounds does not surpass regulatory thresholds for BMF_{fish} ($\log BMF > 3$) or BCF ($\log BCF > 3.3$, (ECHA, 2017)). Despite the inability to observe biotransformation in RTL-W1 cells for the test compounds investigated here, they appear fitting as experimental alternative to experimentation with fish, and as complement to numerical predictions. The latter are based on surrogates of cellular membranes, particularly phospholipids, whereas the cells retain accumulation mechanisms that may not be reflected well by the compounds' D_{OW} or D_{MLW} . Fish cell lines should therefore be further explored as part of gathering weight of evidence and in tiered testing strategies where bioaccumulation assessments in fish remain as a last resort.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envint.2023.107798>.

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